Rapid Method for Isolation of Normal Human Peripheral Blood Eosinophils on Discontinuous Percoll Gradients and Comparison With Neutrophils

By Robert L. Roberts and John I. Gallin

Previous studies on human eosinophils often have used cells from patients with hypereosinophilia syndrome or parasitosis owing to the difficulty in isolating pure populations of eosinophils from normal individuals. In the present study, human eosinophils were isolated with a purity of 97%, with 70% recovery from normal individuals with blood eosinophil counts of less than 3%. Human eosinophils are denser than neutrophils, but the range of densities of the two cell types overlap, making purification of eosinophils by density-gradient centrifugation difficult. However, if neutrophils were exposed to the chemotactic peptide (f-Met-Leu-Phe), which did not stimulate eosinophils, the neutrophils’ density decreased, shifting them away from the density of eosinophils. Whole normal blood anticoagulated with EDTA was incubated at 37 °C for 15 minutes with 10⁸ mol/L f-Met-Leu-Phe and then layered over a discontinuous Percoll gradient (65% and 75% in diluted phosphate-buffered saline) and centrifuged at 400 g for 25 minutes at 22 °C. The cell layer between the 65% and 75% Percoll was collected and washed, and hypotonic lysis was used to remove erythrocytes. This cell layer contained 97.3 ± 0.7% eosinophils (N = 8) with a yield of 4.9 × 10⁹ eosinophils per milliliter of whole blood, or 70% of the total eosinophil count. The isolated eosinophils were in a quiescent state but responded to Escherichia coli endotoxin-activated serum with shape change and chemotaxis, membrane depolarization, and reduced nitroblue tetrazolium (96.0 ± 1.0%), when stimulated with phorbol myristate acetate. In phagocytic assays, 89.3 ± 1.3% of the eosinophils ingested Candida albicans v 96.0% ± 1.0% of neutrophils. In contrast, the eosinophils did not respond chemotactically, alter membrane potential, or reduce nitroblue tetrazolium when treated with f-Met-Leu-Phe, and studies with f-Met-Leu-[³H]Phe showed that normal eosinophils lacked expression of receptors for f-Met-Leu-Phe. In control studies, normal eosinophils that were not exposed to f-Met-Leu-Phe during purification also failed to respond to f-Met-Leu-Phe, indicating intrinsic differences between normal eosinophils and neutrophils. Thus, exposure of whole blood to f-Met-Leu-Phe, followed by separation on Percoll is a simple method for rapid isolation of normal human eosinophils.

REVIOUS STUDIES on human eosinophils often have used cells obtained from patients with parasitosis or the hypereosinophilia syndrome because of the difficulty in getting pure populations of eosinophils from normal individuals. Most methods for isolating eosinophils have used differential adherence properties of eosinophils and neutrophils, or centrifugation through density-gradient materials such as metrizamide or the polyvinylpyrrolidone-coated silica gel, Percoll. The separation on density gradients takes advantage of the greater average density of normal eosinophils compared with other blood leukocytes, which allows for eosinophil enrichment in the heavier layers of the gradient. However, the range of densities for eosinophils and neutrophils overlaps, making the separation of a pure population of eosinophils difficult without greatly reducing the yield.

Pember et al. reported that exposure of human neutrophils to the chemoattractant peptide N-formyl-methionylleucylphenylalanine (f-Met-Leu-Phe) decreased their density, as demonstrated by centrifugation through a continuous gradient of Percoll. In the present study, exposure of anticoagulated (EDTA) whole blood to f-Met-Leu-Phe was also found to decrease the density of the neutrophils when the blood components were later isolated by spinning through a discontinuous Percoll gradient. The eosinophils, which were not activated during purification, remained in a denser layer that was depleted of neutrophils.

Using this method of exposure of whole blood to f-Met-Leu-Phe, followed by a single centrifugation through a discontinuous Percoll gradient, we were able to use blood from normal individuals having a blood eosinophil count of less than 3% to isolate populations of eosinophils with a purity of 97% and a yield of 70%. Similar results were obtained from patients with mild to moderate eosinophilia. The eosinophils isolated were in an unstimulated state, but on stimulation, they ingested yeast, moved toward a chemoattractant, and reduced nitroblue tetrazolium (NBT). This method provides a simple means for rapidly isolating pure populations of eosinophils from peripheral blood.

