Density Heterogeneity of Neutrophilic Polymorphonuclear Leukocytes: Gradient Fractionation and Relationship to Chemotactic Stimulation

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When elicited murine peritoneal exudate cells were subjected to Percoll density gradient centrifugation, polymorphonuclear neutrophils (PMN) were found to distribute over a broad spectrum of buoyant densities (1.10–1.06 g/ml). PMN isolated between approximately 1.10 and 1.085 g/ml were referred to as high density PMN (HD-PMN), and those isolated at approximately 1.085–1.06 g/ml were designated intermediate density PMN (ID-PMN).

Cells were characterized on the basis of morphology and specific markers: PMN by lactoferrin immunocytofluorescence and macrophages by nicotinamide adenine dinucleotide glycohydrase activity. Macrophages banded near the top of the gradient with a peak at 1.04 g/ml. At increasing times following elicitation, the ratio of HD to ID-PMN decreased. Decreased density of either murine HD-PMN or human peripheral blood PMN could be induced in vitro by exposure of the cells to endotoxin-activated serum. A decrease in buoyant density of human PMN was also demonstrated in vitro using the synthetic chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP).

The in vitro interaction of PMN with various chemotactic factors has been shown to result in changes in the relative mean cell volume. Changes in cell buoyant density resulting from interaction with specific chemotactic factors, which may be important in the generation of PMN heterogeneity observed in elicited peritoneal exudate cells. In addition, this approach offers a means of physically separating the cell populations by means of density gradients.

Materials and Methods

Mice

Male and female C57BL/FnLn mice and male BALB/c mice were from our own breeding colonies. Male CF-1 mice were obtained from Charles River Laboratories, Boston, MA. Mice used were 2–5 mo old.

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**Murine Cells**

Leukocyte-rich peritoneal exudate cells (PEC) were elicited by i.p. injection of 2 ml sterile 1% glycogen or 0.4% caseinate in saline and were harvested at the indicated times. Animals were injected (i.p.) with 4 ml deficient Hanks’ balanced salt solution (BSS; Ca²⁺, Mg²⁺-free), and after massage of the abdomen, the cells were carefully removed from the peritoneal cavity using a Pasteur pipette. Nonelicited, resident peritoneal cells were obtained from unstimulated mice in a similar manner.

**Preparation of Human PMN**

Human peripheral blood cells were collected from normal donors using heparin (300 U/20 ml) as an anticoagulant. PMN were found in the upper cell layer after centrifuging the blood at 1,400 g for 20 min at 4°C. These cells were removed and residual red blood cells were lysed at 4°C either by diluting the cell suspension (1 vol) with 9 vol of 0.15 M NH₄Cl, 0.1 mM ethylenediaminetetraacetate (EDTA), and 10 mM NaHCO₃ for 10 min or by hypotonic lysis for 30 sec. Following lysis, cells were centrifuged at 400 g for 5 min and washed once with deficient BSS. Highly purified PMN populations (PMN 97%, eosinophils 3%, determined microscopically on Wright’s stained preparations) were then obtained by density sedimentation using Ficoll-Hypaque. In some experiments, cells were obtained from normal donors undergoing leukapheresis and were further purified (>99%) as described above.

**Chemotactic Factors**

Fresh blood was collected from mice after decapitation, allowed to coagulate, and serum was recovered after centrifugation at 2,000 g for 15 min. A slight modification of the method of Lionetti et al. was used to prepare endotoxin-activated serum. Serum was diluted 1:1 with complete BSS (containing Ca²⁺ and Mg²⁺-free) buffered with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4. Lipopolysaccharide (S. typhosa) was added (10 μg/ml) and the serum was incubated at 37°C for 20 min. Control serum was treated identically, except without the addition of endotoxin. Human endotoxin-activated serum was prepared in the same manner, but autologous serum was diluted 1:4 with complete BSS prior to addition of endotoxin.

