Eosinophil Autofluorescence and its Use in Isolation and Analysis of Human Eosinophils Using Flow Microfluorometry

By Gary J. Weil and Thomas M. Chused

Unstained human eosinophils exhibit unusually bright autofluorescence, which allows them to be distinguished from other leukocytes using fluorescence microscopy. Eosinophil fluorescence is associated with the cytoplasmic granules of the cells. Eosinophil granule extracts, containing an as-yet-undefined eosinophil fluorescence factor, exhibited excitation maxima at 370 nm and 450 nm, with maximum emission at 520 nm. Eosinophils adhering to opsonized parasites in vitro deposit fluorescent material onto the parasite surface. Eosinophil fluorescence was of sufficient intensity to allow the preparation of viable, highly enriched (≥98%) eosinophil suspensions from peripheral blood of normal and eosinophilic donors using a fluorescence-activated cell sorter. Quantitative studies of eosinophil autofluorescence were performed using flow microfluorometry. Fluorescence intensity of blood eosinophils from normal volunteers and eosinophilic patients varied inversely with the log of the donor’s absolute eosinophil count regardless of clinical diagnosis.

Although Paul Ehrlich recognized the clinical significance of blood eosinophilia in the late 19th century, eosinophil function remained a mystery for many years. Interest in these cells has been rekindled by recent studies suggesting important functional roles for the eosinophil in controlling parasitic helminth infections and modulating immediate hypersensitivity reactions.

It has been known for some time that unstained human eosinophils exhibit unusually bright autofluorescence, which allows them to be distinguished from other leukocytes using fluorescence microscopy. In this article, we have defined morphological and spectral characteristics of eosinophil fluorescence. In addition, we have exploited this property of the cells in two ways. First, we used a fluorescence-activated cell sorter (FACS) to separate eosinophils from granulocyte suspensions. This new technique allows the routine preparation of eosinophil suspensions with an unprecedented degree of purity from the blood of both normal and eosinophilic donors. Second, in an analytical application, quantitative studies of eosinophil fluorescence were performed using flow microfluorometry. Cells from normal volunteers and eosinophilic patients were studied in order to define the range of variability of this new parameter. Our studies show that eosinophils from patients with eosinophilia exhibit significantly less fluorescence than eosinophils from normal volunteers.

**Materials and Methods**

**Granulocyte Preparations**

Blood was obtained from normal volunteers and selected patients in the Clinical Center, National Institutes of Health, Bethesda, Md., with eosinophilia associated with helminth infection, clinical allergy, or the idiopathic hyper eosinophilia syndrome. Heparinized venous blood was layered over 5 ml of lymphocyte separation medium (sodium diatrizoate and Ficoll, Litton Bionetics, Kensington, Md.) and centrifuged for 15 min at 800 g (25°C). Mononuclear cells were discarded and buffy coat cells were sedimented in 3% dextran (Sigma Chemical Co., St. Louis, Mo.) in Hanks’ balanced salt solution (HBSS; GIBCO, Grand Island, N. Y.) with 0.5% bovine serum albumin (BSA; Sigma) and 3 mM ethylenediaminetetraacetic acid (EDTA; Sigma) at 37°C for 30 min. After one wash with HBSS-BSA-EDTA, erythrocytes were removed by hypotonic lysis. The cells were washed twice in HBSS-BSA-EDTA, adjusted to 5 x 10⁶/ml, and held at 5°C for later cytometric analysis and sorting. Cell viability was assessed by exclusion of 0.1% trypan blue (GIBCO).

**Eosinophil Granule Extracts**

Granules were isolated with a modification of the method of Chodirker et al. Granulocytes from patients with idiopathic hyper eosinophilia containing greater than 90% eosinophils were repeatedly pipetted in 0.2 M sucrose with sodium heparin (Fisher Scientific Co., Fairlawn, N.J.) 250 U/ml. The viscous cell lysate was liquefied by incubation with deoxyribonuclease I ( Worthington Biochemical Corp., Freehold, N. J.) 50 μg/ml at 37°C for 20 min. The suspension was centrifuged at 800 g for 20 min to remove intact cells and nuclei. The supernatant was centrifuged at 50,000 g for 15 min. The sediment, primarily cytoplasmic granules by phase and fluorescence microscopy, was washed once in phosphate-buffered saline, pH 7.4. The granules were sonicated with four 15-sec bursts with an ultrasonic cell disrupter (Electro Mechanical Instrument Co., Perkasie, Pa.) and frozen (-40°C) and thawed 3 times. The suspension was then centrifuged at 15,000 g for 15 min. Supernatants of this centrifugation were used as granule extracts. Considerable fluorescence remained in the granule sediment after this mild extraction procedure.

