

Mass, charge, and subcellular localization of a unique secretory product identified by the basophil-specific antibody BB1

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Background: BB1 is a basophil-specific mAb (Lab Invest 1999;79:27-38). The identity of the corresponding antigen has not been determined, but it gives a granular appearance on staining and is secreted on activation of basophils.

Objective: We sought to further characterize the basophil-specific antigen identified by BB1.

Methods: Intracellular localization was determined by flow cytometry and by immunogold labeling and electron microscopy. Physical chemical properties were investigated by gel filtration chromatography and preparative isoelectric focusing.

Results: In flow cytometry, permeabilization of cells increased immunofluorescence 100-fold, confirming the predominantly intracellular localization of the antigen. It was further localized to the secretory granules by immunoelectron microscopy. Double labeling with a CD63-specific antibody demonstrated selective binding of BB1 to the granule matrix. Gel filtration chromatography indicated that the antigen is secreted as a complex of approximately 5×10^6 d, which was well resolved from the 210-kd supramolecular complex containing tryptase. The antigen was degraded by pronase. Isoelectric focusing indicated a highly basic protein with an isoelectric point of 9.6.

Conclusion: With its granule localization, release on cell activation, and unique properties, the antigen identified by BB1 could be a novel mediator of allergic disease. We propose the name *basogranulin* for this novel basophil-specific protein. (J Allergy Clin Immunol 2001;107:842-8.)

Key words: Basophil, CD63, tryptase, immunoelectron microscopy, flow cytometry, isoelectric focusing, gel filtration chromatography

Basophils are closely associated with the pathogenesis of allergic disease.¹⁻³ In common with mast cells, they possess high-affinity receptors for IgE, release histamine

Abbreviations used

PAG: Pipes-buffered saline solution containing albumin and glucose

PBS-BSA: PBS containing 1% BSA

pI: Isoelectric point

V_e/V_0 : ratio of elution volume to void volume

and other mediators on cross-linking of these receptors, and stain with basic dyes, such as alcian blue, but are quite distinct in their development, distribution, and behavior.^{4,5} Whereas mast cells are resident in the tissues, basophils are generally confined to the circulation, although they can invade tissues and accumulate at sites of allergic inflammation.^{1,2,6-12} Basophils and mast cells also differ from each other in their possession of different non-IgE-dependent mechanisms of mediator release, the increased releasability of basophils in response to specific cytokines, and the expression of cell surface markers detected by flow cytometry.¹³

On stimulation, basophils release a variety of mediators, including histamine, proteoglycans, enzymes, cytokines, and leukotriene C₄. However, none of the biochemically characterized products identified to date are unique to this cell type.^{2,14-18} In searching for a unique product of basophils, a number of groups have tried the immunologic approach by raising antibodies to whole basophils. Three basophil-specific mAbs have been reported so far: Bsp-1,¹⁹ which recognizes a cell surface marker; and 2D7²⁰ and BB1,²¹ which appear to react with a granule constituent. We have found that BB1 is specific for basophils by immunocytochemistry, dot blots of cell lysates, and flow cytometry, and that the antigen recognized by BB1 appears to be expressed in all basophils.^{8,21} Immunohistochemical studies have failed to detect this antigen in any other recognizable cell type.^{7-12,22}

Although Bsp-1²³⁻²⁵ has been used for flow cytometry and 2D7^{6,18} and BB1^{7-12,22} have been used for immunohistochemistry, the corresponding basophil-specific products remain poorly characterized. The granular appearance of cells stained with BB1 and the secretion along with histamine of its antigen in response to anti-IgE or calcium ionophore suggest that this antigen is localized to the secretory granules.²¹ In this study we confirm this localization by flow cytometry and electron

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microscopy and elucidate some of its physical and chemical properties. We propose the name *basogranulin* for this unique component of the basophil secretory granule.

