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Functional and Phenotypic Characteristics of Percoll-Isolated and Counterflow-Isolated Human Peripheral Blood Monocyte Subpopulations

Jeffrey Bruce Payne

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FUNCTIONAL AND PHENOTYPIC CHARACTERISTICS OF PERCOLL-ISOLATED AND COUNTERFLOW-ISOLATED HUMAN PERIPHERAL BLOOD MONOCYTE SUBPOPULATIONS

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FUNCTIONAL AND PHENOPTYPIC CHARACTERISTICS OF PERCOLL-ISOLATED AND COUNTERFLOW-ISOLATED HUMAN PERIPHERAL BLOOD MONOCYTE SUBPOPULATIONS

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This thesis is dedicated to the memory of my father, who taught me the virtues of hard work, integrity and morality.
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LITERATURE REVIEW

The monocyte-macrophage system is a network of specialized phagocytic cells, including peripheral blood monocytes, Kupffer cells of the liver, microglial cells of the brain, macrophages of lymph node and spleen, alveolar macrophages, other fixed tissue macrophages and fluid phase macrophages (13). Macrophages play a variety of roles in both the afferent and efferent limbs of the immune response. Macrophages are involved in the initiation of immune reactions as antigen-presenting cells, and in the effector phase as inflammatory, tumoricidal, microbiocidal and regulatory cells (11,64). There is evidence that peripheral blood monocytes originate from bone marrow precursor cells and that tissue macrophages are derived from circulating monocytes (22,48). Because macrophages perform many functions, investigators hypothesized that it was unlikely for a single, homogeneous precursor cell population to be responsible for such functional diversity (49). Indeed, evidence suggests that monocyte subpopulations exist which may represent different activation states, different stages of maturation, or distinct, stable subsets (12,21).

Evidence For Macrophage Heterogeneity

The development of monoclonal antibodies to human T lymphocyte surface antigens allowed for the characterization of T lymphocyte subsets (33). The same approach has been used to study
antigenic markers on the surface of tissue and fluid phase macrophages and has revealed surface membrane heterogeneity among macrophages from different anatomical sites (6).

Cell surface antigens have been identified that are present virtually exclusively on human peripheral blood monocytes (5,29), tissue or fluid phase macrophages (1-3,6,57,58,67). In addition, cell surface antigens have been identified that are present on peripheral blood monocytes and tissue or fluid macrophages (29,63). The PHM3 monoclonal antibody detects a surface antigen that is expressed by most peripheral blood monocytes but is expressed only at very low levels on peritoneal macrophages and Kupffer cells (5). Likewise, FMC17 and FMC34 recognize antigens present on circulating monocytes that are lost or markedly decreased in quantity when monocytes enter inflammatory sites (29). On the other hand, Biondi et al. described anti-PAM1 and anti-BMM1, two monoclonal antibodies specific for lung macrophages and breast milk macrophages, respectively (6). Andreesen et al. described a series of monoclonal reagents that recognize MAX antigens which are found only on exudate macrophages residing in serous cavities (1-3), while Dimitriu-Bona et al. reported on M₀P-15 and M₀P-7, which demonstrate moderate and weak immunofluorescent staining, respectively, for the majority of circulating monocytes but intense staining for fluid phase macrophages (14). Moreover, Zwadlo et al. have shown that the monoclonal antibody 27F9 labels most resident tissue macrophages but not circulating monocytes (67). However, PHM2, FMC32, FMC33, and 63D3 persist through all stages of
mononuclear phagocyte differentiation, including end-stage granuloma formation (29,63).

Evidence For Monocyte Functional Heterogeneity

In the last ten years, numerous studies have revealed functional heterogeneity of human peripheral blood monocytes and the presence of subpopulations which may represent both functionally and morphologically different subsets (16, 19,21,25,32,39,44,45,47,56,61,62,66). Countercurrent centrifugal elutriation, Percoll and bovine serum albumin (BSA) density gradients, and adherence have been used to physically separate monocytes into subpopulations.

Monocyte subpopulations differing in \( F_c \) receptor activity have been isolated. Norris and coworkers, using countercurrent centrifugal elutriation to isolate monocyte subpopulations, demonstrated that peripheral blood monocytes could be divided into two subsets, which they designated "small" and "large" (45). Both subsets were phagocytic, glass adherent and nonspecific esterase positive. However, the small monocytes were deficient in \( F_c \) receptor-mediated activity, while the large monocytes were not. This study was flawed, however, since it appears that there was significant lymphocyte contamination of the small monocyte fraction. Melewicz and Spiegelberg, using adherence to isolate monocytes from mononuclear cell preparations, presented evidence that a subpopulation of monocytes from healthy human donors
presumably mediated phagocytosis and lysis of IgE-coated target cells through their $F_c$ receptors for IgE (39).

Functional heterogeneity with respect to colony-stimulating factor (CSF) release has been shown. Yasaka et al. isolated two monocyte subpopulations by countercurrent centrifugal elutriation (66). The two subpopulations exhibited similar functional properties; however, the larger subpopulation was responsible for greater CSF release. Likewise, Tice et al., upon separating monocytes on Percoll gradients, found significantly greater CSF activity in the more dense monocyte fractions than in the least dense fraction (56). Because monocytes were isolated from mononuclear cells by one hour adherence in this study, it is possible that differential activation of the isolated subsets may have occurred, generating artifactual subpopulations.

Monocyte subpopulations differing in hydrogen peroxide ($H_2O_2$), myeloperoxidase and tumoricidal activity have been isolated. Turpin et al. separated monocytes into subpopulations by countercurrent centrifugal elutriation and showed that the large monocytes were responsible for greater $H_2O_2$ production at day one of culture, while by day three, small monocytes were the major producers of $H_2O_2$ (62). Yasaka et al. found higher myeloperoxidase activity in the larger monocyte fraction isolated by countercurrent centrifugal elutriation (66). Finally, Normann and Weiner separated monocytes by elutriation and noted that only the small monocytes appeared responsible for tumoricidal activity (44).

Differential synthesis of prostaglandins by monocyte subpopulations has been reported by several investigators. Goldyne
et al. sedimented human peripheral blood monocytes isolated by adherence through a five step discontinuous BSA density gradient and found significant differences in prostaglandin E₂ (PGE₂) synthesis among the subpopulations (25). However, in this study the stimulus used for monocyte activation was not mentioned. Picker et al., when fractionating mononuclear cell suspensions on discontinuous BSA gradients, showed that a population of low density monocytes produced the majority of PGE (47). Finally, Treves et al. isolated monocytes by adherence to plastic and reported that the loosely adherent monocytes secreted more PGE₂ than the strongly adherent ones (61).