**Spectrofluorometry**

Excitation and emission spectra of eosinophil extracts were determined with an Amino-Bowman spectrofluorometer (American Instrument Co., Silver Spring, Md.).

**Cytometric Analysis and Cell Sorting**

Narrow-angle forward light scatter (2°-15°) and fluorescence were measured, and cells were sorted with a FACS-II (Becton, From the Laboratory of Parasitic Diseases and Laboratory of Microbial Immunity, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

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Dickenson FACS Systems, Sunnydale, Calif.). Fluorescence was excited with an argon ion laser producing 40 mW at 457 nm.

Mean eosinophil fluorescence was defined as the mean of the second peak of the fluorescence histogram (see Fig. 4A). Fluorescence of eosinophils from different donors was expressed as relative eosinophil fluorescence: the ratio of the mean eosinophil fluorescence of donor cells to that of eosinophils from a single individual whose cells were included in every experiment. Relative eosinophil fluorescence was reproducible, varying less than 5% in 4 individuals studied on 2 separate occasions. Data were analyzed with a PDP 11/34 computer (Digital Equipment Corporation, Maynard, Mass.).

**Volume Distributions**

Volume distributions of sorted cells were determined with a Coulter Counter (Coulter Electronics, Hialeah, Fla.).

**Fluorescence Microscopy**

A Leitz Ortholux II microscope was used with mercury vapor lamp epiillumination with a KP500 excitation filter and K510 and BG38 suppression filters.

**Cell Differentials**

Cells air-dried onto glass slides were stained with 1% fast green (Harelco, Gibbstown, N.J.) in methanol for 1 min followed by 1% neutral red (Harelco) in distilled water for 5 min. Two-hundred cells were counted at a magnification of x400. Absolute eosinophil counts were performed on whole blood (Unopette, Becton Dickenson, Rutherford, N.J.).

**Adherence Assays**

Simple opsonic adherence assays were employed to assess the function of sorted eosinophils and to attempt to demonstrate eosinophil deposition of fluorescent material onto opsonized targets in vitro. Complement-dependent adherence of eosinophils onto Sepharose 4B, described by Metcalfe et al.,7 was adapted as follows: 0.1 ml of sorted eosinophils (10⁵/ml in HBSS) were added to 0.1 ml of 0.5% washed Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) in HBSS and 0.05 ml fresh normal human serum or HBSS in plastic tubes. Beads were examined with phase and fluorescence microscopy after a 30-min incubation at 37°C, and cell adherence and bead surface fluorescence were assessed. A minimum of 20 beads were examined, and a positive adherence reaction was defined as ≥3 cells adherent on the bead surface. A second adherence assay, antibody-dependent adherence of leukocytes to microfilariae first described by Pandit et al.,8 was performed as follows: microfilariae of the human pathogen Brugia malayi were isolated from jird (Meriones unguiculatus) peritoneal cavities as previously described. Microfilariae (0.1 ml of a suspension of 10⁶/ml in HBSS), eosinophils, or neutrophils (0.1 ml of a suspension of 3 x 10⁶/ml), and 0.05 ml immune serum were combined in microcentrifuge tubes (Brinkman Instruments, Westburg, N.Y.) and incubated at 37°C for 60 min. Microfilariae with adherent granulocytes were examined with phase and fluorescence microscopy. Immune sera used for these experiments were collected from elephantiasis patients in the Cook Islands (kindly supplied by Dr. Eric A. Ottesen) and contain IgG directed against surface antigens of B. malayi microfilariae (Weil G: unpublished observations).