METHODS

Materials

The basophilic cell line KU812F (ECACC No. 90071804) was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Iscove's modified Dulbecco's medium was purchased from Sigma Chemical Co (Poole, UK), and all other culture media and supplements were purchased from Life Technologies (Paisley, UK). Streptavidin magnetic microbeads and the MACS Basophil Isolation Kit were obtained from Miltenyi Biotec (Bisley, Surrey, UK), and Coomassie protein assay reagent and SuperSignal West Femto and SuperSignal West Pico chemiluminescence reagents were obtained from Pierce and Warriner (Chester, UK). Accugel solution of acrylamide and *N,N'*-methylene-bis-acrylamide (19:1) was purchased from Flowgen Instruments (Lichfield, Staffs, UK), and all other electrophoresis reagents were purchased from Bio-Rad (Hemel Hempstead, UK). The E3/4 anti-IgE mAb was purchased from Serotec (Oxford, UK). All other chemicals were from Sigma or BDH (Poole, UK). AA1 (an antitrypsin mAb),²⁶ BB1 (a basophil-specific mAb),²¹ and rabbit polyclonal antiserum to trypsin²⁷ were produced as previously described. Biotinylated HB121 (anti-IgE mAb) was generously donated by Prof F. K. Stevenson (Southampton, UK). Mouse anti-CD63 mAb 435 was obtained from Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Goat antimouse IgG linked to 10-nm gold particles was from Amer-sham Nederland (Hertogenbosch, The Netherlands).

Biotinylation of BB1

One milliliter of antibody (5 mg/mL⁻¹) in 100 mmol/L sodium carbonate, pH 8.5, was incubated with 25 μ L of 25 mg/mL⁻¹ *N*-hydroxysuccinimidyl 6-(biotinamido) hexanoate (Sigma) for 2 hours at room temperature. The reaction was stopped by the addition of 12 mg glycine, and the biotinylated antibodies were dialyzed extensively against PBS at 4°C.

Cell purification and culture

Basophils were purified from human blood obtained after informed consent from healthy donors (approval No. 307/95, Southampton Joint Ethics Sub-Committee). For flow cytometric studies, basophils of intermediate purity (~20%) were obtained by fractionation of whole blood by Percoll density gradient centrifugation,²¹ and contaminating erythrocytes were removed by hypotonic lysis. For electron microscopy, basophils were purified from buffy coats from 500 mL of blood by successive isopycnic centrifugation and elutriation.¹⁷ Preparations of "untouched" basophils (~80% purity) for production of challenge supernatant for gel filtration studies were produced with the MACS Basophil Isolation Kit (Miltenyi Biotec). For the generation of cell lysates, basophils of greater than 95% purity were isolated by Percoll density gradient centrifugation followed by immunomagnetic selection of basophils with the IgE-specific mAb HB121.²¹ Cells were stained with alcian blue²⁸ and counted in a Neubauer hemocytometer. Cells of the KU812F cell line were grown in Iscove's modified Dulbecco's medium in the presence of 10% FBS, 2 mmol/L L-glutamine and antibiotics.²⁹

Flow cytometry

Cells to be permeabilized were resuspended in 1 mL of ice-cold methanol for 20 minutes and washed in 1 mL of PBS containing 1% (wt/vol) BSA (PBS-BSA). Both permeabilized and nonpermeabilized cells were resuspended in 1 mL PBS-BSA, divided into aliquots, and

incubated on ice for 5 minutes. After centrifugation, the cells were resuspended in 100 μ L of the appropriate antibody dilution in PBS-BSA and incubated on ice for 1 hour. All treatments were performed in duplicate. The cells were washed twice with PBS-BSA and then incubated in 100 μ L of FITC-conjugated secondary antibody (Sigma) for 1 hour on ice. After further washes, the cells were analyzed by FACS (Becton Dickinson, Mountain View, Calif). Cells were gated with the forward scatter and side scatter characteristics of the population of cells showing a strong reaction with anti-IgE.

Electron microscopy

Basophils were fixed in a mixture of 0.5% glutaraldehyde and 4% paraformaldehyde in PBS (pH 7.2) for 2 hours at room temperature and processed for ultrathin cryosectioning.³⁰ Cryosections 45 nm thick were cut at -125°C with diamond knives (Drukker, Cuijk, The Netherlands) in an ultramicrotome (Leica, Vienna, Austria) and transferred with a mixture of sucrose and cellulose onto Formvar-coated copper grids.³¹ The grids were placed on 35-mm Petri dishes containing 2% gelatin. For immunolabeling, the sections were incubated with undiluted BB1 hybridoma culture supernatant for 45 minutes, followed by 45 minutes with rabbit antimouse IgG (dilution 1:40) and then 30 minutes with 10-nm protein A-conjugated colloidal gold (a gift from the Electron Microscopy Laboratory, University of Utrecht, Utrecht, The Netherlands). For double labeling, the cryosections were incubated with BB1 followed by rabbit antimouse IgG and 5-nm protein A-conjugated colloidal gold. The sections were treated with 1% glutaraldehyde for 10 minutes to prevent any interference between the different antibody gold complexes on the sections³² and before incubation with CD63 antibodies³³ and antimouse IgG linked to 10-nm gold. After immunolabeling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM 10 electron microscope (Eindhoven, The Netherlands). For the controls, the primary antibody was replaced by nonrelevant murine antibody.