Differential interleukin-1 (IL-1) elaboration by density-defined human monocyte subpopulations has been noted. Elias et al. divided monocytes into subpopulations using discontinuous Percoll density gradients (16). The denser monocytes produced more IL-1 than the less dense cells. Khansari et al. separated human peripheral blood monocytes on discontinuous BSA gradients and reported production of the majority of PGE₂ and IL-1 by two different monocyte fractions (32). Finally, Treves found that loosely adherent monocytes secreted higher amounts of IL-1 than the strongly adherent monocytes (61).

Nichols et al. demonstrated differential specific activities of PGE₂ and thromboxane B₂ (TxB₂) released by lipopolysaccharide (LPS)-treated human monocytes over a 24 hour period, which suggests the presence of at least two subpopulations of monocytes with distinct secretory responses (42). Because PGE₂ and TxB₂ are derived from a common precursor, arachidonic acid, in the
cyclooxygenase pathway, one would have expected identical specific activities for TxB2 and PGE2 if LPS-treated human monocytes were labeled with arachidonic acid of uniform specific activity. In this study, monocytes were prelabeled for two hours with 0.1 μCi/ml of 3H-arachidonic acid and 14C-arachidonic acid. Cells were then washed to remove unincorporated label and treated continuously with 10 μg/ml Salmonella typhimurium LPS plus 0.1 μCi/ml 14C-arachidonic acid. Parallel unlabeled cultures were treated with LPS to determine immunoreactive PGE2 and TxB2 levels in culture supernatants. The specific activities of 3H- and 14C-PGE2 during the 0-2 hour interval were similar and approximately three times higher than the corresponding TxB2 specific activities. By 24 hours, the specific activities of 3H-PGE2 and 3H-TxB2 converged to a lower level while the 14C-TxB2 specific activity increased to almost equal that of 14C-PGE2.

In addition, this study attempted to examine whether differences in PGE2 and TxB2 specific activities could be attributed to the selective conversion of arachidonic acid from specific sources, either free exogenous arachidonic acid or arachidonic acid incorporated in membrane phospholipids. Monocytes were pulse-labeled for two hours with both 0.1 μCi/ml 3H- and 14C-labeled arachidonic acid. Cells were then washed and treated continuously with medium containing LPS and either 14C-arachidonic acid alone, 3H-arachidonic acid alone, or both 14C-arachidonic acid and 3H-arachidonic acid at 0.1 μCi/ml. 3H/14C ratios for radiolabeled PGE2 and TxB2 in culture supernatants were intermediate between 3H/14C ratios for free arachidonic acid and the major phospholipids
(phosphatidylinositol, phosphatidylcholine and phosphatidyl-
ethanolamine) during the 0-2 and 2-8 hour time intervals regardless
of continuous label used. These experiments showed that both
exogenous and endogenous sources of arachidonic acid serve as
sources of TxB₂ and PGE₂. Therefore, the differences in metabolic
specific activities between PGE₂ and TxB₂ cannot be accounted for
by selective metabolism of either source of arachidonic acid (42).

Even though there is abundant evidence in the literature which
suggests that human peripheral blood monocytes may consist of
functionally different subsets, conclusive evidence is lacking for
several reasons. First, several different techniques were used to
isolate human peripheral blood monocytes, thereby making
interpretation of the literature difficult. In a number of studies,
adherence was used to enrich mononuclear cell preparations.
Monocytes obtained by adherence are often activated by this
isolation procedure. Second, detection of cell surface markers with
monoclonal antibodies was not used to rule out the presence of
other contaminating peripheral blood elements, particularly
lymphocytes, in monocyte cultures. "Pure" monocyte preparations
isolated by adherence often contain plastic adherent, activated
lymphocytes or phagocytic granulocytes. Third, culture conditions
and stimuli used to activate the monocytes were not uniform.
Furthermore, no mention was made of monocyte secretory product
release from unstimulated monocyte cultures in a number of these
studies. Finally, DNA assays to correct for cell number were not
performed in several of these investigations.
Evidence For Monocyte Phenotypic Heterogeneity

Monoclonal antibodies have been developed in an attempt to define distinct monocyte populations. Breard et al. used OKM1 (CD11b), a monoclonal antibody to the C3bi receptor, to define two populations of monocytes that differed with respect to cell size, adherence properties and surface la antigens (9,53). Shen et al. used OKM1 and OKM5 to isolate two functionally distinct human monocyte subsets (54). These investigators identified a rosette negative, OKM1−, OKM5+ subset that triggered the autologous mixed lymphocyte reaction, and a rosette negative, OKM1+, OKM5− subset that could only minimally trigger this reaction (54). Because OKM1 is expressed on greater than 90% of peripheral blood monocytes and OKM5 is expressed on 75% of peripheral blood monocytes, the results of this study have been questioned (36). Furthermore, because 1) OKM1 is expressed by 30% of peripheral blood lymphocytes and most natural killer (NK) cells, neutrophils and eosinophils, and 2) OKM5 is also reactive with platelets, the presence of other peripheral blood elements in monocyte isolates could have had a great impact on the observed results. Finally, Talle et al. demonstrated reactions of OKM3 and OKM6 with subsets of adherent monocytes (55). Unfortunately, these antibodies have never become commercially available and no additional reports on their characteristics have appeared in the literature.

Raff et al. have reported that cells positive for Mac-120 and HLA-DR can function in the genetically restricted presentation of antigen to T cells, while cells negative for Mac-120 and positive for
HLA-DR cannot (50). In addition, this group demonstrated that Mac-120 positive cells were the most effective stimulators of the autologous mixed lymphocyte reaction. However, the staining pattern of Mac-120 does not reveal the presence of subpopulations (26), and the monoclonal antibody and clone responsible for its production are no longer available. This antigen is not generally accepted as a monocyte subpopulation marker.

