

Studies of Cell Adhesion and Flow Cytometric Analyses of Degranulation, Surface Phenotype, and Viability Using Human Eosinophils, Basophils, and Mast Cells

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Products derived from eosinophils, basophils, and mast cells are considered critical to the development of allergic diseases. Studies of the selective recruitment, accumulation, and/or activation of these cells during human allergic inflammatory reactions *in vitro* and *in vivo* have been facilitated by a wide variety of methods. Some have been developed to identify and isolate these cells from a variety of sites, including blood, airway secretions, and surgical or autopsy tissues. Once enriched in purity, assays of cell adhesion to endothelium, epithelium, matrix proteins, and purified, immobilized counterligands for integrins, selectins, or immunoglobulin gene superfamily structures can be performed *in vitro* under both static and flow conditions. Techniques involving flow cytometry, utilizing characteristics of cellular light scatter and immunofluorescence, have permitted the elucidation of cell surface phenotype and have aided in quantification of cellular degranulation and viability. These approaches have yielded new information on the function of human eosinophils, basophils, and mast cells and have suggested unique cell-specific pathways of cell recruitment, activation, and survival that may contribute to the pathogenesis of allergic diseases.

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The initial immunologic events associated with the onset of tissue-specific symptoms of allergic dis-

ease involve the interaction of allergen with specific immunoglobulin E (IgE) bound to the surface of mast cells, with subsequent release of preformed and newly generated inflammatory mediators. These immediate events, although clearly IgE- and mast cell-dependent, are felt to differ mechanistically from events that occur in chronic forms of allergic disease such as allergic rhinitis, atopic dermatitis, and asthma. Even though these latter immunopathologic changes are viewed as requiring mast cells and IgE, large numbers of eosinophils and basophils accumulate at these sites (1). Experimental models involving local allergen challenge of sensitive individuals have provided additional evidence that IgE-dependent recruitment of eosinophils and basophils accompanies the allergic response. For example, in the lung, eosinophils can account for up to 60% of the cells entering the lumen of the airways after introduction of antigen (2). Basophil numbers in the airway also increase, often by more than 100-fold, and can account for as many as 3% of the cells (3). Recently, an appreciation has grown for the direct role that these cells and their products play in producing allergic inflammation. Eosinophils and basophils increase in the airways during asthma exacerbations, and eosinophil numbers correlate with the degree of airway hyperresponsiveness and disease severity (1). Release of eosinophil-derived products, such as

leukotriene C₄ (LTC₄) and the toxic major basic protein (MBP), are now felt to contribute to the destruction of airway epithelium, smooth muscle contraction, sensitization of airway nerves, vascular leak, and associated phenomena (4). Basophils contain preformed mediators such as histamine and are a potent source of newly generated cytokines, such as interleukin-4 (IL-4), that are critical in allergic inflammation (5). In addition, the hypothesis that eosinophils and basophils play central roles in allergic diseases is supported by the identification of eosinophil- and basophil-derived mediators at sites of allergic inflammatory responses and the reduction of eosinophils, basophils, and their mediators during the resolution of allergic reactions (1).

Since eosinophils and basophils are preferentially attracted into extravascular inflammatory sites, where they contribute to disease pathophysiology, mechanisms must exist that favor their mast cell- and IgE-dependent recruitment into and persistence within the affected tissues (6). To better understand how eosinophils, basophils, and mast cells emigrate to specific extravascular sites and ultimately participate in the allergic response, methods have been developed to isolate these cells in sufficient numbers from biological fluids or tissues to permit *in vitro* study. Using strategies that compare one cell type to another, these cells have now been extensively investigated with respect to their adhesive and migratory functions. Conditions have been defined under which these cells display prolonged survival and resist apoptosis. All of these studies could not have been possible without reliable methods to analyze and compare the surface phenotype of these cells. The purpose of this review is (1) to summarize methods in current use for the isolation of eosinophils, basophils, and mast cells, (2) to describe how these cell suspensions are then functionally evaluated in a variety of adhesion-based assays, and (3) to outline how flow cytometry is employed for analysis of surface marker expression, viability, and degranulation.