Cell activation studies

Basophil-enriched cell suspensions were washed in PIPES (piperazine-*N,N'*-bis-2-ethanesulfonic acid)-buffered saline solution containing albumin and glucose (PAG) and resuspended in PAG containing 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂. Cells were incubated with 0.3 μ g/mL E3/4 anti-IgE mAb at 37°C for 30 minutes. After centrifugation, the supernatants were removed and centrifuged again, and the second supernatant was taken for analysis by either dot blot or gel filtration chromatography. Histamine was measured by a glass fiber-based fluorometric assay,³⁴ by use of 96-well microtiter plates coated with a glass-fiber matrix (Lundbeck Diagnostics, Copenhagen, Denmark).

Gel filtration chromatography

Samples (~1.0 mL) were applied to either a 66.5 $\times$ 2.6-cm column of Sephacryl S-300 or a 68 $\times$ 1.6-cm column of Sephacryl S-500 equilibrated with PBS containing 0.02% NaN₃ and then eluted with the same buffer. The elution profiles of blue dextran (2000 kd), thyroglobulin (660 kd), apoferritin (340 kd), β -amylase (200 kd), BSA (66 kd), and carbonic anhydrase (30 kd) were monitored by absorbance at 280 nm, whereas that of human skin trypsin (134 kd) was monitored by assay with pyroglutamyl-Pro-Arg-p-nitroanilide.³⁵ The elution profiles of basophil products were monitored by dot blotting.

Dot blotting

Samples were applied to a 96-well dot blot apparatus (Bio-Rad) according to the manufacturer's instructions. The blot was removed, blocked with 5% skim milk powder, and probed with 0.5 μ g/mL⁻¹

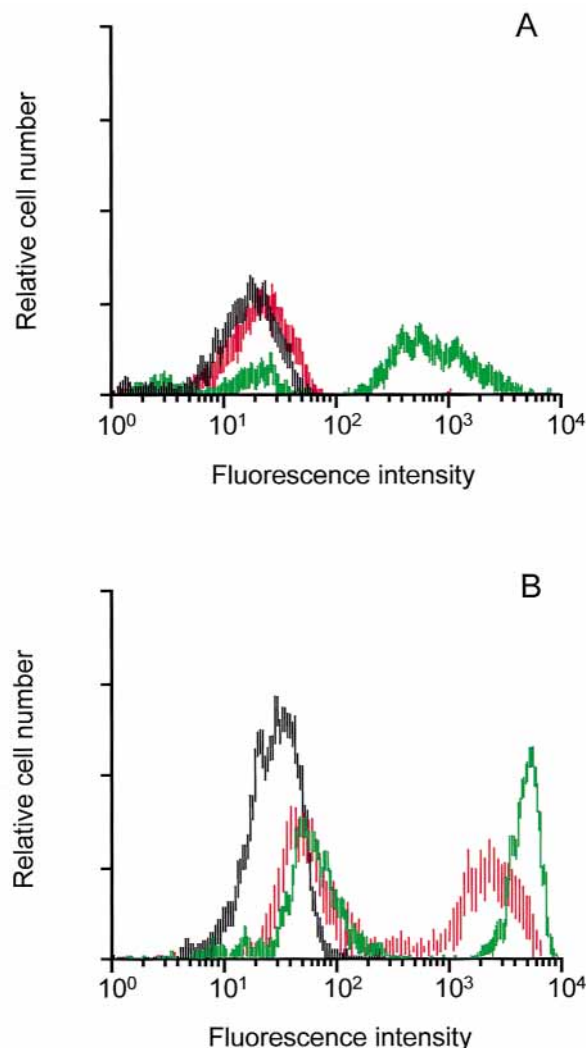


FIG 1. Flow cytometric analysis of partially purified human basophils. Percoll density gradient fractions containing 20% basophils were incubated on ice for 1 hour with $10 \mu\text{g/mL}^{-1}$ control isotype IgG_{2a} antibody (black trace), with $10 \mu\text{g/mL}^{-1}$ anti-IgE antibody HB121 (green trace), or with $10 \mu\text{g/mL}^{-1}$ BB1 (red trace). **A**, Nonpermeabilized basophils. **B**, Basophils permeabilized with methanol. Cells were gated by forward scatter and side scatter to select the population enriched in cells reacting strongly with anti-IgE antibody.