Several groups over the last few years have identified cell surface antigens that are expressed only in response to monocyte activation by either LPS, phorbol esters, or muramyl dipeptide, a structurally defined fragment of bacterial peptidoglycan (18,30,59). Although these monoclonal antibodies have not yet been reported to identify a subset that has greater functional activity, these antibodies do serve as excellent markers of monocyte activation. Todd et al. produced a monoclonal antibody, anti-Mo3e, whose expression is increased by LPS (59). A more detailed description of this monoclonal antibody will follow. Furthermore, Ewan et al. developed a monoclonal antibody, A1-3, which binds selectively to LPS-stimulated human peripheral blood monocytes and is associated with procoagulant activity (18). In an immunohistochemical study using this antibody, Hancock et al. found restriction of A1-3 binding to monocytes in inflammatory tissues and not in normal tissues (30). Similarly, Zwadlo et al. produced a monoclonal antibody, 27E10, that detects a surface antigen found only on macrophages in inflammatory tissues and not in normal tissues, and detects an antigen that is present on only 20% of peripheral blood monocytes (68). 27E10 antigen density is increased by stimulating monocytes
in culture with γ-interferon (IFN), 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and LPS. Interestingly, Zwadlo et al. have also described a surface antigen, RM3/1, that is down-regulated by LPS, γ-IFN, and TPA and appears to be associated with the healing phase of the inflammatory process (69).

At this time, however, no monoclonal antibody is uniformly accepted as a marker of a distinct monocyte subpopulation.

**Characteristics of Mo3e and Leu-M3**

Freshly isolated adherent human monocytes express barely detectable amounts of the Mo3e antigen on their cell surfaces (59). However, following an 18-24 hour incubation at 37°C in RPMI 1640 medium containing *E. coli* LPS, monocytes demonstrate a significant increase in surface Mo3e expression (59). On the other hand, monocytes cultured for the same duration in LPS-free medium express surface Mo3e levels comparable to those observed for freshly isolated cells (59). In addition, muramyl dipeptide, phorbol myristate acetate (PMA) and other biologically active phorbol compounds cause a dose-dependent increase in surface Mo3e expression by monocytes cultured for 18-72 hours in medium containing these agents (59). Mo3e has been termed an activation-associated antigen because it is selectively expressed by activated macrophages and monocytes. However, γ-IFN has no effect on Mo3e expression. The Mo3e antigen is specific for monocytes and macrophages and it is identified by an IgM monoclonal antibody, anti-Mo3e (35,59).
The Mo3e antigen is a protein determinant and is, therefore, a protease-sensitive antigen (60). Mo3e expression is temperature dependent since incubation of monocytes at 4°C prevents its expression, which suggests that Mo3e expression depends on active cellular metabolism. Furthermore, protein synthesis inhibitors such as cycloheximide and puromycin block Mo3e expression by LPS-stimulated monocytes. Finally, pre-treatment of monocytes with the anti-Mo3e antibody blocks their response to migration inhibitory factor (MIF). This effect is specific since other IgM monoclonal reagents fail to block MIF activity (35). These data suggest that Mo3e is the MIF receptor (35).

The Leu-M3 antigen (CD 14) was first described by Dimitriu-Bona et al. (14). The MøP-9 clone is responsible for the IgG2b monoclonal antibody that recognizes Leu-M3, a pan-monocytic marker that is present on 70-90% of human peripheral blood monocytes, 77-90% of pleural or peritoneal fluid phagocytic cells, and 86-92% of synovial mononuclear phagocytes (Becton-Dickinson technical bulletin). The Leu-M3 antigen is found at high densities on monocyte and macrophage cell surfaces. Leu-M3 is present at very low antigen densities on a minimal fraction of peripheral blood granulocytes (14), and does not react with lymphocytes, erythrocytes, or platelets.
RATIONALE AND SPECIFIC OBJECTIVES

The independent synthesis of TxB₂ and PGE₂ by counterflow-isolated human monocytes suggests the presence of monocyte subsets with different secretory responses (42). Furthermore, although inconclusive, there are numerous papers in the literature which suggest that monocyte subpopulations exist and differ with respect to PGE₂ and IL-1β release, Fc receptor expression, colony-stimulating activity, H₂O₂ production and tumoricidal capacity (16,25,32,44,45,47,56,61,62,66).

It was reported that peripheral blood monocytes from patients with severe periodontitis secreted two to three times more PGE₂ in response to LPS from Bacteroides gingivalis, B. intermedius, Actinobacillus actinomycetemcomitans or Salmonella typhimurium than peripheral blood monocytes of sex-, age-, and plaque-matched patients without attachment loss (24). However, monocyte secretion of IL-1β did not significantly differ between the two patient groups. A PGE₂ hypersecretory response has also been observed in peripheral blood monocytes isolated from patients with Hodgkin's disease when compared to monocytes isolated from healthy control patients (17,27).

Because monocyte subpopulations that differ with respect to PGE₂ release may exist, it is tempting to speculate that the hypersecretory responses observed in severe periodontitis and Hodgkin's disease patients may be a result of hypersecretion of a distinct subset. In addition, the differential specific activities of TxB₂ and PGE₂ may be a reflection of different secretory responses
from distinct monocyte subpopulations. It is the purpose of this thesis to assess PGE$_2$, TxB$_2$ and IL-1$\beta$ release from LPS-stimulated monocyte subsets generated by either countercurrent centrifugal elutriation or discontinuous Percoll density centrifugation, the major methods that have been used in previous investigations to fractionate peripheral blood monocytes into subpopulations. However, no study in the literature has evaluated monocyte subpopulations generated by both methods and no investigation has combined the two techniques to generate monocyte subsets. Counterflow centrifugal elutriation was selected to enrich mononuclear cell preparations for monocytes because elutriation, as opposed to adherence, does not activate monocytes and allows for rapid isolation of peripheral blood monocytes in high purity (20,28,37,52). In addition, Mo3e expression was examined to determine if differences in cell surface expression of this activation antigen could be correlated with differences in functional responses between subpopulations. Mo3e expression was examined relative to expression of Leu-M3, the pan-monocytic marker which served as a positive control.
ABSTRACT

The purpose of this investigation was to separate counterflow-isolated human peripheral blood monocytes into subpopulations by either discontinuous Percoll density gradients or countercurrent centrifugal elutriation and to assess PGE$_2$, TxB$_2$ and IL-1$\beta$ release by each of the subsets in response to *Salmonella typhimurium* LPS. In addition, surface antigen expression of Leu-M3, a panmonocytic marker, and Mo3e, an LPS-associated antigen, was examined to determine if Mo3e expression could be correlated with differences in functional responses. PGE$_2$, TxB$_2$ and IL-1$\beta$ levels in culture supernatants were determined by radioimmunoassay.