Stock solutions (1 mM) of the synthetic peptides N-formylmethionyl-leucyl-phenylalanine (FMLP), and N-formyl-(methionyl)-methionine (FM) were prepared in dimethylsulfoxide. N-formylmethionyl-phenylalanine (FMP) and carbobenzoxy-phe

**Density Gradient Fractionation of Murine PEC**

Unless otherwise stated, all operations were carried out at 4°C. PEC were initially pelleted by centrifugation at 250 g for 7 min and washed once with deficient BSS. Contaminating erythrocytes were lysed by suspending the cells in NH₄Cl solution as described above. After 6 min, the suspension was underlayered with 1 ml of 0.25 M sucrose and centrifuged 7 min at 250 g. The pellet was then resuspended in deficient Dulbecco’s phosphate-buffered saline (PBS; Ca²⁺, Mg²⁺-free). To remove residual debris and obtain a single cell suspension, the cells were passed through a 200-mesh stainless steel screen. Cell number was determined by electronic counting (Couler Counter, Model Z2). After centrifugation at 250 g for 7 min, the pellet was resuspended in deficient PBS at a concentration of 6–7 x 10⁶ cells/ml.

Density gradient centrifugation of PEC was performed using polyvinylpyrrolidone-treated silica (Percol, Pharmacia, Piscataway, NJ). Densities were determined by constructing a standard curve of refractive index (Abbe refractometer, Bausch and Lomb) versus Percoll solutions of known densities and by use of calibrated density beads (Pharmacia). Percoll was adjusted to pH 7.5 using 1 N HCl and made isotonic with 10-fold concentrated PBS. Three milliliters of the cell suspension were thoroughly mixed with 6 ml of isotonic Percoll. The mixture was then placed in acid-washed siliconized glass tubes (15 ml, Corex) and centrifuged in a Sorvall RC2-B centrifuge using an SS-34 fixed-angle rotor for 25 min at 18,000 rpm (10,000 g · min). The self-generated gradients were fractionated using an automatic system consisting of a peristaltic pump (LKB, model 2120) and fraction collector. Cell fractions were diluted with 2 vol of deficient BSS and centrifuged 10 min at 400 g. Recovery of cells from the gradient was 75–85% of total cells. Viability of the cells as determined by trypan blue exclusion was >90% for PMN and 80–90% for macrophages.

In addition to determining density by refractive index, the buoyant density of cells within the gradients was also determined by measuring the distance from the center of the cell band to the bottom of the centrifuge tube and relating this to the position of standardized colored density marker beads (Pharmacia), which were run in a separate tube or were added, in some cases, to the test gradient after centrifugation. There was unavoidable mixing when gradients were fractionated (refractive index), while use of unrefractionated gradients (density beads) for density determination minimized the problem of mixing.

**Refractionation of Murine PMN Following Treatment With Chemotactic Factors**

PMN recovered after Percoll density gradient centrifugation of elicited PEC (5 x 10⁶ to 1 x 10⁷ cells) were incubated in 1 ml of HEPES-buffered (20 mM, pH 7.4) complete BSS with 20% activated or control serum. The cell suspensions were gently agitated in plastic tubes at 37°C in a metabolic shaker water bath for 30 min. In some experiments, PMN were exposed to endotoxin-activated serum after pretreatment with cytochalasin-B (5 μg/ml, 10 min). Cells exposed to endotoxin-activated serum with and without cytochalasin-B, as well as control cells, were centrifuged on Percoll density gradients to assess changes in cell buoyant density. Cells were pelleted after incubation with serum at 400 g for 5 min at 4°C, resuspended in 3 ml ice-cold PBS, and mixed with 6 ml isotonic Percoll. Centrifugation and fractionation of the gradients were as described above.

**Fractionation of Human PMN Following Treatment With Chemotactic Factors**

Human PMN (4 x 10⁷) were incubated in 20 ml of RPMI 1640 culture medium containing 4 ml of activated or control serum. Cells were incubated in plastic Petri dishes for varying periods of time at 37°C in a humidified atmosphere containing 7.5% CO₂/air. Alternatively, in experiments using the synthetic chemotactic peptides, the peptide was incubated at the desired concentration with 2 ml of human PMN suspension (10⁷ cells/ml) in complete BSS containing 20 mM HEPES buffer, pH 7.4 at 37°C. The suspensions were gently agitated at 15 ml siliconized Corex tubes using a metabolic shaker water bath. In experiments using CBZ-PM, the antagonist was preincubated with cells for 5 min before addition of FMLP. In experiments using colchicine, it was preincubated with the cells for the indicated period of time before treatment with FMLP. Activi-
tion of cells was terminated by addition of an equal volume of ice-cold deficient PBS containing 2 mM EDTA followed by centrifugation at 400 g for 5 min at 4°C. Cells were resuspended in PBS containing 1 mM EDTA and centrifuged at 400 g for 5 min (4°C). Cells were then diluted in an appropriate volume of ice-cold PBS without EDTA to give a concentration of 2 × 10⁷ cells in a final volume of 3 ml. Cells were passed through a 200-mesh stainless steel screen to remove any debris before assessment of buoyant density changes were made by density gradient centrifugation. Control and stimulated cells were mixed with 6 ml of isotonic Percoll, and density gradients were generated, fractionated, and cells isolated as described above. Differences in measured cell densities from experiment to experiment appeared to be accounted for by changes in the density of untreated control cells (1.100 g/ml ± 0.006, mean ± SD, 18 experiments) and by differences in the method used to measure the density (see above). Recovery of cells from the gradients was 75%–90% of the cells applied, and recovered cells were >95% viable. Some nonviable material was occasionally found at the extreme top of the density gradients.