METHODS FOR ISOLATION OF HUMAN EOSINOPHILS, BASOPHILS, AND MAST CELLS

Because eosinophils, basophils, and mast cells have distinctive tinctorial properties and phenotypic

markers, they can easily be identified and enumerated even when present in low purity (7–9). However, biological studies of these cells often requires removal of other cells to ensure that effects seen are a result of direct activation of the cell of interest. Therefore, a variety of methods have been developed to enrich the purity of eosinophils, basophils, and mast cells. These methods take advantage of several conserved characteristics among each cell type, the most important of which are density (specific gravity) and surface phenotype. Examples of techniques commonly used to purify eosinophils and basophils from peripheral venous blood after anticoagulation are listed in Table 1. The levels of starting and final purity range from <1% to >99%; the higher the final purity, the lower the yield. In addition to peripheral blood, these cells have been successfully obtained for study, and in some cases enriched, from extravascular sites including the airways (from nasal (10, 11) or bronchoalveolar lavage (3, 12, 13)) and skin blisters (14, 15). Density gradient centrifugation is by far the most common and useful method for initial cell enrichment. The specific gravity of basophils overlaps with that of monocytes and lymphocytes (16), while eosinophils are more dense, overlapping with neutrophils and erythrocytes. Therefore, reagents such as Percoll (Pharmacia, Piscataway, NJ), diluted to specific gravity <1.079 g/ml with buffer, can be used to separate cells of lower density (lymphocytes, monocytes, and basophils) from cells of higher density (neutrophils, eosinophils and erythrocytes, specific gravity >1.080) following centrifugation at 130*g* for 20 min. Further enrichment of the latter cell types usually requires a method for erythrocyte removal such as hypotonic lysis (e.g., exposure of erythrocyte-containing cell pellets to ice-cold water for 30 s, followed by restoration of tonicity with an appropriate amount of 10× concentrated buffer solution). When cells are needed at extremely high purity, flow cytometry can be used (17, 18), although currently preferred methods employ immunomagnetic negative removal of contaminating cells because these methods enhance yields and minimize artifactual *in vitro* cell activation. For eosinophils, this involves labeling and removal of neutrophils using CD16 antibody, while for basophils, contaminating monocytes, T cells, B cells, and NK cells are removed using a cocktail of CD3, CD4, CD8, CD14, CD16, and/or CD19 antibodies (19, 20). Examples of useful immunomagnetic reagents cited frequently in publications for these purposes include

those available from Dynal, Inc. (Lake Success, NY) and Miltenyi Biotec (Sunnyvale, CA). In some situations, positive selection may be acceptable. This is especially true in circumstances where purified cells will be lysed (e.g., for mRNA extraction), and where possible cellular activation during the purification procedure will not adversely affect results. Examples of surface markers that could be targeted with antibodies to positively select basophils among mixed cell populations include IgE and Fc ϵ RI α . Similarly, CD9 or CD49d can be used to positively select eosinophils from a mixture containing neutrophils. A newly identified antibody to CCR3, the receptor for the eosinophil-specific eotaxin receptor, might also prove useful in this situation (21). While positive and negative selection methods are more laborious and expensive, they appear to be the best strategies for obtaining enough cells at high purity for further *in vitro* biochemical analysis.

One potential artifact that has received relatively little attention is that of platelet activation and contamination during the process of phlebotomy and cell isolation. Virtually all low-purity preparations of basophils contain adherent platelets, detected microscopically as well as by flow cytometry, where varying patterns of "expression" of platelet-specific markers, such as CD41, are easily observed. This is not seen in basophils that have undergone more

extensive purification, either by elutriation or by negative selection (see Fig. 1). While this appears to be less of a problem for eosinophils, caution must be exercised in experiments where conditions leading to platelet activation, with secondary basophil activation, might occur. Furthermore, detection of basophil "release" of "platelet-specific mediators" might mistakenly occur. This phenomenon may explain, in part, why in some circumstances differences in mediator release responses are seen in low-purity versus high-purity basophils.

Methods for the isolation of mast cells have provided additional challenges. Because they are tissue-resident cells, protocols had to be developed to generate cell suspensions from which mast cells could then be enriched. Methods are now available for the isolation of human mast cells from several tissues including lung (22), skin (23), gut (24), and uterus (25). The methods require initial mechanical disruption of the tissue (e.g., chopping with a pair of scissors) followed by incubation with proteases (e.g., collagenase). Once cell suspensions have been generated, further enrichment can be achieved using cell culture, density gradient centrifugation, and/or affinity purification steps to yield preparations of higher purity (26, 27). As with eosinophils and basophils, positive selection may be useful; in addition to markers shared with basophils (IgE or Fc ϵ RI α), antibodies to