MATERIALS AND METHODS

The following materials were obtained: polyvinylpyrrolidone-treated silica (Percoll; Pharmacia Fine Chemicals, Piscataway, NJ); 3-μm cellulose nitrate filters (Neuroprobe, Potomac, Md); Hanks’ balanced salts solution (HBSS), pH 7.2, with or without calcium and magnesium.

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magnesium; 0.85% phosphate-buffered saline (PBS; Whittaker Corp., Walkersville, Md); Gey's tissue culture medium, pH 7.2, containing 2% bovine serum albumin, penicillin, and streptomycin (Microbiological Associates, Bethesda, Md); cytochalasin B (Aldrich Chemical Co, Inc, Milwaukee); the peptide f-Met-Leu-Phe (Peninsula Labs, Inc, San Carlos, Calif); the croton oil derivative phorbol myristate acetate (PMA; Consolidated Midland Corp., Brewster, NY); 50% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa); NBT (Sigma Chemical Co, St Louis); and Escherichia coli 0123:B endotoxin (lipopolysaccharide B; Difco Laboratories, Detroit); di-O-C12(3) was obtained from Allan Waggoner, Amherst College, Amherst, Mass. Candida albicans used for the phagocytosis assay were obtained from Dr John Bennett of the National Institute of Allergy and Infectious Diseases.

All water-insoluble compounds were dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific Co, Fairlawn, NJ) to make appropriate stock solutions such that the final concentration of DMSO in the reaction mixtures was not greater than 0.1% (vol/vol). This concentration affects neither the assays performed nor the viability of cells as measured by trypan blue exclusion or lactic dehydrogenase release.

Separation of Neutrophils and Eosinophils

Peripheral blood obtained from normal volunteers by venipuncture was anticoagulated with EDTA (0.3% final concentration). The blood was then transferred to 50-ml tubes (15 to 20 ml of blood per tube), and f-Met-Leu-Phe (10^{-6} to 10^{-7} mol/L) was added to those tubes that would be used for isolating eosinophils (Fig 1). The final concentration of DMSO (the solvent for f-Met-Leu-Phe) was 0.01%, which does not affect the subsequent separation of cells in the absence of f-Met-Leu-Phe. Concentrations of DMSO of 0.1% or higher were noted to cause hemolysis of erythrocytes when incubated at 37 °C, although it did not affect the separation of cells. The tubes of blood with and without f-Met-Leu-Phe were then incubated at 37 °C in a rocking water bath for 15 minutes.

Discontinuous Percoll gradients were prepared with modifications of recently described techniques.7 Percoll solution (Pharmacia) with a density of 1.130 (referred to as 100%) was diluted with concentrated (ten times) PBS to obtain an osmolarity of 310 to 320 mosm/kg and then further diluted with regular PBS to obtain concentrations of 65% and 75%. A 5-mL vol of the 65% solution was placed in a 15-mL conical tube (Falcon 2095, Oxnard, Calif), and then 5 mL of the 75% Percoll was layered below the 65% layer by means of a narrow catheter placed at the bottom of the tube. Whole blood (5 mL) that had been incubated at 37 °C was then layered over the Percoll gradient. The tubes were then spun at 1,500 rpm (400 g) at 22 °C for 25 minutes, resulting in the formation of one band of leukocytes above the 65% layer (band I) and another between the 65% and 75% layers (band II). When the blood had not been exposed to f-Met-Leu-Phe, band II was predominately neutrophils (96% to 99%), with the remainder of cells being eosinophils (1% to 4%) or lymphocytes (0% to 1%). When blood exposed to f-Met-Leu-Phe was used, band II was highly enriched in eosinophils, with neutrophils shifting to band I (see Results for further details). The erythrocytes pelleted to the bottom of the tube, but hypotonic lysis was required to remove residual erythrocytes from band II. The cells were washed twice in PBS, and Wright's stain was used to differentiate cell types.

Phase Microscopy

After isolation, the cells were resuspended in the particular buffer as indicated by the experimental condition. Test reagents were then added, and the tubes were placed in a 37 °C rocking water bath for the time indicated in the particular experiment. Immediately after the tubes were removed from the bath, glutaraldehyde was added (0.5% final concentration) to each, and the cells were allowed to fix for at least ten minutes at room temperature.8 The cells were examined by phase microscopy (Carl Zeiss, Inc, New York) with a 100 x phase objective and photographed.