Relative mean cell volume was measured using a Coulter Counter (model ZBI) equipped with a 100-channel volume channelyzer as described by O’Flaherty et al. 12 Latex particles (5 and 10μ) were used for calibration.

Identification of Cell Types

PMN were distinguished by characteristic morphology and the presence of lactoferrin, a specific marker of this cell type (myelocytes and older). 15,16 Slides were prepared using a cytocentrifuge (Shandon Southern Instruments, Sewickley, PA), and light microscopy was performed using Wright’s stain. The cellular localization of peroxidase activity was determined following fixation with 10% formalin-ethanol (v/v) using 3,3-diaminobenzidine as described by Graham and Karnovsky, 22 except that 10 mM sodium phosphate buffer, pH 7.0, was employed. Lactoferrin was demonstrated by an immunochromogenic procedure using the tetramethylrhodamine-labeled F(ab’)2 portion of rabbit IgG directed against murine lactoferrin as described previously. 23 Macrophages were identified in Wright’s stained preparations on the basis of morphology, and biochemically by their content of nicotinamide adenine dinucleotide glychydrase (NADase) (100 U/5 × 10⁶ cells). NADase activity was determined by the cyanide procedure 24 in cell homogenates prepared in 0.25 M sucrose. The assay mixture contained 0.5 mM NAD, 10 mM sodium phosphate buffer, pH 7.2, and 0.1 μl of sample in a final volume of 0.5 ml. Incubation was for 30 min at 37°C and the reaction was stopped by the addition of 2.5 ml of 1.0 M KCN. Several minutes were allowed for formation of the stable NAD-CN complex, after which the decrease in absorbance at 325 nm was measured together with the appropriate controls. The millimolar extinction coefficient used for the NAD-CN complex was 6.3. 25 Monocyte and macrophage nonspecific esterase activity was demonstrated as described by Ornstein and coworkers 26,27 using α-naphthylbutyrate as the substrate after fixation in formalin vapor for 1 min. Histochemically stained cells were observed with a Leitz Orthplan microscope. Fluorescence was viewed with the same microscope equipped with a 100W mercury lamp and epillumination.

Solutions and Chemicals

Balanced solutions used in experiments with murine cells were adjusted to correspond to an osmolarity of 308 mosmole. The contribution to total osmolarity was calculated from tables for each component in Ca²⁺ and Mg²⁺-deficient Hanks’ and Dulbecco’s saline solutions. The osmolarity was then adjusted by the addition of the appropriate amount of NaCl. Adjusted solutions were Hanks’: KCl, 0.40 g/liter; KH₂PO₄, 0.06 g/liter; NaCl, 8.82 g/liter; NaH₂CO₃, 0.35 g/liter; Na₂HPO₄, 0.048 g/liter; glucose, 1.0 g/liter; pH 7.4. Dulbecco’s: KCl, 0.20 g/liter; KH₂PO₄, 0.20 g/liter; NaCl, 8.64 g/liter; Na₂HPO₄, 1.14 g/liter; pH 7.5. When complete Hanks’ was used it contained, in addition, 0.14 g/liter CaCl₂, 0.10 g/liter MgSO₄ · 7H₂O, and 0.10 g/liter MgCl₂ · 6H₂O. When Percoll was made isotonic, ideal (nonadjusted) Dulbecco’s phosphate-buffered saline was used, since Percoll contributes to the osmolarity of the suspension.