**RESULTS**

**Microscopic Studies**

Fluorescence microscopy demonstrated that eosinophil autofluorescence was confined to the specific cytoplasmic granules of the cells (Fig. 1). Eosinophils are known to degranulate in association with complement-dependent adherence to Sepharose 4B. However, no fluorescent material was observed on the surface of Sepharose beads with adherent eosinophils when the beads were examined with fluorescence microscopy. Therefore, a second adherence system was employed using parasite targets opsonized with human immune serum. Fluorescence microscopic examination of microfilariae with adherent eosinophils revealed that the parasites were coated with fluorescent material not observed when microfilariae were incubated with immune serum alone (Fig. 2) or immune serum plus neutrophils (not shown in figure).

**Spectral Properties**

The absorbance and emission spectra of eosinophil granule extracts are shown in Fig. 3. Excitation peaks at 370 nm and 450 nm and a single emission peak at 520 nm were observed. Identical patterns were observed using extracts derived from three different donors.
Cytometric Analysis and Cell Sorting

Cytometric analysis of unstained granulocyte suspensions consistently revealed the presence of two cell populations (Fig. 4). One population, shown by sorting to be eosinophils, exhibited greater fluorescence and less narrow-angle forward light scattering than the other, which was comprised of neutrophils. The absolute intensity of eosinophil fluorescence was modest, equivalent to the fluorescence of 30,000–50,000 molecules of fluorescein per cell under optimal conditions. Fluorescence intensity, expressed as relative eosinophil fluorescence, varied inversely with the log of the absolute eosinophil count of the donor ($r = -0.68, p < 0.01$), i.e., eosinophils from noneosinophilic donors tended to fluoresce with the greatest intensity (Fig. 5).

Fluorescence and light scatter were used to separate eosinophils from neutrophils using a cell sorter. Sorting windows were selected to exclude cells in the region of overlap in the fluorescence distributions of neutrophils and eosinophils. The size of the excluded eosinophil fraction (25% ± 2.7%, range 16%–41%) varied with the brightness of the eosinophils being...
Fig. 4. Flow microfluorometric analysis of unstained human granulocytes. Granulocyte preparation contained 9% eosinophils, 91% neutrophils. (A) Fluorescence histogram. (B) Contour plot of light scatter versus fluorescence.

Fig. 5. Relative fluorescence of eosinophils from normal and eosinophilic subjects. Linear regression of eosinophil fluorescence versus log_{10} absolute eosinophil count \( n = 31; r = -0.68; p < 0.01 \).

Fig. 6. Flow microfluorometric analysis of unstained human granulocytes. Granulocyte preparation contained 9% eosinophils, 91% neutrophils. (A) Fluorescence histogram. (B) Contour plot of light scatter versus fluorescence.

sorted. Due to coincidence losses, the FACS is able to sort 85% of input cells. Thus, the average net yield of the sorting procedure was about 65% of input eosinophils. Results of representative sorting experiments are shown in Table 1. Generally, when eosinophils comprised >5% of input cells, the sorted fluorescent ("bright") cells were almost entirely eosinophils (≥98%), and the nonfluorescent ("dull") cells were almost entirely neutrophils. When the eosinophil fraction of input cells was low, an initial high-speed (20,000 cells/sec) enrichment run followed by a low-speed, high-resolution run (4000 cells/sec) yielded eosinophils with purity approaching 100%. Sorted eosinophils were morphologically normal by light and electron microscopy, and cell viability was >95%. In addition, as mentioned above, sorted eosinophils performed well in a simple assay of opsonic adherence. After cells and Sepharose beads were incubated in the presence of fresh human serum, >90% of the beads had three or more adherent eosinophils. No adherence was observed when serum was omitted from the reaction.

Coulter volume determinations were performed on purified eosinophils and neutrophils to investigate the meaning of the observed difference in narrow-angle forward light scatter. The distributions of light scatter and Coulter volume of sorted neutrophils and eosinophils from the same donor are shown in Fig. 6. Although eosinophils scatter less light than neutrophils, the volume distributions of the two cell populations were essentially superimposable, suggesting that the difference in light scatter is due to differences in refractive index rather than size.