Assays of Cell Function

Neutrophil and eosinophil locomotion was assessed using the morphologic assay described by Zigmund and Hirsch,9 but with modified Boyden chambers. A Zeiss photomicroscope 2 and an image analyzer (Optomax CPU-2, Micromerasurement, Cambridge, England) interfaced with a Hewlett-Packard 9815 calculator and 7225A plotter were used to automate the reading of filters. The number of cells was determined in 1.16 x 10^{3}μm^2 cross-sections taken at 10-μm intervals. For each experimental condition, two "cores" were measured throughout the depth of the individual filters in each of four replicate filters. The duplicate readings for each filter were averaged, and the variability (SE) among the four replicate filters was determined. The average distance migrated by the population of cells was used as the unit of measurement. The chemoattractants used were f-Met-Leu-Phe and endotoxin-activated serum (5% vol). Spontaneous, nondirected migration was assessed with buffer alone as the stimulus. Bovine serum albumin (final concentration 2%) was added to all buffers used in suspending cells or diluting chemoattractants.

Specific binding and the number of f-Met-Leu-[^3H]Phe receptors on eosinophils were determined as described previously.10 Isolated neutrophils and eosinophils were incubated with 10^{-6} mol/L f-Met-Leu-[^3H]Phe alone, or with a 1,000-fold excess of nonradioactive f-Met-Leu-Phe to determine nonspecific binding. In a separate experiment, a granulocyte preparation (isolated on layers of 65% and 75% Percoll without f-Met-Leu-Phe) was exposed to 10^{-7} mol/L f-Met-Leu-[^3H]Phe for 15 minutes at 37 °C and then layered over the 65% Percoll. After centrifugation, the activity of the cells in the band above the 65% layer and the pellet was measured.

A modification of the technique described by Patterson-Delafield and Lehrer18 was used to assay for phagocytosis of heat-killed C albicans that had been opsonized with fresh serum. The cells and Candida were then centrifuged at 1,500 rpm for five minutes at room temperature, and the undisturbed pellet was allowed to incubate at 37 °C for 15 minutes. A drop of trypan blue dye was added to each tube, and the cells were then examined by phase microscopy. Heat-killed Candida ingested by cells do not take up trypan blue, whereas extracellular Candida stain blue; this differential staining was the criterion for determining whether or not the yeast had been

Fig 1. Separation of normal human eosinophils from whole blood by centrifugation on discontinuous Percoll gradients.

The cells were collected by aspiration, washed and resuspended in modified balanced salt solution (MBSS) with 2% bovine serum albumin and 10% fresh serum. The cells and MBSS were then centrifuged at 1,500 rpm for five minutes at room temperature, and the undisturbed pellet was allowed to incubate at 37 °C for 15 minutes. A drop of trypan blue dye was added to each tube, and the cells were then examined by phase microscopy. Heat-killed Candida ingested by cells do not take up trypan blue, whereas extracellular Candida stain blue; this differential staining was the criterion for determining whether or not the yeast had been...

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ingested. At least 100 cells were examined for each experimental condition. Cells with candidae were also examined by Wright’s staining.

NBT reduction was performed using 0.1% solution in HBSS as previously described.11 Specimens were counterstained with safranine, and the percentage of NBT-positive cells was noted.

The membrane potential of eosinophils and neutrophils was determined using the indirect probe di-0-C₃(3) as previously described.12

Statistical Analysis

Except where indicated, Student’s t test (two-tailed) was used to compare the means of different experiments.