biotinylated BB1 antibody. After treatment with ExtrAvidin-horseradish peroxidase conjugate (Sigma), spots were visualized on radiographic film with SuperSignal West Pico or West Femto chemiluminescent substrate. In some experiments the blot was then incubated overnight in blocking buffer containing 0.05% (wt/vol) NaN_3 to inactivate any peroxidase associated with BB1 and reprobed with rabbit antiserum to tryptase. The blots were developed with peroxidase-conjugated secondary antibodies and SuperSignal West Femto as above. Spot intensities were quantified with an Epson GT-8000 scanner and Phoretix 1-D Advanced (version 4.01) image-analysis software.

Isoelectric focusing

Frozen cells (2.1×10^6 basophils, 96% purity, or 16×10^6 KU812F cells) were resuspended in 1.0 mL PBS, sonicated, and centrifuged. The supernatant was mixed with 16 mL 2% Bio-Lyte

3/10 ampholyte and subjected to preparative isoelectric focusing in a Rotaphor apparatus (Bio-Rad) according to the manufacturer's instructions. The pH of each fraction was measured with a pH Boy-P2 field effect transistor pH meter (CamLab, Cambridge, UK), and the position of basogranulin was determined by dot blotting.

RESULTS

Flow cytometry

We have previously reported that treatment of intact basophils with BB1 produced a small but significant increase in fluorescence.²¹ Permeabilization of cells by methanol fixation produced a 100-fold greater response (Fig 1), confirming the predominantly intracellular localization of the antigen. The magnitude of the response seen with BB1 is comparable to that seen with the anti-IgE mAb HB121. The bimodal distribution of immunofluorescent cells can be attributed to the presence of non-HB121-reactive cells (nonbasophils) within the selection window. The control isotype antibody did not produce any increase in fluorescence.

Localization of the BB1 antigen by immunoelectron microscopy

Immunostaining of basophils with BB1 and gold-labeled secondary antibody resulted in the dense localization of the gold particles almost exclusively to the secretory granules (Figs 2 and 3, A). The contents of basophilic granules were frequently partly or totally dissolved as has been previously described.³⁶ In well-preserved granules, the gold particles tended to be evenly distributed throughout. No immunogold label was seen over the granules of neutrophils, eosinophils, lymphocytes (not shown), or monocytes (Fig 2). Double labeling with an antibody to CD63, a marker present on the membranes of secretory granules in basophils and other granulocytes,^{33,37} confirmed the identity of the membrane-bound compartments containing the BB1 antigen as secretory granules. CD63 was present on the granule membrane, whereas the BB1 antigen was on the matrix (Fig 3, B). Granules with partly dissolved contents were poorly labeled with BB1 but retained membrane-bound CD63 (Fig 3, B). No labeling was found in the controls of each experiment, where the specific antibody was replaced by a nonrelevant murine antibody (results not shown).

Molecular mass determination

When the supernatant of anti-IgE-challenged basophils was applied to a column of Sephacryl S-300, the antigen identified by BB1 eluted in the void volume (Fig 4, A). It thus displayed a molecular mass greater than or equal to 2,000,000 d and was well resolved from the macromolecular complex containing tryptase, which eluted at 210,000 d. The reaction on the dot blot was specific for the secreted antigen because omission of BB1 did not lead to a reaction product. Also, identical results were obtained whether the primary antibody was biotinylated and detected with ExtrAvidin-peroxidase or whether non-biotinylated BB1 was used, followed by detection with peroxidase-conjugated rabbit antimouse IgG.