Table 1. FACS Separation of Eosinophils: Percentage Eosinophils* Input and Sorted Cells

<table>
<thead>
<tr>
<th>Input Cells</th>
<th>Sorted Cells†</th>
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<tr>
<td>38</td>
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<td>2</td>
<td>93</td>
<td>0</td>
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*Based on 200 cell differentials.
†Single sorting run, 4000 cells/sec.
Fig. 6. (A) Light scatter distributions of highly enriched (>99%) eosinophils (solid line) and neutrophils (dashed line) sorted from the granulocyte preparation described in Fig. 4 (gated to exclude dead cells and erythrocytes). (B) Coulter volume distributions of the same sorted eosinophils and neutrophils.

DISCUSSION

We have defined morphological and spectral characteristics of eosinophil autofluorescence and have used this information to develop new techniques for the isolation and analysis of these cells. The compound responsible for eosinophil autofluorescence is at present unknown. Since eosinophil secondary granules contain a major basic protein, which comprises 50% of total granule protein content, as well as large amounts of eosinophil peroxidase, these compounds were prime candidates. However, preliminary results indicate that fluorescence activity does not coelute with any of the major protein peaks when granule extracts are subjected to gel filtration chromatography. Pending further identification of the fluorescent compound, we shall refer to it as eosinophil fluorescence factor. A possible function of this factor is suggested by the observation that it is deposited onto opsonized, nonphagocytosable parasite surfaces (microfilariae) in association with eosinophil adherence and degranulation. Eosinophil peroxidase and major basic protein are also deposited on parasite surfaces in association with eosinophil adherence, and the cytotoxic properties of eosinophils have been ascribed to these compounds. 

Eosinophil fluorescence factor may contribute to the cytotoxic potential of eosinophils, but we have not yet tested this hypothesis. Interestingly, surface fluorescence was not observed when eosinophils adhered to opsonized Sepharose 4B. Adhesion of eosinophil fluorescence factor to a target may depend on the chemical nature of the target. However, other variables, such as the nature of the opsonin or the form of cell degranulation may also affect adhesion.

The isolation of normal eosinophils has been a technical obstacle in the past, and for this reason, most studies of eosinophil structure and function have used cells from eosinophilic donors. FACS separation of eosinophils is superior to any previously reported technique in the purity of the eosinophil suspensions obtained. This method necessarily excludes cells that are weakly fluorescent, but it avoids selection problems associated with other methods of separation. For example, although differences in cell density allow eosinophil enrichment using density gradient centrifugation, the density distributions of eosinophils and neutrophils overlap so that only the most dense eosinophils can be separated with any purity. Other techniques enrich for eosinophils by negative selection on the basis of their relative lack of membrane opsonic receptors but systematically exclude the important subset of eosinophils that is receptor positive. While the functional capacity of FACS-sorted eosinophils has not been fully evaluated, the cells have normal morphology, exclude trypan blue, and function in a simple assay of opsonic adherence.

Quantitative studies of eosinophil fluorescence using flow microfluorometry were performed to define the range of variability of this new parameter and to determine whether this variability could be related to clinical syndromes associated with eosinophilia. Eosinophil fluorescence varied greatly among the individuals studied and varied inversely with the log of the absolute eosinophil count of the donor. This interesting relationship was independent of the underlying disease causing eosinophilia and probably reflects variability in fluorescence factor content per cell. Systematic variation in the number of granules per cell, fluorescence content per granule, or the concentration of fluorescence-quenching substances in the cells could account for this finding. Eosinophils from patients
with Loeffler's cardiomyopathy have been reported to contain decreased numbers of secondary granules, and a recent study found decreased amounts of the granule-associated protein, arylsulfatase B, in eosinophils of patients with filariasis during the period of increased eosinophilia observed after drug therapy. The issue of whether circulating eosinophils in these clinical settings are immature (granules not fully developed) or “spent” (granules discharged) is at present unresolved.

Recent studies have shown that eosinophils exhibit heterogeneity in a number of other ways that may relate to cell maturation or activation. For example, eosinophils from eosinophilic patients have increased membrane receptors for the opsonins IgG-Fc and C3b and have increased antiparasite cytotoxic activity in vitro. However, difficulties with existing methods of cell separation and analysis have impeded progress in the study of eosinophil heterogeneity, an issue that may be important in understanding the function of these enigmatic cells. Flow microfluorometric separation and analysis of eosinophils should significantly facilitate future studies of eosinophil heterogeneity and function.

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