Oyster glycoprotein (type II), nicotinamide adenine dinucleotide, and colchicine were purchased from Sigma Chemical Co., St. Louis, MO. Lipopolysaccharide (S. typhosa) was purchased from Difco Laboratories, Detroit, MI. Percoll was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Wright’s stain was manufactured by Harleco, Philadelphia, PA. Chemotactic peptides and carb benzoxyl-phenylalanil-methionine were obtained from Sigma Chemical Co. or Vega Chemical Co., Tucson, AZ. RPMI 1640 culture medium was obtained from Gibco, Grand Island, NY.

RESULTS

Composition of Peritoneal Exudates

A variety of agents has been used to elicit a sterile inflammatory response in the peritoneum of experimental animals for isolation of leukocytes. Since the nature of the stimulator, concentration, and time of harvest following elicitation are important in determining the composition of the exudate, a first step in these studies was to examine the composition of cells populating the peritoneum of mice at various times following injection (i.p.) with 1% glycogen in saline.

Table 1 shows the results of such experiments with C57BL/FnLn mice, where total cell yields and cell differentials were compared at different times postinjection. When exudates were taken 5 hr after injection, greater than 10⁷ cells/mouse were obtained, with the greatest overall percentage of PMN. When male BALB/c and CF-1 mice were used, PMN in the exudate could be enriched to over 90% with a correspondingly lower percentage of eosinophils (<1%). Lower eosinophil percentages resulted, in part, from using male mice exclusively, since female mice show higher variability in eosinophil number. 28 Beyond 24 hr following glycogen injection, the cell differentials changed quickly, with mononuclear cells becoming the predominant cell type in all strains used. Based on these studies, exudates taken from male mice 5 hr postinjection contained an optimal percentage of PMN. The same time of harvest was found to be optimal for the percentage of PMN (90%) in exudates elicited with 0.4% caseinate in saline.

Density Heterogeneity, Fractionation, and Characterization of Peritoneal Exudate Cells

The procedure adopted for investigating possible physical changes in elicited PMN, as well as for
purification of the major cell types, involved density gradient centrifugation using Percoll. Two representative fractionation experiments with PEC from C57BL/FnLn mice are presented in Fig. 1. When nonelicited resident cells populating the peritoneum (mostly macrophages, Table 1) were separated using this technique, the macrophages banded in the low density region of the gradient with a peak at about 1.04 g/ml. Also depicted in Fig. 1 is the fractionation of PEC harvested 2 hr after glycogen injection when PMN have begun to populate the peritoneum (45% total cells at 2 hr). In this experiment, macrophages again banded with a peak at 1.04 g/ml. PMN exhibited a pronounced density heterogeneity, banding over a rather broad spectrum of densities. For the purpose of simplifying these initial investigations, the distribution of PMN populations was divided into two groups: (1) high density PMN (HD-PMN) ranging in density from 1.10 to 1.085 g/ml, with a peak at about 1.09 g/ml—these cells accounted for 80% of the total recovered PMN; (2) intermediate density PMN (ID-PMN) ranging in density from 1.08 to 1.06 g/ml, with a peak usually around 1.06 g/ml—cells in this region of the gradient accounted for 20% of the total recovered PMN.

Elicited exudates harvested at 2, 5, and 9 hr post-stimulation contained different percentages of PMN (see Table 1). Figure 2 shows that when each of these exudate populations was fractionated by density gradient centrifugation, the percent PMN that banded in the HD region did not correlate directly with the percent PMN in the starting population. Rather, there

### Table 1. Yields and Compositions of Peritoneal Exudate Cells at Different Times Following Glycogen Stimulation

<table>
<thead>
<tr>
<th>Harvest Time (hr-post injection)</th>
<th>Cells/Mouse</th>
<th>PMN</th>
<th>M-M</th>
<th>Lymph</th>
<th>EO</th>
<th>Other</th>
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<td>70</td>
<td>22</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.80 x 10⁷</td>
<td>46</td>
<td>38</td>
<td>11</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
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<td>77</td>
<td>11</td>
<td>8</td>
<td>3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>9</td>
<td>1.10 x 10⁷</td>
<td>56</td>
<td>32</td>
<td>8</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>96</td>
<td>1.20 x 10⁷</td>
<td>1</td>
<td>60</td>
<td>37</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Data represent mean values from at least two experiments.
†Figures are based on pooled exudates from five C57BL/FnLn male and female mice.
§Cell differentials are based on counts of at least 300 cells from a pool of 5 exudates and are expressed as percentages.
¥M-M, Lymph, and EO are monocytes/macrophages, lymphocytes, and eosinophils, respectively.
DENSITY HETEROGENEITY OF NEUTROPHILIC PMN LEUKOCYTES