TABLE 1

Methods for Isolation of Human Eosinophils and Basophils from Peripheral Blood

Method (reference)	Cells eliminated	Purity (%)	Notes
Eosinophils			
DGC (12)	P, L, M, B	1–30	Low purity but rapid
Discontinuous DGC (13)	P, L, M, B, RBC, N	80–100	High purity only with normodense cells
DGC plus immunomagnetic negative selection (61)	P, L, M, B, RBC, N	95–100	Currently preferred method for high purity
Basophils			
Dextran sedimentation (62)	P, RBC	<1	Rapid
Single-step DGC (16)	P, RBC, N, E	1–10	Modest purity, rapid
Discontinuous DGC (63)	P, RBC, N, E	2–20	Higher purity
Positive selection (64)	L, M, B, RBC, N	50–100	Requires passive sensitization with specific IgE and an affinity column
DGC plus immunomagnetic negative selection (19, 20)	L, M, B, RBC, N	50–100	Requires a mixture of mAb to remove cells
DGC plus elutriation (65)	L, M, B, RBC, N	50–100	Requires leukophoresis packs as starting material

Note. DGC, Density gradient centrifugation; P, platelets; L, lymphocytes; M, monocytes; B, basophils; RBC, red blood cells; N, neutrophils.

c-kit (the stem cell factor receptor, CD117) have been used to positively select mast cells among mixed cell populations (19).

CELL ADHESION ASSAYS

Virtually every step in the allergic cellular recruitment cascade depends upon the expression and function of cell adhesion molecules. These molecules are subdivided into families (integrins, immunoglobulin-like structures, selectins, and sialylated carbohydrate counterligands for selectins) based on shared structural characteristics and functions (reviewed in (28)). Leukocyte recruitment results from a complex process that involves the interaction of adhesion molecules on the leukocyte with molecular counterligands on vascular endothelial cells, extracellular matrix proteins, and other tissue structures (29). During this process, circulating leukocytes undergo tethering and rolling on the endothelial surface, events involving the formation of numerous weak,

reversible bonds, primarily between selectins and their carbohydrate-containing counterligands. This initial binding may be followed by the induction of firm adhesion mediated by leukocyte integrins interacting with endothelial adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (29). Leukocytes subsequently transigrate between the vascular endothelial cells into the tissue.

Until recently, relatively little was known about the mechanisms of eosinophil, basophil, and mast cell adhesion. Great strides have been made as a result of the development of *in vitro* assays that mimic conditions of cell adhesion under shear stress as well as under static conditions. These assays involve the quantitation of the interactions between these enriched cell types and specific target ligands. Adhesion is quantitated using several approaches, depending on the purity of the cells being used. In the simplest approach, microscopic counts of adherent cells are performed. Another frequently employed technique that can be used with highly pure cells is to label them with a fluorescent dye, such as carboxyfluorescein diacetate (CFDA, 20 μ g/ml; Molecular Probes, Eugene, OR) (30, 31), or a radioactive tracer, such as ^{51}Cr (e.g., 100 μ Ci sodium chromate per 2×10^6 cells) (32). If the cells have unique biochemical properties, this may permit the use of other types of assays as well as the use of lower purity cell preparations. For example, eosinophil adhesion can be quantitated in mixed cell populations by assaying for eosinophil peroxidase activity among adherent cells (33) and comparing this activity to a separate aliquot of cells, yielding an estimate of percent adhesion. A similar approach, using the histamine content liberated by lysing adherent cells with 1.6% perchloric acid, has been successfully employed to quantify basophil or mast cell adhesion (34, 35). These latter techniques are valid only under adhesion conditions that do not result in degranulation. If the stimulus being tested has secretagogue activity, calcium-free buffers containing magnesium or manganese can be substituted to provide a source of divalent cations for adhesion while at the same time preventing calcium-dependent histamine secretion (36, 37).

Many types of adhesion ligands can be studied. These can be matrix proteins (e.g., fibronectin, laminin) or recombinant adhesion molecules (e.g., ICAM-1, VCAM-1, E-selectin, P-selectin) immobilized on a plastic surface (e.g., by incubation of Nunc MaxiSorb