After DNA correction, PGE$_2$, TxB$_2$ and IL-1$\beta$ release did not differ significantly between LPS-stimulated monocyte fractions isolated by either countercurrent centrifugal elutriation or Percoll density centrifugation. In addition, although the specific activities of PGE$_2$ and TxB$_2$ differed throughout the 24 hour LPS treatment period, the specific activities did not differ between counterflow-isolated subpopulations. Furthermore, Leu-M3 and Mo3e antigen expression on the monocyte cell surface did not significantly differ at 0, 8, or 24 hours between subpopulations isolated by either separation technique. These data suggest that human peripheral blood monocyte subpopulations separated by physical means are not heterogeneous with respect to PGE$_2$, TxB$_2$ and IL-1$\beta$ release as has been previously reported and that the different specific activities of TxB$_2$ and PGE$_2$ may be due to independent metabolism within a single
population of cells rather than by different monocyte subpopulations.
INTRODUCTION

Numerous investigators in the past decade have physically separated human peripheral blood monocytes into subpopulations on the basis of density or size in an attempt to determine whether monocyte subsets with different functional capabilities can be isolated. Counterflow centrifugal elutriation has been used to fractionate monocytes into subpopulations that differ in Fc receptor activity (45), CSF release and myeloperoxidase activity (66), H2O2 production (62) and tumoricidal activity (44). Centrifugation through discontinuous Percoll and BSA density gradients has yielded fractions exhibiting differences in colony-stimulating activity (56), PGE release (4,25,32,47) and IL-1 production (16,32). Finally, adherence to plastic or glass has been used to separate monocytes into subpopulations and Treves et al. found that loosely adherent monocytes secreted higher amounts of IL-1 than strongly adherent monocytes (61). However, a number of these studies have been criticized because either significant lymphocyte contamination of monocyte cultures existed, activation of monocytes by the isolation technique occurred or DNA assays were not performed to correct for cell number.

The capacity of unfractionated counterflow-isolated human peripheral blood monocytes to independently synthesize TxB2 and PGE2 when stimulated with bacterial LPS has been previously demonstrated (42). Independent metabolism was confirmed by showing that TxB2 and PGE2 had different specific activities within each time interval during a 24 hour culture period. These data
suggest that distinct monocyte subpopulations with different secretory responses may exist within an unfractionated isolate of cells. It is uncertain whether these differential specific activities are due to the existence of physically separable monocyte subsets. Indeed, such monocyte functional heterogeneity may reflect the existence of discrete cell lineages, subsets representing different activation states or maturation states (21) or distinct metabolic processes within a single subset of cells.

Attempts have been made to define discrete monocyte subpopulations using monoclonal antibodies (9,50,54,55). However, conclusive evidence that subpopulations differ with respect to cell surface antigen expression is lacking. Because elaboration of inflammatory mediators by monocytes is dependent on activation of these cells by LPS, lymphokines, and other soluble factors, it was important to identify activation-associated antigenic determinants on the monocyte cell surface. Monoclonal antibodies to LPS-associated antigenic determinants are now available (18,30,35,59,60) and may be important in identifying monocyte subsets in different stages of activation. Todd et al. have characterized the Mo3e antigenic determinant and have found that freshly isolated monocytes express barely detectable amounts of Mo3e on their cell surfaces (35,59). However, after a 24 hour exposure to LPS, a significant increase in Mo3e expression is seen relative to monocytes exposed to LPS-free medium.

It is the purpose of this present investigation to determine whether physical separation of counterflow-isolated monocytes into subpopulations by either discontinuous Percoll density gradients or
countercurrent centrifugal elutriation yields subpopulations with distinct secretory responses. PGE\textsubscript{2}, TxB\textsubscript{2} and IL-1\textbeta release by LPS-treated human monocytes were assessed. Il-1\textbeta production was investigated because 1) recent evidence suggests that the release of IL-1 and PGE\textsubscript{2} from monocytes is interrelated (34) and 2) 90% of the IL-1 produced by LPS-stimulated human monocytes is IL-1\textbeta (46). In addition, Mo3e expression relative to the expression of Leu-M3, a panmonocytic marker that served as a positive control, was examined to assess whether differences in surface Mo3e expression among the subpopulations could be correlated with differences in functional responses.
MATERIALS AND METHODS

Materials

\[^{3}\text{H}\]Arachidonic acid (sp. act., 100 Ci/m mole), \[^{3}\text{H}\]TxB\(_2\), and \[^{3}\text{H}\]PGE\(_2\) were obtained from New England Nuclear (Boston, MA). Ficoll, TxB\(_2\), PGE\(_2\), penicillin, streptomycin, 4',6-diamidino-2-phenyl-indole (DAPI), FITC-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-mouse IgM, mouse IgG\(_{2b}\) (MOPC 141), and mouse IgM (TEPC 183) were obtained from Sigma Chemical Co. (St. Louis, MO). Hypaque was obtained from Winthrop-Breon Laboratories (NY,NY). Fluoresceinated beads were obtained from Flow Cytometry Standards Corporation. Falcon plastic culture dishes (16 mm) were obtained from Becton Dickinson Labware (Lincoln Park, NJ). Anti-PGE\(_2\) and Anti-TxB\(_2\) were obtained from Advanced Magnetics, Inc. (Cambridge, MA) and fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT). The IL-1\(\beta\) radioimmunoassay kit was obtained from Cistron Biotechnology (Pine Brook, NJ). LPH-K thin-layer plates (10x20 cm, precoated 200 \(\mu\)m, silica gel G) were purchased from Whatman International LTD. (Maidstone, England). Bacterial lipopolysaccharide (Salmonella typhimurium) was obtained from Difco Laboratories (Detroit, MI) and anti-Leu-5b (isotype IgG\(_{2a}\)), anti-Leu-12 (isotype IgG\(_1\)) and anti-Leu-M3 (isotype IgG\(_{2b}\)) were obtained from Becton-Dickinson (Mountain View, CA). Anti-Mo3e (isotype IgM) was kindly provided by Dr. Robert Todd (University of Michigan School of Medicine).
Cell Isolation