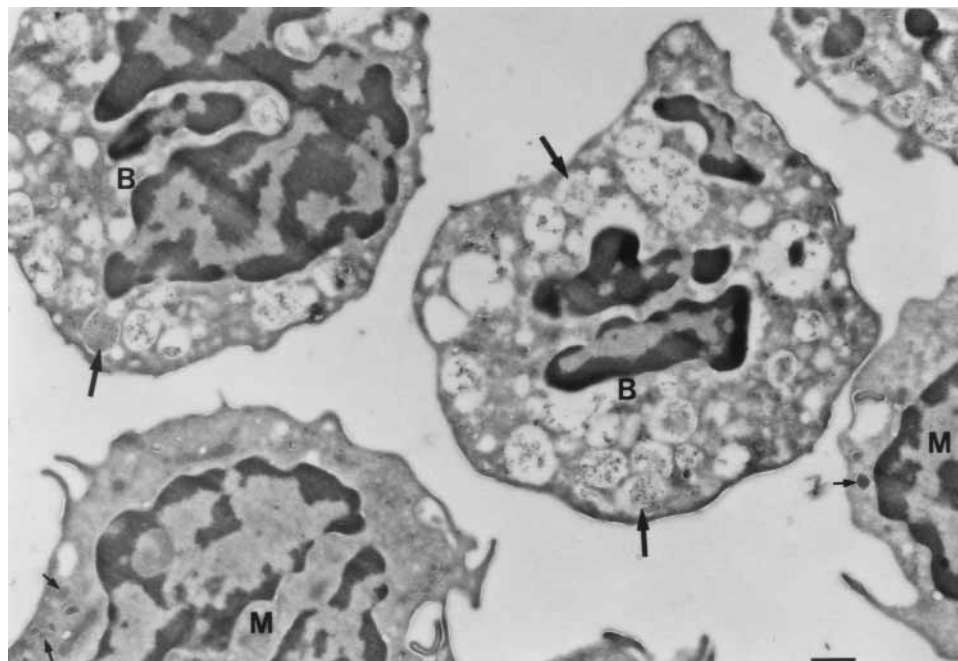


FIG 2. Localization of BB1 binding in basophils. Survey electron micrograph of basophils isolated from peripheral blood buffy coat. Cryosections were incubated with BB1 antibody and 10-nm gold conjugate. Basophils (*B*) show abundant labeled granules (*large arrows*). Note that the content of most of these granules is partly dissolved. Monocytes (*M*) show small unlabeled granules (*small arrows*) (*bar* = 500 nm).

The antigen identified by BB1 antibody eluted in the void volume whatever its source, whether from basophils or KU812F cells or whether from challenge supernatants or cell lysates. These results contrast with those we had previously obtained by nondenaturing PAGE, where a molecular mass of 124 kd for the antigen derived from KU812F cells had been estimated,²¹ although the smearing of bands from the top of the gel had suggested an equilibrium with a much larger complex. The basophil-derived antigen never entered the gels under any conditions tested. Attempts to disrupt this large complex into smaller units by altering chromatographic conditions were unsuccessful: similar elution profiles were seen whether the column was equilibrated with PBS or with 2 mol/L NaCl and 50 mmol/L Mops, pH 6.8 (results not shown).

A better estimate of the molecular mass was sought by chromatography on the larger porosity matrix Sephacryl S-500. The antigen identified by BB1 antibody eluted after the void volume (identified by genomic DNA) but before blue dextran (Fig 4, *B*). It eluted as 2 peaks, a narrow minor one at a ratio of elution volume to void volume (V_e/V_o) of 1.19 and a broad major one at a V_e/V_o of 1.66 (1.51-1.78). There are no suitable protein markers for this matrix, but the manufacturer estimates a fractionation range of 50,000 to 100,000,000 d for globular proteins. If the void volume is taken as 100×10^6 d and skin tryptase and blue dextran are used as standards, a plot of log (molecular mass) versus V_e/V_o gives molecular mass values of 46×10^6 d for the minor peak and 5×10^6 d for the major one. Incubation of the challenge supernatant with 10

$\mu\text{g/mL}^{-1}$ pronase for 1 hour at 37°C before chromatography eliminated both peaks. No immunoreactive fragments were detected as seen with the pronase-digested basophil tryptase (results not shown), which is consistent with BB1 recognizing a conformational rather than linear epitope.²¹

Determination of isoelectric point

When a basophil lysate was subjected to isoelectric focusing, the antigen identified by BB1 concentrated at the basic end of the pH gradient (not shown). Because the gradient was rather steep at this point, the last 5 fractions were pooled and resubmitted to isoelectric focusing without any additional ampholytes. An isoelectric point value (pI) of 9.6 was obtained (Fig 5, *A*). The profile suggests that there might be additional minor isoforms with pIs between 8.8 and 9.4. In marked contrast lysates of KU812F cells appeared to contain 3 major isoforms with pIs of approximately 7.3, 7.8, and 8.9 (Fig 5, *B*).