Fig. 2. Representative Percoll density gradient centrifugation profiles of PEC harvested at different times following i.p. injection of 1% glycogen. Two hours, ••-••; 5 hr., A-A; 9 hr., O-O. The inset describes the decrease with time of PMN in peak A (1.09 g/ml) as a fraction of the total number of PMN in the starting exudate.

appeared to be a first-order rate of disappearance of cells from the HD region (inset, Fig. 2) and a corresponding increase in the population of PMN distributing within the ID region. These results indicated that the observed distribution of PMN was not artifactual, but corresponded to a true shift in density of the cells.

The HD population of PMN was routinely recovered at greater than 97% purity, regardless of sex or strain of mice used, or the time at which the PEC were harvested following stimulation. In contrast, the purity of ID-PMN was dependent on the mouse strain and time at which the PEC were harvested. For example, using C57BL/FnLn mice, the percentage of PMN in the ID cell population could be enriched to a purity of about 75%, the remainder being predominantly mononuclear cells resulting from contamination in this region of the gradient. When 5-hr exudates from either CF-1 or BALB/c male mice were used, the starting population of PMN in the exudate was optimized at 90% or greater. When these PEC were fractionated, PMN in the ID region of the gradient could then be obtained at about 90% purity.

In PEC harvested and fractionated at sequentially longer times following injection of glycogen (> 16 hr), both the HD and ID regions of PMN essentially disappeared. This corresponded to significant increases in the percentage of mononuclear cells and decreases in PMN in the total exudate (see Table 1).

Macrophages were generally recovered at about 90% purity up to 2 hr postelicitation, as judged by morphology and content of NADase activity. The NADase activity in this cell fraction decreased with increasing time of harvest, reflecting increased numbers of monocytes and lymphocytes.

The characteristics and purity of the cell fractions obtained by density gradient centrifugation of different PEC populations are summarized in Table 2. PEC elicited with other agents, such as casein, could also be fractionated using this technique, but good separation was dependent on the concentration of casein used. Satisfactory separation was obtained with 0.4% caseinate in saline; however, 1% caseinate in

<table>
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<tr>
<th>Density of Cell Fraction (g/ml)</th>
<th>Time Postinjection (hr)</th>
<th>Mouse Strain</th>
<th>Histochemistry (Major Cell Type)</th>
<th>NADase†</th>
<th>Cell Differential‡</th>
</tr>
</thead>
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<tr>
<td>1.04</td>
<td>0</td>
<td>C57</td>
<td>– –</td>
<td>97.4</td>
<td>0 94 6 &lt;1 0</td>
</tr>
<tr>
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<td>– –</td>
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<tr>
<td>1.04</td>
<td>15</td>
<td>C57</td>
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<td>C57</td>
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<tr>
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<td>5</td>
<td>CF-1</td>
<td>+ +</td>
<td>2.0</td>
<td>90 7 2 &lt;1 &lt;1</td>
</tr>
<tr>
<td>1.09 (HD)</td>
<td>2</td>
<td>C57</td>
<td>+ +</td>
<td>2.8</td>
<td>97 &lt;1 2 &lt;1 0</td>
</tr>
<tr>
<td>1.09</td>
<td>5</td>
<td>CF-1</td>
<td>+ +</td>
<td>1.9</td>
<td>98 &lt;1 1 &lt;1 0</td>
</tr>
</tbody>
</table>

†Units/5 x 10⁶ cells; 1 U is that amount of enzyme that splits 1 nmole NAD in 5 min.
‡Cell differentials were determined on Wright's stained preparations and are expressed as percentages based on a minimum of 300 cells; M-M, monocytes/macrophages; EO, eosinophils; Lymph, lymphocytes.
§Diaminobenzidine, electron donor for histochemical demonstration of peroxidase.
¶Immunocytofluorescence; rhodamine-labeled rabbit IgG anti-mouse lactoferrin. There was an exact correspondence between positively stained cells and the percent PMN as shown under Cell Differential.
||Confirmed by nonspecific esterase positivity (α-naphthylbutyrate).
saline yielded exudates that separated poorly, forming large clumps of cells. PMN elicited with 0.4% caseinate in saline also exhibited density heterogeneity.