Healthy random donors served as the sources of whole blood. Subjects who had taken non-steroidal anti-inflammatory drugs within the past week were excluded from the study. Citrated fresh human blood, obtained by venipuncture, was layered over Ficoll-Hypaque (1.077g/ml) (8) and sedimented at 400 x g (35 min., 10°C). The mononuclear leukocyte fraction was collected, diluted and washed in phosphate-buffered saline (PBS) containing 0.27 mM EDTA (2x, 125 x g, 15 min., 10°C). This fraction was then subjected to counterflow centrifugation (Beckman J2-21 centrifuge with JE-6B rotor, Palo Alto, CA) to remove lymphocytes and platelets and yield a highly enriched monocyte population, as previously described (51). Elutriation was monitored by electronic counting (Coulter ZM-counter, Coulter Electronics, Hialeah, Florida). Following counterflow isolation, monocytes were pelleted in PBS containing 1% autologous plasma (200 x g, 15 min., 10°C). Monocytes were diluted in MCDB 104 medium (38) supplemented with 1% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, at a concentration of 10^6 cells/ml. Cells were plated at 10^6 cells/well in 16 mm plastic wells. All incubations were carried out at 37°C in a H₂O-saturated 5% CO₂ atmosphere. After two hours, the medium was exchanged to remove any nonadherent cells. At this time (time zero), 10 μg/ml of Salmonella typhimurium LPS was added to half the wells. The other wells served as controls. Medium was removed at 2, 8, 16 and 24 hours and was replaced with fresh identical medium, and the supernatants were collected and
frozen. For experiments evaluating labeled metabolite release, labeled arachidonic acid (0.1 μCi/ml) was added to culture medium either during the two hour adherence period only for pulse-labeled cultures or during the two hour adherence period and the 24 hour LPS treatment period for continuously-labeled cultures.

**Incubation of Nonadherent Monocytes**

Some media samples removed at 0, 2, 8, and 16 hours were transferred to different culture wells and incubated until the 24 hour time point. These supernatants included nonadherent monocytes (cells that had detached from the plates) and eicosanoids produced by adherent cells during either the 0-2, 2-8 or 8-16 hour time intervals. This additional incubation was designed to determine the contribution of the nonadherent monocytes to overall eicosanoid production.

**Separation of Monocytes into Subpopulations**

Fractionation of monocytes into subpopulations by elutriation was achieved by varying the flow rate of the eluting buffer. After the lymphocytes were eluted (mean=11 ml/min) the flow rate was increased (mean=13.8 ml/min) until the small monocytes began to elute. Once the small monocytes eluted, the superior portion of the Sanderson chamber clarified and a new interface formed. At this time, the flow rate was once again increased (mean=15 ml/min) until the intermediate-sized monocytes began to elute. After these
monocytes were collected, the remaining subpopulation was eluted from the chamber by terminating centrifugation and flushing cells from the rotor.

Fractionation of monocytes into subpopulations by discontinuous density centrifugation was achieved by layering the whole counterflow-isolated monocyte preparations over 10, 40, 45 and 50% Percoll (40, 45 and 50% Percoll corresponded to specific gravities of 1.048, 1.056, and 1.063 g/ml, respectively). Attempts were made to use density gradients of 35 and 55% Percoll; however, too few cells were isolated from the interfaces with these gradients. Monocyte subpopulations were aspirated from density interfaces and pelleted in PBS to remove any remaining Percoll (2x, 200 x g, 15 min., 4°C) prior to placing them into culture.

Radioimmunoassay for PGE_2, TxB_2 and IL-1β

Levels of PGE_2 and TxB_2 in culture supernatants were determined by radioimmunoassay using immunoadsorption to dextran-coated charcoal. Levels of IL-1β in culture supernatants were determined by a radioimmunoassay which utilized a polyethylene glycol second antibody to precipitate antibody-antigen complexes (according to Cistron technical bulletin). All determinations were carried out in duplicate and the average counts for each pair were used to calculate PGE_2, TxB_2 and IL-1β levels. Regression coefficients for logit-log-transformed standard curves were generally greater than 0.990.
Labeled Arachidonic Acid Metabolite Extraction and Separation

Arachidonic acid metabolites were extracted by adjustment of the pH of culture supernatants to 3.5 with formic acid (70%) followed by 3 3-ml washes with ethyl acetate. The combined organic extracts were evaporated to dryness under vacuum and stored at -20°C under nitrogen. Prior to thin-layer chromatography, arachidonic acid (5 μg), PGE₂ (5 μg) and TxB₂ (5 μg) were added to each sample and the contents were dried under a stream of nitrogen. Individual samples were dissolved in 10 μl of chloroform, mixed vigorously, and spotted on silica gel G plates. Three additional washes with 5 μl of chloroform were then performed and each wash was plated immediately below the previous application. Plates were then developed in one dimension with chloroform/methanol/acetic acid/H₂O (90:8:1:0.8, vol/vol) (65). After drying, the plates were sprayed with H₂O and the lipid spots were visualized. Arachidonic acid metabolites were identified by co-migration with authentic standards (Rf values were 0.80 for arachidonic acid, 0.512 for PGE₂ and 0.40 for TxB₂). The plates were then scraped and counts determined.

Determination of Cell DNA Content

DNA content of adherent monocytes was determined at 0, 8 and 24 hours. Cells were treated with 0.5 ml of 5 mM NaOH and sonicated briefly. After a one week incubation with NaOH to insure
complete solubilization of the DNA, 100 µl from each well was assayed for DNA content by the 4',6-diamidino-2-phenyl-indole fluorometric assay of Brunk et al. (10).

**Flow Cytometric Analysis**

One million monocytes isolated by counterflow centrifugal elutriation or Percoll density centrifugation were placed into 12 X 75 mm siliconized glass test tubes. At either 0, 8, or 24 hours, the suspension was centrifuged at 200 x g (5 min., 4°C). Saturating doses of anti-Leu-M3 or anti-Mo3e were incubated with the pellet (30 min., 4°C). Following two washes with PBS containing 5 mM EDTA, the test cells were incubated with fluorescein-conjugated goat anti-mouse immunoglobulin of the appropriate isotype in antibody excess (30 minutes, 4°C). The cells were then washed twice and fixed in 10% buffered formalin. Prior to FACS analysis, cells were centrifuged at 200 x g (5 min., 4°C) and suspended in 0.5 ml Hematall. Ten thousand cells were examined by the FACS Analyzer (Becton Dickinson, Mountain View, CA) and the channel number representing the mean fluorescence intensity for each monoclonal antibody was determined. The FACS Analyzer was calibrated each day with fluoresceinated beads and the photomultiplier tube setting was held constant for each experiment (400 V at maximum resonance). Cell viability was evaluated by propidium iodide exclusion and always exceeded 90%. The extent of T and B lymphocyte contamination was assessed with anti-Leu-5b and anti-
Leu-12, respectively. The irrelevant monoclonal antibodies MOPC 141 (IgG₂b) and TEPC 183 (IgM) served as negative controls.