DISCUSSION

In our first report on BB1 antibody,²¹ we suggested that its cognate antigen might be localized to the secretory granule on the basis of 2 lines of evidence: the granular appearance of cells stained with BB1 and the secretion of the antigen in response to stimulation with anti-IgE or the calcium ionophore A23187. Additional evidence has come from the present study to support this hypothesis. Flow cytometry of nonpermeabilized and permeabilized cells indicated that more than 98% of the

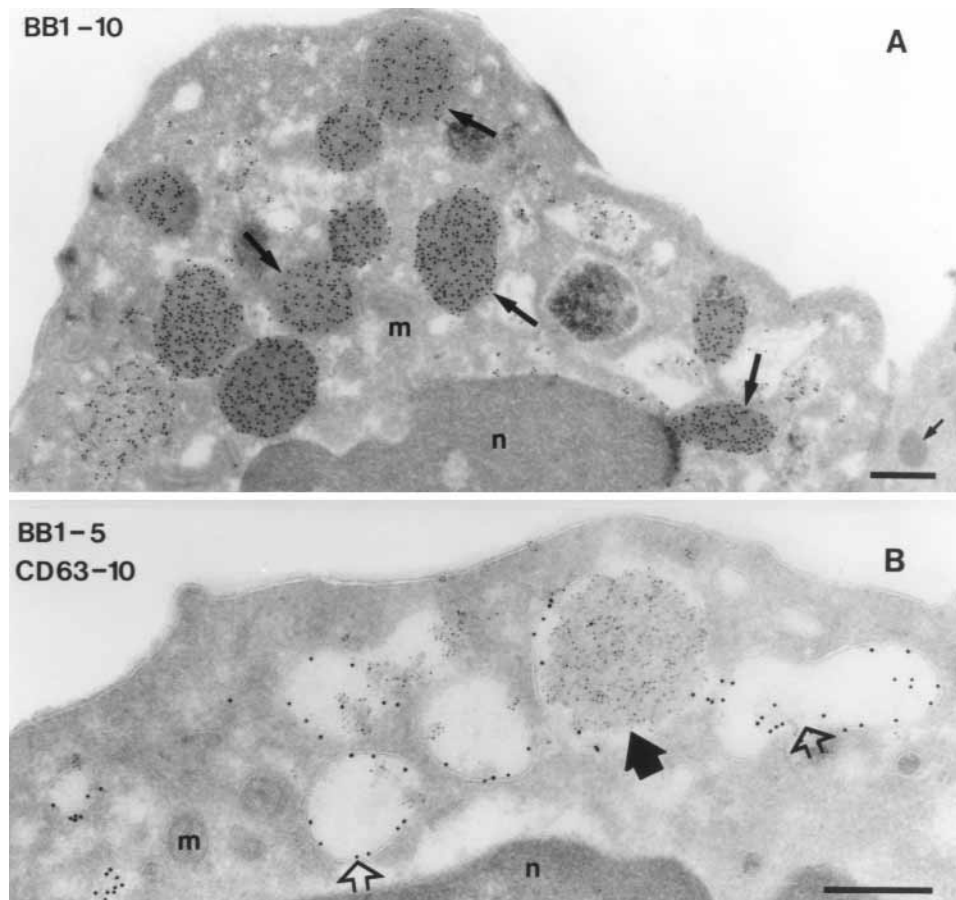


FIG 3. A, Detail of basophil showing BB1 binding. Same preparation as in Fig 2. All of the granules are highly labeled (*large arrows*). On the *right*, an area of a monocyte is shown, and no labeling is observed on the granule (*small arrow*). *N*, Nucleus; *m*, mitochondria. **B,** Characterization of the BB1-positive granules. Cryosections of basophils were double labeled for BB1 (5-nm gold) and CD63 (10-nm gold). An area of a basophil showing granules labeled for both CD63 on the membrane and BB1 on the matrix. *Closed arrow* shows a granule with a full content highly labeled for BB1; *open arrows* pointed to granules with partly dissolved contents clearly showing labeling for CD63 on granule membranes (*bars* = 400 nm).

antigen is located intracellularly, whereas immunoelectron microscopy has shown it to be confined to the secretory granules. In view of its specificity for basophils and its localization within the secretory granules, we propose that this antigen be called *basogranulin*.