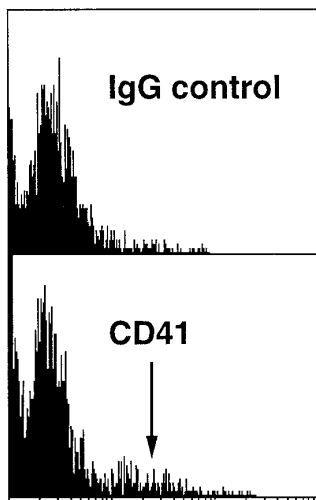


FIG. 1. Flow cytometric detection of adherent platelets on the surface of partially purified human basophils. Basophils were enriched by discontinuous Percoll density gradient centrifugation (63) and labeled using dual-color immunofluorescence for "expression" of several platelet-specific markers such as CD41 and CD42. A representative histogram is shown, illustrating that while most of the basophils are negative, variable levels of positive fluorescence is seen, suggesting the presence of variable numbers of adherent platelets. This can easily be confirmed by direct visualization using light microscopy.

immunoplates overnight at 4°C in PBS, followed by blocking with a 1% solution of heat-denatured bovine serum albumin (BSA) for 2–4 hours at 22°C (35, 38–42), or monolayers of cells stimulated or engineered to express specific adhesion molecules. Examples include assays testing adhesion to umbilical vein endothelial cells, respiratory epithelial cells, or transfected cell lines expressing specific adhesion molecules (32, 37, 42, 43). The specificity of adhesion must always be verified using specific antagonists, such as adhesion molecule-blocking antibodies. Because eosinophils, basophils, and mast cells express Fc γ receptors, such as CD32, blocking antibodies that attach to the ligand on the plate should be used as F(ab')₂ or Fab fragments to avoid providing inadvertent ligands for the added cells via Fc.

If adhesion via selectins and their carbohydrate ligands are being investigated, assays are often performed under conditions of flow to approximate shear forces that are felt to influence these interactions *in vivo*, although results with these selectin-dependent assays and eosinophils have yielded similar results under both static and nonstatic conditions (37, 41, 42, 44–47). In addition to shear forces, conditions that can be explored in these adhesion assays include effects of time, temperature (critical for integrin function, less so for selectin function), and divalent cations (critical for essentially all adhesive functions, requiring Ca²⁺ and/or Mg²⁺). Other conditions that greatly alter adhesion have been identified. For example, results of various studies indicate that the functional state of integrins can be regulated in addition to the number of integrin molecules on the sur-

face of a cell. Using these approaches it has been demonstrated that $\alpha 4\beta 1$ integrins on eosinophils and mast cells exist in a state of partial activation and can be maximally activated for adhesion to ligands such as fibronectin and VCAM-1 after exposure to Mn²⁺ or integrin-activating antibodies, conditions that do not change their total cell surface expression ((48, 49) and see Fig. 2). In contrast, several stimuli reduce eosinophil adhesion by inducing shedding of an important adhesion molecule, such as L-selectin (44), while others, such as IL-5 or inhibitors of tyrosine kinases (e.g., genistein, tyrphostin) downregulate function with little or no change in the total amount of integrin expressed (48–50). Another important aspect of these methods is that they provide functional assays for drug discovery by helping to screen for effects of adhesion molecule antagonists (51, 52).

FLOW CYTOMETRIC METHODS FOR IDENTIFYING EOSINOPHILS, BASOPHILS, AND MAST CELLS AND ANALYZING THEIR SURFACE MARKER EXPRESSION, VIABILITY, AND DEGRANULATION

Methods involving flow cytometry have become commonplace throughout cell biology. Not surprisingly, this technology has been used for the study of eosinophils, basophils, and mast cells. Protocols for their labeling and analysis have essentially been

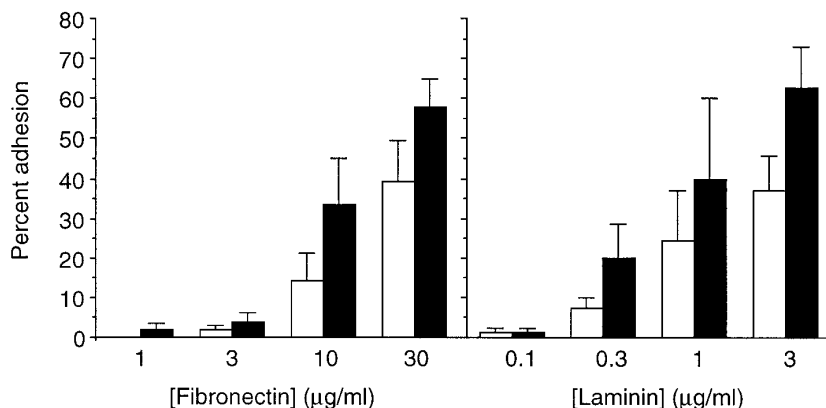


FIG. 2. Human skin mast cell adhesion to immobilized fibronectin (left, $n = 4$) or laminin (right, $n = 3$) is enhanced by the $\beta 1$ integrin activating antibody 8A2. Cells were preincubated with (black bars) or without (open bars) 1 μ g/ml 8A2, and adhesion was assessed as previously described (35, 49).