**Data Analysis**

Monocyte subpopulation means for PGE₂, TxB₂ and IL-1β release after DNA correction were compared by two-way ANOVA, with treatment and fraction as the independent grouping-variables. Monocyte subpopulation means for PGE₂ and TxB₂ specific activities were compared by three-way ANOVA, with treatment, fraction and time interval as the independent grouping-variables.
RESULTS

Distribution of Monocytes into Subpopulations

The mean distribution of monocytes isolated by discontinuous Percoll density centrifugation was as follows: Fraction 1, harvested from the density interface between 10 and 40% Percoll, contained 12% of the cells, while Fraction 2, harvested at the density interface between 40 and 45% Percoll, contained 30% of the cells, and Fraction 3, harvested at the density interface between 45 and 50% Percoll, contained 58% of the cells. The small, medium, and large monocytes isolated by counterflow centrifugal elutriation represented 27, 31, and 42%, respectively, of all recovered monocyte isolates from six donors.

Coulter Profiles for Percoll-Isolated and Counterflow-Isolated Monocyte Subpopulations

Percoll fractionation resulted in a substantial separation of monocytes by relative cell size (Figure 1). The three subpopulations isolated by elutriation differed markedly with respect to elution properties, although their relative cell sizes were not as disparate as monocyte fractions isolated by discontinuous Percoll gradients (Figure 2).
PGE\textsubscript{2}, TxB\textsubscript{2} and IL-1\textbeta Production by Percoll Fractions

In general, PGE\textsubscript{2} release from fractions 2 and 3 was similar for all time intervals (Figure 3). However, monocytes from fractions 2 and 3 secreted two to three times more PGE\textsubscript{2} than monocytes from fraction 1. Even though the same number of monocytes was plated initially for all fractions, fraction 1 cultures retained only half as much DNA per well as fractions 2 and 3 when compared at 8 and 24 hours (Table 1). Two-way ANOVA revealed no statistically significant difference between fractions for PGE\textsubscript{2} release, after DNA correction.

Similarly, TxB\textsubscript{2} release from fractions 2 and 3 was virtually identical for all time intervals (Figure 4). However, cells from fraction 1 secreted two to four times less TxB\textsubscript{2} than cells from the other two fractions. As for PGE\textsubscript{2}, TxB\textsubscript{2} release after DNA correction did not differ significantly between fractions when assessed by two-way ANOVA (Table 1). Furthermore, all three fractions exhibited the typical pattern of immunoreactive TxB\textsubscript{2} release: peak release during the 2-8 hour interval followed by a diminution in TxB\textsubscript{2} release at the 16-24 hour interval to levels below those observed for the 0-2 hour interval.

The vast majority of IL-1\textbeta release occurred during the 2-8 hour interval. For this culture interval, IL-1\textbeta release from fractions 2 and 3 was virtually identical. However, IL-1\textbeta secretion by Fraction 1 was two and one-half times less than IL-1\textbeta secretion by the other two fractions (Figure 5). After DNA correction, there was
no statistically significant difference between fractions with respect to IL-1\(\beta\) release (Table 1).

LPS-stimulated eicosanoid and IL-1\(\beta\) release was always significantly greater than the control release of these mediators for all fractions for all time intervals.

**PGE\(_2\), TxB\(_2\) and IL-1\(\beta\) Production by Elutriator Fractions**

PGE\(_2\) release did not differ between fractions for any of the time intervals (Figure 6). In contrast to Percoll fractions, DNA correction did not change this finding because the DNA remaining per well at 24 hours was virtually identical for each fraction and for both control and LPS-stimulated cultures (Table 2). In addition, TxB\(_2\) and IL-1\(\beta\) release did not differ between fractions for each time interval (Figures 7 and 8). Likewise, DNA correction did not alter these findings (Table 2).

**Specific Activities of PGE\(_2\) and TxB\(_2\) for Pulse-Labeled Elutriator Fractions**

The specific activity of PGE\(_2\) for the 0-2 hour time interval was three and a half times higher than for TxB\(_2\) (Figures 9 and 10). The specific activities of both PGE\(_2\) and TxB\(_2\) converged to a lower level by the 16-24 hour interval, although PGE\(_2\) still maintained a specific activity that was twofold higher than that of TxB\(_2\). However, even though TxB\(_2\) and PGE\(_2\) had different specific activities throughout the 24 hour time course, the specific activities of PGE\(_2\)
and TxB$_2$ did not differ between fractions. Control values are not included in Figures 9 and 10 because the amount of immunoreactive eicosanoid produced by monocytes not exposed to LPS was so low that specific activity calculations were not meaningful.

**Specific Activities of PGE$_2$ and TxB$_2$ for Continuously-Labeled Elutriator Fractions**

PGE$_2$ had a specific activity that was four and a quarter times greater than that of TxB$_2$ during the 0-2 hour interval (Figure 11 and 12). However, during the 24 hour time course, the specific activity of PGE$_2$ fell while the specific activity of TxB$_2$ rose so that by the 16-24 hour time interval, the specific activity of TxB$_2$ actually overtook that of PGE$_2$. Once again, the specific activities of both TxB$_2$ and PGE$_2$ did not differ between fractions for any of the time intervals.

**Leu-M3 and Mo3e Expression on Percoll-Isolated Monocyte Subpopulations**

A representative dot plot and FACS profiles are illustrated in Figure 13. Mo3e and Leu-M3 expression did not differ significantly between fractions at 24 hours (Table 3). Mo3e expression on the cell surface of the third Percoll fraction (the smallest monocytes) was 27% less than Mo3e expression on the cell surface of the first Percoll fraction (the largest monocytes). Although the mean fluorescence intensity for Mo3e was highest for the largest
monocytes, it is likely that relative Mo3e antigen density is comparable between fractions because the largest cells also have the greatest cell surface area.