The association of basogranulin with the matrix rather than with the membrane of these granules was confirmed by double labeling with antibodies to CD63. This latter marker, of uncertain function, is a member of the tetraspanin or TM4 superfamily of surface receptors.³⁷ It is found on the membranes of secretory granules of basophils,³³ the specific granules of eosinophils,¹⁶ and various secretory vesicles in other cell types.^{30,38,39} On cellular activation, the membranes of these vesicles fuse with the plasma membrane, resulting in a marked increase³³ in the surface expression of CD63 (20- to 100-fold in the case of basophils³³). Basogranulin was found in all CD63⁺ granules. This is in contrast to the Charcot-Leyden crystal protein, which is present in only a sub-

population of these granules.¹⁶ Its presence in all granules suggests that basogranulin is always secreted on basophil degranulation, thus making it a candidate for a diagnostic marker of basophil activation.

The massive size of basogranulin is quite remarkable. No single polypeptide chain of this size is known, so it almost certainly exists as a multisubunit complex. Determination of its subunit structure by SDS-PAGE followed by Western blotting was not possible because BB1 does not react with denatured antigen,²¹ and silver staining of gels was not sensitive enough to reveal the protein composition of this complex (results not shown). This macromolecular complex is separate from that containing tryptase, which at 210 kd is very similar in size to the tryptase-proteoglycan complex released by mast cells.^{40,41} This does not preclude basogranulin from being complexed with proteoglycans because mast cells were found to secrete chymase and carboxypeptidase in proteoglycan-containing complexes distinct from those containing tryptase.

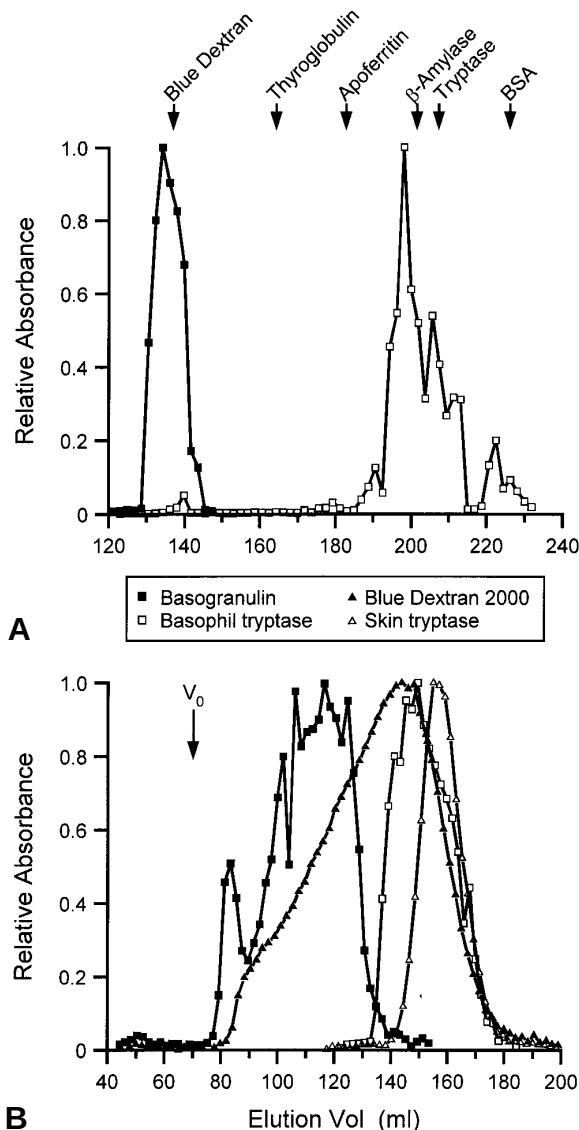


FIG 4. Gel permeation chromatography of supernatant from basophils challenged with anti-IgE. **A**, Elution profile from a column of Sephacryl S-300. The elution positions of molecular mass markers are indicated by arrows. **B**, Elution profile from a column of Sephacryl S-500. V_0 indicates the void volume as determined with sonicated pig heart DNA. All peak heights have been normalized to the same value (1.00). Basophils (0.3×10^6 at 73% purity in 0.5 mL reaction volume and 2.0×10^6 at 80% purity in 0.8 mL reaction volume for **A** and **B**, respectively) were incubated with $0.3 \mu\text{g/mL}^{-1}$ anti-IgE mAb (clone E3/4) for 30 minutes at 37°C . Under these conditions, 71% and 51% of the total histamine was released from these cells in **A** and **B**, respectively.