adapted from those for other cells (53–55). Indeed, analysis of highly pure cell suspensions is extremely easy, and even when these cells are expressed at lower purity, they are easily identified using parameters such as light scatter and dual color surface labeling, taking advantage of unique patterns of expression of various cell surface markers ((56) and see Table 2). Also, cell degranulation is associated with shape change, and, for basophils, these changes translate into detectable alterations in light scatter characteristics as well as actin polymerization, both of which can be quantified by flow cytometry (57, 58). Recent studies have also used acridine orange labeling to detect changes that appear to be degranulation (58). The use of appropriate gating strategies that exclude cells with markedly unusual light scatter patterns is also helpful in eliminating cellular fragments or aggregates from analysis. Because eosinophils and basophils express CD32 (FcγRII), many antibody labeling protocols incorporate human IgG (4 mg/ml) to reduce non-specific Fc region binding of mouse monoclonals (especially IgG₁ isotypes) to these IgG receptors. Using these methods, eosinophils and basophils have been readily identified and phenotyped in peripheral blood (54, 59) and after experimental allergic inflammation in the nose (10, 11) and lower airways (3, 12, 13).

Because of the availability of fluorescent probes that detect membrane permeability by binding to intracellular nucleic acids (e.g., propidium iodide, used at 2 μg/ml, available from Clontech, Calbiochem, R&D Systems, and others, or Via Probe [7-aminoactinomycin D, from Pharmingen], especially

useful for three-color work because of its fluorescence in the far red range beyond that of phycoerythrin), intracellular biochemical activity (e.g., CFDA, used at 20 μg/ml, from Molecular Probes), or cell surface expression of phosphatidylserine associated with apoptosis (e.g. using FITC-conjugated annexin V, used at 1 μg/ml, available from several sources including Clontech, R&D Systems, and Chemicon), flow cytometry has also been used to assess cell viability and its alteration by necrosis versus apoptosis. Because of the advantages of dual-color or even three-color labeling, the viability of a particular cell subpopulation in a mixed leukocyte sample can be readily assessed. Most of these methods have been successfully applied to the study of eosinophils and basophils. For example, from these types of analyses it has been determined that eosinophil survival can be prolonged by cytokines such as IL-3, IL-5, and GM-CSF, as well as by engagement of α4 integrins during adhesion (40). IL-3 has this same property for basophils, while stem cell factor prolongs mast cell differentiation and survival (9). In contrast, in the absence of these cytokines, cells rapidly undergo apoptosis, not necrosis; for eosinophils, this process can be accelerated by engagement of cell surface Fas antigen (CD95; (60)).

In conclusion, the generation of information regarding the mechanisms of selective recruitment, accumulation, and/or activation of human eosinophils, basophils, and mast cells has been facilitated by a wide variety of *in vitro* methods. Because of methods that can reliably identify and isolate these cells from a variety of sites, assays testing cell adhesion to a

TABLE 2
Distinguishing Characteristics Useful for Flow Cytometric Identification of Human Eosinophils, Basophils, or Mast Cells

Characteristic or surface marker	Eosinophils	Basophils	Mast cells
Light scatter	Similar to neutrophils	Between lymphocytes and monocytes	Like monocytes except higher 90° scatter
CD9	Bright; negative on neutrophils	Bright	Bright
CD16	Negative; bright on neutrophils	Negative	Negative
CD17	Negative; medium bright on neutrophils	Bright	Negative
CD49d	Medium bright, negative on neutrophils	Medium bright	Medium bright
CD117 (c-KIT)	Negative	Weakly positive	Bright
IgE	Negative	Positive ^a	Weakly positive
FcεRIα	Negative	Positive ^a	Weakly positive

^a Intensity of staining correlates with serum IgE levels and ranges from undetectable to brightly positive (53, 66).

wide variety of substrates can be performed under both shear and static conditions. Techniques involving flow cytometry, utilizing characteristics of cellular light scatter and immunofluorescence, have permitted the elucidation of cell surface phenotype and have aided in quantification of cellular degranulation, necrosis, and apoptosis. These approaches have yielded new data on the function of human eosinophils, basophils, and mast cells and have suggested unique cell-specific pathways of cell recruitment, activation, and survival that may contribute to the pathogenesis of allergic diseases.

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