RESULTS

Eosinophil Recovery

Exposure of whole blood to 10⁶ to 10⁷ mol/L f-Met-Leu-Phe altered the density of almost the entire neutrophil population, resulting in a band of cells over the 75% Percoll (Fig 1, band II) that was greatly enriched for eosinophils. When concentrations of 10⁷ mol/L f-Met-Leu-Phe or greater were used, more than 95% of the cells in band II were eosinophils (Table 1), and the majority of neutrophils had then shifted to band I (over 65% Percoll) with the mononuclear cells. After 10⁶ mol/L f-Met-Leu-Phe treatment, the cell counts done on band II showed that 4.9 ± 0.7 x 10⁶ eosinophils were recovered per milliliter of whole blood separated. This was approximately 70% of the total eosinophil count based on leukocyte differentials of the donors tested. The remainder of the eosinophils were in the lower portion of the 65% Percoll layer, which also contained a small number of neutrophils. We were able to obtain similar eosinophil recoveries by using 50-mL conical tubes (Falcon 2070, Becton Dickinson, Oxnard, Calif) for separation and by increasing the volumes of the two Percoll layers and the blood layer to 15 ml per layer. When whole blood was stimulated with E coli endotoxin-activated serum (10% vol/vol), PMA (20 ng/mL), or calcium ionophore A23187 (10⁶ mol/L), both eosinophils and neutrophils decreased their density in approximately proportional numbers.

Wright’s-stained eosinophils recovered from band II were round or ovoid and appeared to have retained their granules. Eosinophils that were fixed with 0.5% glutaraldehyde after separation and examined by phase microscopy also appeared round in more than 90% of the cells examined, with no obvious morphologic evidence of activation (Fig 2A). After f-Met-Leu-Phe treatment, the neutrophils in band I were irregular in shape and were sometimes noted to have a shift of the nucleus to one end of the cell. Approximately 50% of the f-Met-Leu-Phe–exposed neutrophils in band I showed pseudopod formation when examined by phase microscopy after fixation. Aggregates of neutrophils, monocytes, and platelets also

Table 1. Normal Eosinophil Enrichment From Whole Blood by f-Met-Leu-Phe Treatment

<table>
<thead>
<tr>
<th>Concentration (mol/L)</th>
<th>No.</th>
<th>Eosinophils in Band II (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻⁶</td>
<td>5</td>
<td>20.8 ± 13.1% (4-73)</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>4</td>
<td>67.5 ± 7.0% (52-83)</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>5</td>
<td>96.2 ± 1.4% (92-99)</td>
</tr>
<tr>
<td>5 x 10⁻⁷</td>
<td>11</td>
<td>95.7 ± 1.0% (90-99)</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>8</td>
<td>97.3 ± 0.7% (93-100)</td>
</tr>
</tbody>
</table>

*Percentage of eosinophils in band II of Percoll gradient after treatment of whole blood with concentration of f-Met-Leu-Phe indicated in first column. The individuals studied had less than 3% eosinophils in their peripheral blood and total WBC counts of 5,000 to 10,000. Data are the mean ± SEM of the indicated number of experiments.

Fig 2. Phase contrast microscopy of eosinophils (A) and control neutrophils (C) fixed with 0.5% glutaraldehyde separation as described in the text. Other panels show eosinophils (B) and control neutrophils (D) that were separated, incubated with endotoxin-activated serum for 15 minutes at 37 °C, and then fixed. Cells were stained in suspension with Wright’s stain after fixation.
were often present in band I after f-Met-Leu-Phe exposure.

This method was also effective in obtaining purified populations of eosinophils from patients with mild to moderate eosinophilia with recovery rates for eosinophils of 54% to 72% (Table 2). Whole blood was diluted with PBS (50%) for patients with elevated eosinophil counts to prevent overloading the Percoll gradient, which makes recovery more difficult. In some studies, this method was used for patients with the hypereosinophilia syndrome, but recovery was much less than in normals owing to a large percentage of eosinophils from these patients that have a density less than neutrophils.

In attempts to isolate large numbers of eosinophils using fewer Percoll gradients, purified granulocytes (neutrophils and eosinophils at 10 x 10^6/mL) that had been prepared by centrifugation on Ficoll-Hypaque and dextran sedimentation were exposed to 10^-6 mol/L f-Met-Leu-Phe at 37 °C and then centrifuged over the 65% and 75% Percoll gradients. As with whole blood, the Ficoll-Hypaque neutrophils shifted to band I after f-Met-Leu-Phe exposure but were prone to aggregate, and visible clumps of cells sometimes could be seen in band I after centrifugation. When large aggregates did occur, few cells migrated to band II, and microscopic examination revealed the presence of eosinophils in these neutrophil aggregates. Thus, because of the aggregation of neutrophils and the subsequent trapping of eosinophils in these aggregates, separation of eosinophils from granulocytes purified on Ficoll-Hypaque was less reliable.