**Leu-M3 and Mo3e Expression on Counterflow-Isolated Monocyte Subpopulations**

Mo3e antigen expression on counter-flow-isolated monocyte subpopulations was not detectable at time zero, since its mean fluorescence intensity was virtually the same as the mean fluorescence intensity of the negative control antibody. However, after an 8 hour exposure to LPS, Mo3e antigen expression was significantly increased (Table 4). A twofold increase in mean fluorescence intensity was seen between 8 and 24 hours. Nevertheless, no differences between fractions with respect to mean fluorescence intensity were observed for any of the time intervals. Greater than 80% of the monocytes were positive for the Mo3e antigen at 8 hours and greater than 90% of the monocytes were positive for Mo3e at 24 hours.

Leu-M3 antigen expression on counterflow-isolated monocyte subpopulations also did not differ between fractions (Table 5). The mean fluorescence intensity for the Leu-M3 antigen actually diminished at 8 hours for all three fractions but increased by 24 hours to levels that were 68% higher than at 0 hours.
DISCUSSION

In contrast to previous reports (16,25,32,47,56,61), this study demonstrated that Percoll-isolated and counterflow-isolated human peripheral blood monocyte subpopulations do not differ with respect to PGE₂, TxB₂ and IL-1β release. It is likely that our findings differ from those results previously reported for three reasons. First, counterflow centrifugal elutriation, which allows rapid isolation of human peripheral blood monocytes in high purity, was used rather than adherence to enrich mononuclear cell preparations. Second, indirect immunofluorescence confirmed negligible lymphocyte contamination of our monocyte preparations. Finally, DNA assays were performed to correct for cell number. This paper also represents the first report in the literature to examine relative antigen expression of monocyte markers on different subpopulations; Leu-M3 and Mo3e antigen expression did not differ between subsets isolated by either Percoll density centrifugation or counterflow centrifugal elutriation.

Monocyte purity was confirmed by showing that 70-90% of counterflow-isolated monocytes expressed the Leu-M3 antigen and that negligible numbers of cells expressed Leu-12 or Leu-5b. Microscopic examination of cells using Wright's stain revealed the absence of polymorphonuclear leukocytes and eosinophils in culture wells. Minimal platelet contamination of the monocyte preparations was observed by phase contrast microscopy. It has been demonstrated that PGE₂ and TxB₂ release from monocytes incubated with a tenfold excess of platelets is comparable in control and LPS-
stimulated cultures (42). In addition, PGE\textsubscript{2} and TxB\textsubscript{2} release from monocytes cultured with this excess of platelets is maximal during the 8-16 hour time interval, rather than the 2-8 hour time interval. Had there been substantial platelet contamination of the monocyte cultures, this nonspecific elevation and different pattern of eicosanoid release would have been observed (42).

The anti-PGE\textsubscript{2} antibody demonstrates 100% cross-reactivity with PGE\textsubscript{1}, 9% with 15-HETE, 6% with PGA\textsubscript{2}, 3% with PGA\textsubscript{1}, 1.3% with PGF\textsubscript{2}, and less than 1% cross-reactivity with other related eicosanoids at 50% bound/total (42). However, it has been shown using gas chromatography-mass spectroscopy that 97% of PGE production by human monocytes in response to bacterial LPS is PGE\textsubscript{2} (25, 42). In addition, LPS-treated monocytes release relatively small amounts of eicosanoid products capable of cross-reacting in the PGE radioimmunoassay (41). Furthermore, the anti-TxB\textsubscript{2} antibody demonstrates no significant cross-reactivity (less than 0.1%) with any of the aforementioned products. Finally, the IL-1\textbeta antiserum used in the IL-1\textbeta radioimmunoassay is specific for IL-1\textbeta and exhibits no cross-reactivity with IL-1\textalpha, IL-2, TNF or γ-IFN (Cistron technical bulletin). Therefore, based on this information, the PGE, TxB\textsubscript{2} and IL-1\textbeta radioimmunoassays were considered acceptable to quantitate the release of immunoreactive PGE\textsubscript{2}, TxB\textsubscript{2} and IL-1\textbeta.

The use of Percoll density centrifugation resulted in reduced cell yield and higher control eicosanoid release when compared to the elutriator fractions. Because the cell yield was limited, DNA corrections at 8 hours and determinations of mean fluorescence
intensity at 0 and 8 hours could not be determined in every experiment. However, for those experiments when there was enough cellular material, the ratio of DNA remaining between fractions 2 or 3 and fraction 1 at 8 hours was virtually the same as the ratio at 24 hours. In addition, the mean fluorescence intensity did not differ significantly between fractions at eight hours and monocytes from all three fractions expressed barely detectable levels of the Mo3e antigen at time zero.

Previous studies (16, 25, 56) have demonstrated that higher density monocytes produce greater amounts of PGE2 and IL-1. Our lowest density fraction corresponded to the same specific gravity and represented the same percentage of total monocytes as the lowest density fraction described by Tice et al. (56). However, because Fraction 1 never entered the gradient and only half as much DNA was retained in its culture wells at 24 hours than in the other two fractions, it is likely that this fraction represented a damaged cell population. Furthermore, the nonadherent cells (cells which were not retained in the culture wells), although positive for Leu-M3, did not produce appreciable amounts of TxB2, PGE2, or IL-1β at any time point.

Consistent with previous reports, Mo3e expression on the monocyte cell surface was significantly increased with exposure to LPS for both Percoll-isolated and counterflow-isolated subsets (35, 59). Indeed, Mo3e was not expressed on the cell surface at 0 hours and an increase in its expression was seen after an 8 and 24 hour exposure to LPS for all subsets, although monocytes in LPS-free medium for 24 hours did not demonstrate cell surface Mo3e
expression. Mo3e antigen expression was even higher after 48 and 72 hours of LPS exposure; however, PGE\(_2\) and TxB\(_2\) release between 24 and 72 hours in culture was not significantly higher than control levels (23). Leu-M3 expression also increased after a 24 hour exposure to LPS, although there was actually a diminution in mean fluorescence intensity at 8 hours, which suggests shedding or internalization of the Leu-M3 antigen between 0 and 8 hours. Although both Leu-M3 and Mo3e expression differed markedly between 0 and 24 hours, no significant differences in Leu-M3 or Mo3e expression were observed between subpopulations isolated by either Percoll density centrifugation or counterflow centrifugal elutriation at any time point.