**Density of PMN Decreased by In Vitro Exposure to Endotoxin-Activated Serum**

To investigate the possibility that the time-dependent generation of density heterogeneity observed in elicited PMN might be related to interaction with chemotactic factors, in vitro model experiments using both murine and human neutrophilic PMN were conducted. Elicited murine PEC were initially fractionated by density gradient centrifugation to yield a population of HD-PMN. Half of these cells were incubated under essentially nondegranulating conditions, with endotoxin-activated serum as a source of serum-derived chemotactic activity, while the remainder of the cells were incubated in the absence of activated serum and served as control cells. Cells were then recentrifuged on Percoll density gradients. The results of such an experiment are given in Fig. 3A. Stimulation of HD-PMN with endotoxin-activated serum resulted in a substantial shift of the cells toward lower density relative to unstimulated control cells. When HD-PMN were induced to degranulate as a result of pretreatment of cells with cytochalasin-B followed by endotoxin-activated serum, a slightly smaller shift in density was observed (Fig. 3B). In some experiments, degranulated HD neutrophils exhibited a density shift equivalent to, but never greater than, that of cells incubated with endotoxin-activated serum alone.

The experiments described above indicated that chemotactic activation of HD-PMN resulted in a shift of the density of the cells toward lower values and suggested that this phenomenon could occur in the absence of significant degranulation. However, because elicited PEC have been shown to be degranulated relative to unelicited cells, and because it was impossible to monitor and/or control the in vivo peritoneal milieu, similar experiments were carried out using purified human peripheral blood PMN. The
results of such experiments are presented in Fig. 4. Control PMN typically banded in the gradient at a density of about 1.100 g/ml, whereas PMN incubated for 30 min with serum-derived chemotactic agents banded at a lower density of about 1.085 g/ml. Treatment of PMN with endotoxin alone (20 μg/ml) had no effect, clearly demonstrating the involvement of serum components. The time course of the density shift in response to 20% endotoxin-activated serum is summarized in the inset to Fig. 4. The maximum shift in density occurred after a 30-min incubation. At longer times of incubation (60–180 min), there was often a slight return toward higher densities, although never returning to the density characteristic of control cells. These results demonstrated that under more controlled in vitro conditions, shifts in PMN density toward lower values were dependent on interaction of the cells with endotoxin-activated serum. Because of the complex nature of the serum factors involved in generating this response, we decided to study the effect of FMLP, a well characterized, synthetic, chemotactic peptide, in order to gain a clearer understanding of the role of chemotactic agents in inducing density shifts.

**Time and Dose Response of FMLP-Induced Density Changes**

Incubation of PMN with FMLP caused a shift in the density of the cells toward lower values, similar to that observed using endotoxin-activated serum. The data of Fig. 5 show representative kinetic and dose–response profiles. Minimal responses were elicited using 10^{-9} M FMLP, and no effect was seen with 10^{-11} M FMLP, while a maximum response was observed using 10^{-8} M peptide. Increasing concentrations of FMLP (10^{-7} M to 10^{-5} M) led to an apparent inhibition of the response. At all doses tested, the maximum response occurred after about 30 min of incubation with the chemotactic agent. Longer incubation periods resulted in a decrease in the maximum density shift, suggesting a partial reversal of the effect with time similar to that observed with endotoxin-activated serum. No differences were observed between PMN obtained during leukapheresis and routine collection using heparinized tubes. In general, a significant broadening of the cell band (6.9 mm ± 1.7, mean ± SD, n = 7) appeared to accompany the maximum change in density. In contrast, rather narrow bands (1.7 mm ± 0.6, n = 6) were observed with control cells or cells incubated with suboptimal concentrations of FMLP. Between different experiments, the same relative potencies for a given range of FMLP concentrations were always observed and the shape of the curves was always the same.

**Specificity and Characteristics of the FMLP Response**

Aggregation of PMN has been shown to result from the interaction of these cells with FMLP. However, under the experimental conditions employed in our studies, and as reported by others, aggregation did not occur to any significant extent; this was confirmed by viewing aliquots of neutrophil-Percoll suspension using phase-contrast microscopy and by measuring changes in the reactive mean cell volume (see below). Thus, changes in buoyant density of the PMN were not due to an aggregation phenomenon.