Comparison of Normal Eosinophils and Neutrophils

The following series of experiments were performed to evaluate the function of the purified normal eosinophils in comparison with neutrophils. These experiments show that normal eosinophils do not express receptors for f-Met-Leu-Phe and exclude the possibility that eosinophils are deactivated by exposure to f-Met-Leu-Phe during purification, as has been reported for neutrophils.

Chemotaxis. (Table 3.) Normal eosinophils and neutrophils were able to migrate in response to endotoxin-activated serum. Both eosinophils and neutrophils developed pseudopods and formed aggregates in the presence of endotoxin-activated serum when examined by phase microscopy after glutaraldehyde fixation (Fig 2B and D). However, while f-Met-Leu-Phe did not stimulate eosinophil chemotaxis, it did induce neutrophil chemotaxis (Table 3).

Two studies were performed to test the possibility that eosinophil chemotactic responsiveness to f-Met-Leu-Phe was selectively deactivated during purification. First, eosinophils and neutrophils from a patient with the hypereosinophilia syndrome were isolated to 94% pure eosinophils by discontinuous Percoll gradients sedimenting above a 65% Percoll layer and mononuclear cells sedimenting above a 54% layer without exposure to f-Met-Leu-Phe. The purified eosinophils were then tested for chemotactic responsiveness (Fig 3). Like normal eosinophils, patient eosinophils failed to respond to f-Met-Leu-Phe but responded to activated serum. In contrast, neutrophils from this patient responded to both stimuli (Fig 3).

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**Table 2. Eosinophil Purification in Eosinophilic Patients**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Peripheral Blood</th>
<th>After Isolation*</th>
<th>Recovery†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (11)</td>
<td>1.6 ± 0.3</td>
<td>95.7 ± 1.0</td>
<td>70</td>
</tr>
<tr>
<td>Chronic granulomatous</td>
<td>4.2 ± 0.3</td>
<td>99.2 ± 0.3</td>
<td>72</td>
</tr>
<tr>
<td>disease (6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperimmunoglobulin E</td>
<td>5.5 ± 1.5</td>
<td>98.5 ± 1.5</td>
<td>54</td>
</tr>
<tr>
<td>syndrome (2)</td>
<td>40.0 ± 2.0</td>
<td>100.0 ± 0.0</td>
<td>67</td>
</tr>
</tbody>
</table>

*Percentage of eosinophils in band II of Percoll gradient after treatment of whole blood with 5 x 10^-7 mol/L f-Met-Leu-Phe.
†Numbers in parentheses indicate number of subjects.
††Percentage of eosinophils recovered from whole blood.

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**Table 3. Normal Eosinophil and Neutrophil Chemotaxis**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Average Distance Migrated (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eosinophils</td>
</tr>
<tr>
<td>Buffer</td>
<td>17.5 ± 1.0</td>
</tr>
<tr>
<td>Activated serum</td>
<td>34.6 ± 0.9†</td>
</tr>
<tr>
<td>f-Met-Leu-Phe (2 x 10^-6 mol/L)</td>
<td>18.4 ± 0.6</td>
</tr>
</tbody>
</table>

*Normal eosinophils and neutrophils were purified from the same blood sample by the new method described. The data represent the mean ± SEM of four replicate chemotactic chambers in a representative experiment.
†P < .02 vs buffer.

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Fig 3. Chemotactic response of eosinophils and neutrophils from a patient with the hyper eosinophilia syndrome. Cells were separated on discontinuous Percoll gradients without prior exposure to f-Met-Leu-Phe. The data represent the mean ± SEM of four replicate chemotactic chambers. O—O, Buffer; □—□, activated serum; ●—●, f-Met-Leu-Phe.
In the second study, normal granulocytes (eosinophils and neutrophils) that had not been exposed to f-Met-Leu-Phe during the separation procedure (discontinuous Percoll gradient) were incubated with 10^{-7} mol/L f-Met-Leu-Phe at 37°C for 15 minutes, after which they were fixed with glutaraldehyde. The cells were then stained in suspension with Wright’s stain, which made eosinophils readily distinguishable from neutrophils. Ninety-five percent to 98% of the eosinophils retained their mound shape, whereas more than 90% of the neutrophils developed a highly polarized shape with pseudopod formation (Fig 4A). Thus, it appears that eosinophils are unable to respond to f-Met-Leu-Phe with chemotactic responsiveness or shape change regardless of previous exposure to the peptide.