Basogranulin appears to be a protein because antigenicity was lost on digestion with pronase. No other protein has been described with this combination of cell specificity, charge, and mass, so it is likely to represent a novel gene product. The alternative possibility, that the epitope is present on a novel oligosaccharide or other posttranslational modification attached to a known pro-

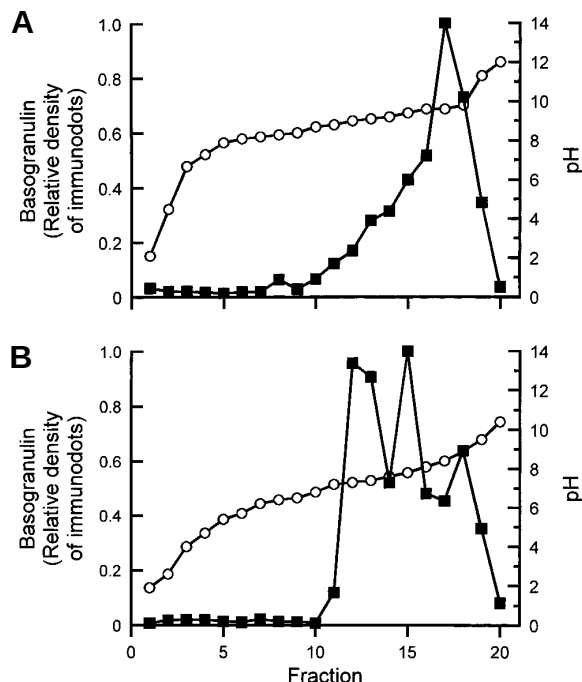


FIG 5. Preparative isoelectric focusing. **A**, Fractions 16 through 20 of a whole cell lysate of peripheral blood basophils that had been fractionated on a pH 3 to 10 gradient were pooled and refractionated without additional ampholytes to produce a more shallow gradient (pH ~8-10). **B**, Whole cell lysate of KU812F cells was fractionated on a pH 3 to 10 gradient. Basogranulin was detected by BB1 antibody on dot blots.

tein, is less likely because it would be highly unusual for a modification to be specific for a single product of a single cell type. Confirmation of the novelty of basogranulin by N-terminal sequence analysis must await the availability of very much greater quantities of material. Judging from relative reactivities with different chemiluminescent substrates and from the very weak response on silver-stained gels, this study dealt with picogram, possibly low nanogram, quantities of basogranulin. In comparison, standard N-terminal sequencing techniques require microgram quantities of pure protein.

The extraordinary size of the basogranulin complex, together with its high pI, helps explain the difficulties encountered in estimating its molecular mass by nondenaturing gel electrophoresis.²¹ The intact complex is too large to enter even low-porosity gels and, with a net positive charge, would have moved away from the gel, not into it. The expression by KU812F cells of isoforms with lower pIs explains their ability, under certain conditions, to enter the gel, although much of the immunoreactive material still remained at the top of the gel, suggestive of a dynamic equilibrium between associated and disassociated forms of this protein. The factors affecting this equilibrium are unclear and require further investigation. The chemical basis for the lower pIs seen in the KU812F-derived material remains to be investigated, but differences in post-translational processing could be responsible.

The secretion of basogranulin on basophil activation strongly suggests a role in allergic inflammation. The biologic function will be affected by its massive size, which will severely restrict its rate of diffusion. For basophils that have left the circulation and accumulated in tissue, the effect of this antigen would, consequently, be highly localized. Moreover, the positive charge would favor adsorption to proteoglycans, either on cell membranes or on structural elements, or free in the circulation. As a unique secretory product of the basophil, basogranulin should be valuable as a discriminating marker of cell activation. Elucidation of the biologic function of this novel antigen remains an important challenge.

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