FMLP-induced functional changes in PMN appear to be mediated by a specific membrane receptor. In order to assess whether the changes in buoyant density induced by FMLP were also dependent on interaction

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**Fig. 5.** Time and dose dependence of FMLP-induced human PMN density changes. Error bars indicate the standard error of the mean of determinations usually carried out at least in triplicate. All points were made using separate density gradients, and all data were normalized to a control density of 1.097 g/ml.
Fig. 6. Dose-response curves for PMN density changes induced by FMLP (■—■), FMLP in the presence of 10^{-4} M CBZ-PM (○—○), and FMP (▲—▲). The curve for FM was essentially identical to that obtained using FMLP. All incubations were for 30 min and used 2 × 10^7 cells.

with this same receptor, we investigated the effect of CBZ-PM, a known competitive antagonist of FMLP. As shown in Fig. 6, the dose–response curve for FMLP was uniformly shifted toward higher concentrations by an order of magnitude in the presence of 10^{-6} M CBZ-PM. The apparent dissociation constant ($K_a$) for CBZ-PM was calculated from the following equation:

$$\frac{A_i}{A_0} - 1 = \frac{B}{K_a}$$

where $B$ is the concentration of the antagonist (CBZ-PM), $K_a$ is the apparent dissociation constant of the antagonist, $A_i$ is the concentration of agonist (FMLP) eliciting a given response in the absence of the antagonist, and $A_0$ is the concentration of agonist stimulating the same magnitude of response in the presence of the antagonist. Using the concentrations of FMLP that elicited a maximum density change in the presence and absence of 10^{-4} M CBZ-PM, the calculated value of $K_a$ for CBZ-PM was 1.1 × 10^{-5} M. This value was essentially the same as the $K_a$ calculated by others for CBZ-PM inhibition of other FMLP-induced functional responses.

Further support for the receptor-mediated nature of the density changes came from studies using two synthetic agonists, FMP and FM. Relative to FMLP, FMP is less potent (5,000-fold) as a PMN chemotactic factor or as a stimulator of lysosomal enzyme release, while FM is comparable with respect to these two activities. As can be seen from the data presented in Fig. 6, FMP produced density changes similar to those seen using FMLP, but in concentrations that were approximately four orders of magnitude higher. On the other hand, FM produced density changes with a dose–response relationship similar to that of FMLP (data not shown).

Chemotaxis is dependent on extracellular Ca^{2+}. In order to explore the possibility that extracellular divalent metal ions might be necessary for the density effect, incubations were performed in BSS containing 1 mM EDTA with cells that had been previously washed in Ca^{2+}- and Mg^{2+}-free medium. No differences were observed in the maximum response to FMLP (10^{-8} M, 30 min) compared to PMN incubated in the presence of Ca^{2+} and Mg^{2+}. However, shorter periods of incubation in the presence of FMLP in Ca^{2+}-, Mg^{2+}-free medium produced smaller density shifts compared to identical time points in the presence of Ca^{2+} and Mg^{2+}. These observations indicated that the rate of density change, but not the absolute extent, was dependent on extracellular Ca^{2+} and Mg^{2+}.

In order to investigate the possibility that cytoskeletal components might be involved in the observed density changes, cells were preincubated with 10^{-5} M colchicine for various periods of time (5–60 min) prior to interaction with FMLP. No effect was seen on FMLP-induced density changes under these conditions, suggesting that intact microtubule structure did not appear to be necessary for this phenomenon.

**Correlation of Chemotactic-Factor-Induced Density Changes With Changes in Relative Mean Cell Volume**

PMN undergo time-dependent changes in mean cell volume when exposed to chemotactic factors in vitro. A direct relationship between our observations of chemotactic-factor-induced density changes and cell volume might therefore be predicted. To test this possibility, human PMN were incubated in the presence of various concentrations of FMLP, and after 30 min, relative mean cell volume was determined using a Coulter Counter (model ZBI) equipped with a volume channelyzer. As seen in Fig. 7, increasing cell volume was dose-related to interaction with FMLP. The maximum increase of PMN relative mean cell volume was inhibited at 10^{-7} M and 10^{-8} M FMLP, similar to apparent inhibition of maximum density changes observed at higher peptide concentrations. In fact, these results directly correlated with the decrease in PMN density observed at 30 min, as indicated in the inset to Fig. 7.