Membrane potential changes. The membrane potential of normal eosinophils and neutrophils was compared using the membrane potential sensitive probe di-O-C(3) (Fig 5). Eosinophils and neutrophils took up the di-O-C(3) to comparable extents, indicating a similar resting membrane potential for the two cell types. Whereas neutrophils decreased di-O-C(3) fluorescence after f-Met-Leu-Phe exposure (apparent depolarization), eosinophils did not respond. In contrast, the eosinophils did respond to PMA with decreased di-O-C(3) fluorescence (apparent depolarization), although to a lesser degree than neutrophils.

f-Met-Leu-[^{3}H]Phe binding. Specific binding of f-Met-Leu-[^{3}H]Phe to eosinophils prepared by neutrophil depletion after f-Met-Leu-Phe exposure was determined. In one study, specific f-Met-Leu-[^{3}H]Phe binding to eosinophils was 20 cpm of f-Met-Leu-[^{3}H]Phe per 10^6 eosinophils and 2,720 cpm of f-Met-Leu-[^{3}H]Phe per 10^6 neutrophils prepared under identical conditions. In a different experiment, normal granulocytes (neutrophils and eosinophils obtained by centrifugation over the 65% and 75% Percoll layers in the absence of f-Met-Leu-Phe) were treated with 10^{-7} mol/L f-Met-Leu-[^{3}H]Phe for 15 minutes at 37°C and then layered over a single 65% Percoll layer. The cell layer above the 65% Percoll was 92% neutrophils and 8% eosinophils with specific f-Met-Leu-[^{3}H]Phe binding of 4,434 ± 155 cpm/10^6 cells. The resulting cell pellet was 78% eosinophils and 22% neutrophils with 667 ± 33 cpm of f-Met-Leu-[^{3}H]Phe bound per 10^6 cells. The small amount of radioactivity in the eosinophil-enriched cell pellet could be accounted for by the presence of contaminating neutrophils. Thus, there is little or no specific binding of f-Met-Leu-[^{3}H]Phe to normal eosinophils regardless of prior exposure of the cells to f-Met-Leu-Phe.

NBT reduction. When purified eosinophils were stimulated with PMA, more than 90% were able to reduce NBT (Table 4). The distribution of reduced NBT in the eosinophils was similar to that of the neutrophils, with dark granules scattered throughout the entire cytoplasm. Normal eosinophils did not reduce NBT when stimulated with f-Met-Leu-Phe (10^{-6} mol/L) in the presence or absence of cytochalasin B (5 μg/mL), whereas neutrophils will reduce NBT when stimulated with f-Met-Leu-Phe in the presence of cytochalasin B.

Granulocytes (neutrophils and eosinophils not exposed to f-Met-Leu-Phe during purification) were stimulated with f-Met-Leu-Phe plus cytochalasin B in

![Image](image_url)
the NBT assay and stained with safranine, which enables differentiation of eosinophils from neutrophils by nuclear morphology. The cells were then counted after staining, and only 1% to 4% of eosinophils had reduced NBT compared with more than 80% of the neutrophils (Fig 4B). These results indicated that the lack of eosinophil responsiveness to f-Met-Leu-Phe was intrinsic to the cells and not because of deactivation by previous exposure to the peptide.

Palmblad et al recently reported that eosinophils from a patient with hypereosinophilia syndrome would respond to f-Met-Leu-Phe in chemiluminescence assays. We therefore studied NBT reduction from eosinophils purified in various ways from a patient with the hypereosinophilia syndrome (Table 5). Percoll gradient-purified normal neutrophils reduced NBT in response to PMA and f-Met-Leu-Phe, as did unfractionated patient granulocytes. Patient neutrophils and eosinophils exposed to f-Met-Leu-Phe during isolation on Percoll and sedimenting corresponding to band I of Fig 1 also reduced NBT. In contrast, pure preparations of patient eosinophils prepared by f-Met-Leu-Phe exposure and Percoll gradient centrifugation and sedimenting at band II (Fig 1) did not respond to f-Met-Leu-Phe (P < .01). Preliminary experiments measuring superoxide production gave similar results with regard to f-Met-Leu-Phe responsiveness of different populations of this patient’s eosinophils (data not shown). These experiments point out the heterogeneity of responsiveness of eosinophils from patients with the hypereosinophilia syndrome as well as differences in patient eosinophils and normal eosinophils. In addition, the data also indicate that the failure of f-Met-Leu-Phe-purified normal eosinophils to respond to f-Met-Leu-Phe described in Table 4 is not due to either suppressive effects of cytochalasin B or secreted products from neutrophils.