**DISCUSSION**

The present studies have demonstrated that the major cell types present in elicited murine PEC can be isolated and purified using Percoll density gradient
Density heterogeneity of neutrophilic PMN leukocytes

DENSITY HETEROGENEITY OF NEUTROPHILIC PMN LEUKOCYTES

Density heterogeneity among elicited PMN. In addition, degranulation of PMN can result from exposure to chemotactic factors, and in vitro studies have also shown that PMN preexposed to chemotactic factors can acquire hyperresponsive superoxide production when cells are reactivated.

The data presented in this article demonstrate for the first time that a change in buoyant density is another measurable response of PMN that results from interaction with certain chemotactic factors. Density changes in both human and murine PMN were directly demonstrated in vitro following interaction of the cells with factors generated in endotoxin-activated serum and with synthetic chemotactic peptides, such as FMLP. Using FMLP and human PMN, density changes were shown to be dose and time dependent. Thus, cellular interactions involving chemotactic factor(s) within the peritoneum would seem a reasonable component in the in vivo generation of density heterogeneity among elicited PMN. Moreover, the spectrum of densities obtained in vivo could result, in part, from differences in relative degree of exposure (time and concentration) of cells to a chemotactic stimulus within the bulk PMN population.

FMLP-induced changes in PMN appear to be mediated through a specific cell membrane receptor. Our data provide strong support for the idea that FMLP-induced density changes are also receptor mediated. Using the competitive antagonist CBZ-PM, the apparent dissociation constant \( K_d \) for the inhibition of FMLP-induced density change \( (1.1 \times 10^{-5} M) \) was in close agreement with published values calculated for inhibition of FMLP-induced superoxide production \( (0.8 \times 10^{-5} M) \). PMN aggregation, locomotion, and lysosomal enzyme release \( (1.0 \times 10^{-5} M) \). Moreover, the relative potencies of two agonists, FMP and FM, for induction of maximum density changes were similar to those reported for chemotaxis and degranulation.

We observed that FMLP-induced decreases in PMN density directly correlated with FMLP-induced increases in relative mean cell volume. The concentration dependence of the density response was similar to other cellular responses mediated by this agent. Although PMN chemotaxis and superoxide production occurred at lower concentrations of FMLP than that required for density changes, optimum responses for each of these processes occurred at about \( 10^{-5} M \) FMLP. Furthermore, reports describing inhibition of chemotaxis at higher FMLP concentrations were in the same range where inhibition of the density response occurred \( (10^{-7} M-10^{-5} M) \).

The time course of FMLP-induced density changes was also similar to that of sustained changes in mean cell volume reported for human and rabbit PMN.

Fig. 7. Changes in the relative mean cell volume of human PMN following exposure to the chemotactic peptide FMLP. Cell volumes were measured using a Coulter Counter (ZBI) equipped with a volume channelizer. Untreated control; \( 5 \times 10^{-8} M \) FMLP. The inset shows the relationship between FMLP-induced changes in relative mean cell volume and FMLP-induced changes in the buoyant density as measured by Percoll density gradient centrifugation (see Fig. 5). Density values were measured at the midpoint of the cell band following centrifugation, and relative mean cell volumes were measured at that channel number containing the maximum number of cells in the frequency distribution. Correlation coefficient, \( r = 0.9968 \).
treated with FMLP or bacterial chemotactic factors. Moreover, our observation that the absence of extracellular calcium decreased the rate at which FMLP-stimulated density changes occurred was consistent with a similar effect of this ion on the rate and extent of swelling of rabbit PMN induced by bacterial chemotactic factors. Takamori K, Yamashita T: Biochemical properties of polymorphonuclear neutrophils from venous blood and peritoneal exudates of rabbits. Infect Immun 29:395-400, 1980

In addition to establishing density change as a new response of PMN to certain chemotactic factors, the data presented here offer a means of physically separating cytotaxin-activated PMN into subpopulations that may have subtle biochemical differences. Such preparations could include cells from inflammatory sites as well as cells isolated from in vitro chemotaxis systems. Percoll fractionation of cytotaxin-stimulated cells should provide an added refinement to the study of biochemical events surrounding PMN activation and movement.

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