Eosinophils from five patients with chronic granulomatous disease also were isolated on Percoll and stimulated with PMA in the presence of NBT. The patients’ eosinophils failed to reduce NBT, as did their neutrophils, suggesting that the same defect in oxidative metabolism was present in both neutrophils and eosinophils as previously reported. Eosinophils and neutrophils from the mother of a patient with chronic granulomatous disease also were stimulated with PMA (20 ng/mL). Forty-six percent of her eosinophils and 47% of her neutrophils reduced NBT, indicating the same degree of heterogeneity in both cell populations.

Phagocytosis. In phagocytic assays, 90.0% ± 1.4% of the purified eosinophils ingested one or more candidae, whereas 96.8% ± 1.0% of the neutrophils from the same donors ingested one or more candidae. (N = 4, P < .02). Neutrophils ingested more organisms per cell: 91.3% ± 1.7% of neutrophils had four or more candidae per cell, whereas 71.0% ± 3.6% of the eosinophils had four or more candidae per cell (P < .01 for neutrophils vs eosinophils).

DISCUSSION

Separation of normal human eosinophils by exposure of whole blood of f-Met-Leu-Phe, followed by centrifugation on a discontinuous Percoll gradient provides a rapid means for obtaining pure populations of eosinophils from normal donors. Eosinophils in greater than 95% purity with 70% recovery could readily be obtained from normal subjects with less than 3% eosinophils in their peripheral blood. The Percoll is maintained at a physiologic osmolality, minimizing injury to the cells, and the procedure does

Table 4. NBT Reduction by Neutrophils and Eosinophils

<table>
<thead>
<tr>
<th>Donor and Stimulus</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>'Buffer'</td>
<td>1.3 ± 0.6</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>'PMA (20 ng/mL)'</td>
<td>95.2 ± 1.3</td>
<td>91.6 ± 3.3</td>
</tr>
<tr>
<td>'f-Met-Leu-Phe (10⁻⁷ mol/L)' and cytochalasin B (5 μg/mL)</td>
<td>81.8 ± 7.8</td>
<td>3.7 ± 2.3</td>
</tr>
<tr>
<td>CGD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>'PMA (20 ng/mL)'</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CGD heterozygote*</td>
<td>47 ± 4</td>
<td></td>
</tr>
</tbody>
</table>

CGD, chronic granulomatous disease
*One subject.

Table 5. NBT Reduction by Eosinophils From a Patient With Hypereosinophilia Syndrome

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Buffer</th>
<th>PMA (20 ng/mL)</th>
<th>f-Met-Leu-Phe (10⁻⁴ mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal neutrophils</td>
<td>2.0 ± 0.1</td>
<td>97 ± 3</td>
<td>75 ± 5</td>
</tr>
<tr>
<td>Unfractionated patient granulocytes (eosinophils plus neutrophils)*</td>
<td>26 ± 1</td>
<td>95 ± 2</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>f-Met-Leu-Phe-purified patient eosinophils plus patient neutrophils†</td>
<td>22 ± 3</td>
<td>92 ± 2</td>
<td>70 ± 4</td>
</tr>
<tr>
<td>f-Met-Leu-Phe-purified patient eosinophils‡</td>
<td>6 ± 5</td>
<td>87 ± 3</td>
<td>7 ± 1</td>
</tr>
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Peripheral blood WBC count was 83,000 cells per cubic millimeter with 55% eosinophils. Data are mean ± SEM of two determinations.
*Patient granulocytes contained 90% eosinophils and 10% neutrophils.
†Patient eosinophils (90%) and neutrophils (10%) prepared in presence of f-Met-Leu-Phe were recovered from band I (Fig 1).
‡Patient eosinophils purified in presence of f-Met-Leu-Phe (99% eosinophils) (band II Fig 1).
not require any specialized equipment. The method was also effective for isolating eosinophils from patients with mild to moderate eosinophilia (Table 2). Larger tubes (50 mL) may be used to hold the gradients in order to process larger volumes of blood.

To establish that the normal eosinophils recovered were undamaged, several criteria were used. Almost 90% of the normal eosinophils were able to ingest opsonized candidae, although neutrophils engulfed more organisms per cell. The chemotactic response of eosinophils to endotoxin-activated serum was comparable to that of neutrophils, and the eosinophils were also able to reduce NBT when stimulated with PMA.

Previous methods for separating normal eosinophils have often used metrizamide, taking advantage of the greater average density of eosinophils compared with that of other blood leukocytes. However, because of the overlapping densities of eosinophils and neutrophils, it is difficult to achieve a highly pure population of eosinophils without greatly reducing the yield. In addition, eosinophils prepared by metrizamide have impaired chemotactic responsiveness to a variety of stimuli (J.I.G., unpublished observations). Several investigators have used eosinophils from patients with eosinophilia, but these cells differ from normal eosinophils both functionally and metabolically. Other methods have used adherence steps to remove neutrophils, and the autofluorescence of eosinophils has been used to separate them with flow cytometry. These procedures require more time or sophisticated equipment, and the purified eosinophils can be either damaged or activated by the procedures themselves. Newer techniques using Percoll generally have given the best purity, but there are several steps in these procedures before isolating the cells. Some of the techniques using Percoll have also required the presence of fetal calf serum, which may activate the cells. The present method requires only a single centrifugation of whole blood through isosmotic Percoll in order to isolate the eosinophils from other blood components and does not activate the eosinophils during the process.

In the present study, we show that f-Met-Leu-Phe had no effect on normal eosinophil morphology, was not a chemoattractant for normal eosinophils, did not stimulate normal eosinophil NBT reduction, did not change normal eosinophil membrane potential, and did not specifically bind to normal eosinophils. The purified normal eosinophils had a round morphology characteristic of unstimulated cells, and the resting membrane potential of purified normal eosinophils was the same as normal neutrophils not exposed to f-Met-Leu-Phe. The latter data provide evidence that the normal eosinophils had not been activated during purification. Furthermore, the failure of purified normal eosinophils to respond to f-Met-Leu-Phe was not due to an effect of f-Met-Leu-Phe on eosinophils during purification, because normal eosinophils that had not been previously exposed to f-Met-Leu-Phe were also unresponsive to the peptide (Figs 4 and 5).

Other investigations also have reported that oxygen consumption, chemotaxis, and enzyme release by eosinophils are not affected by f-Met-Leu-Phe. On the other hand, studies also have reported that f-Met-Leu-Phe will stimulate eosinophil chemotaxis, superoxide production, hexose uptake, and release of platelet-activating factor, but the eosinophil response was considerably less than that in neutrophils. In the latter studies, however, the investigators used either cells from hypereosinophilia patients, whose eosinophils may be more activated, or normal eosinophil preparations contaminated with neutrophils, which may have been the responding cells. Palmblad et al recently reported that eosinophils from a patient with the hypereosinophilia syndrome were hyperactive for chemiluminescence, but the cell preparations were contaminated with 15% neutrophils.

Eosinophils isolated from patients with the hypereosinophilia syndrome differ morphologically and functionally from normal eosinophils. We have noted heterogeneity in the density of eosinophils from patients with this syndrome, with the less mature eosinophils having a lower density and the more mature appearing eosinophils having the higher density of normal eosinophils. In one patient, the less dense eosinophils responded to f-Met-Leu-Phe in terms of NBT reduction (Table 5), but 10% neutrophils were present in these preparations. This patient’s purified eosinophils, which had the same density as normal eosinophils, did not respond to f-Met-Leu-Phe (Table 5). However, studies in a different patient with the hypereosinophilia syndrome, using eosinophils sedimenting above the 65% Percoll layer in the absence of f-Met-Leu-Phe, did not respond to f-Met-Leu-Phe in a chemotaxis assay. The great variations in eosinophil number, density, and function encountered in these patients with the hypereosinophilia syndrome make it difficult to use these cells to make statements about normal eosinophil function. These difficulties emphasize the need for a rapid method, such as that described in this article, for purifying eosinophils from normal individuals.

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Rapid method for isolation of normal human peripheral blood eosinophils on discontinuous Percoll gradients and comparison with neutrophils

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