This study reveals no human monocyte subpopulation primarily responsible for PGE\(_2\), TxB\(_2\) or IL-1\(\beta\) release. Such subpopulations may exist, but current means of separating monocytes into subpopulations based on density and/or size did not allow for subset detection. Our inability to define distinct monocyte subpopulations is in agreement with a recent study by Dransfield et al. (15). However, the results of this experiment suggest that the independent synthesis of TxB\(_2\) and PGE\(_2\) observed in our laboratory may be due to distinct metabolic processes within a single population of monocytes and not due to the existence of discrete subpopulations. Differential release of immunoreactive TxB\(_2\) and PGE\(_2\) could result from independent activities of the thromboxane synthetase and prostaglandin isomerase enzymes, but the disparate specific activities cannot be explained in this way. Differentially labeled arachidonic acid pools and independent cyclooxygenase
metabolic compartments within a single subset of cells may be responsible for the differential specific activities of TxB₂ and PGE₂ released from LPS-activated monocytes. Confirmation of the divergent specific activities of PGE₂ and TxB₂ by gas chromatography-mass spectroscopy and exploration of the possibility of independent cyclooxygenase metabolic compartments will be the focus of future studies. Finally, the data in this study suggest that the PGE₂ hypersecretory response observed in patients with severe periodontitis (24) and in patients with Hodgkin's disease (17,27) may reflect hypersecretion of the entire monocyte population rather than only a particular subset.
This investigation demonstrated that Percoll-isolated and counterflow-isolated monocyte subpopulations do not differ with respect to PGE$_2$, TxB$_2$ and IL-1$\beta$ release or Leu-M3 and Mo3e surface antigen expression. In the majority of previous studies, adherence was used to enrich mononuclear cell preparations for monocytes. It is possible that this isolation technique coupled with the physical removal of monocytes from plastic culture wells prior to assessing functional activity activated the whole monocyte population, and possibly some monocytes more than others. This phenomenon is likely since some monocytes are more adherent and, therefore, may be activated by the scraping procedure to a greater extent than less adherent monocytes. Furthermore, in each of these studies, monocyte purity was assessed by nonspecific esterase staining, morphology or phagocytosis of latex particles. Immunofluorescence was not used to confirm minimal contamination of monocyte cultures with lymphocytes and other peripheral blood elements and immunofluorescence was not used to confirm monocyte identity. However, a number of these studies were conducted before the availability of Leu-M3 and other monoclonal reagents that could have confirmed monocyte purity. Because countercurrent centrifugal elutriation was used in our experimental protocol to enrich mononuclear cell preparations for monocytes and the elutriation process was monitored by electronic sizing, the cells placed in culture in this study were virtually all monocytes. In previous investigations, monocytes were cultured in RPMI 1640 medium at
37°C for one hour with, in all likelihood, a fourfold excess of lymphocytes. Lymphokines elaborated by the lymphocytes may have played a role in the activation of monocytes in culture. Interestingly, in the majority of previous investigations, the functional properties of monocytes in unstimulated cultures were not mentioned.

The differential specific activities of PGE$_2$ and TxB$_2$ may be accounted for by compartmentalization of cyclooxygenase enzymes and differentially labeled arachidonic acid pools. However, compartmentalization of cyclooxygenase enzymes has never been demonstrated. The PGE$_2$ and TxB$_2$ specific activity differences will first be confirmed by gas chromatography-mass spectroscopy (GC-MS) before exploring this possibility, since GC-MS represents the best method for specific estimation of nanogram quantities of prostanoids in the presence of interfering substances.

The patterns of immunoreactive TxB$_2$ and IL-1β release are similar over the 24 hour time course. In addition, between 24 and 72 hours, their release continues to decline and actually equals control levels. However, although peak release for PGE$_2$ also occurs during the 2-8 hour interval, another rise in PGE$_2$ release occurs during the 16-24 hour interval following a decline between 8 and 16 hours. It has been shown that IL-1 can regulate its own production through PGE$_2$, a self-induced inhibitor (34). IL-1 alone can elevate endogenous PGE$_2$ levels and the dose-dependent suppressive effect of exogenous PGE$_2$ on IL-1 production by LPS-stimulated monocytes has been demonstrated (34). These phenomena may be responsible
for the diminution in IL-1β release after 8 hours and the increase in PGE₂ release during the 16-24 hour interval.

Percoll and countercurrent centrifugal elutriation are accepted techniques for separating monocytes into subpopulations, although such separation may be artifactual. This investigation represents the first report in which both techniques were used to fractionate monocytes into subpopulations and in which elutriation was used to isolate monocytes from mononuclear cells prior to Percoll density centrifugation. Even though both techniques resulted in monocyte subpopulations that differed with respect to relative cell size, no functional or phenotypic heterogeneity was noted between subsets. Cell sorting on the basis of cell size and shape may represent another technique that can be used to evaluate potential monocyte heterogeneity, but this method has several limitations that may preclude its implementation. First, 10-90% of the counterflow-isolated monocytes may be lost during cell sorting (31). Second, monocytes may be activated by this technique. Third, since the cell sorter can only process 10⁷ cells per hour, it would take approximately five hours to sort an average donor's monocytes (31). Cells isolated at one hour may differ functionally from those isolated at the termination of cell sorting, merely because of the long isolation procedure.

It has been noted that inter-individual differences with respect to PGE₂ secretion by LPS-stimulated human monocytes far exceed the intra-individual differences observed for replicate blood donations up to six months apart (40, 43). This phenomenon needs to be explored more carefully, particularly since it appears that high
and low responders to LPS may exist, using PGE\(_2\) release as the marker of LPS-responsiveness (17,24). In addition, it would be worthwhile to investigate relative expression of activation-associated antigens on the cell surface of human peripheral blood monocytes in different donors to determine if 1) differences exist between healthy individuals and 2) immunophenotypes remain stable over time. It has been reported that immunophenotypes of healthy individuals examined on a weekly basis for 13 weeks differed less than 5% between donations with respect to T4 and T8 antigen expression (7).

Heterogeneity certainly exists among fluid macrophages. The multitude of functions that these macrophages perform coupled with the evidence for restriction of cell surface markers to macrophages of defined anatomical locations confirms this fact. Whether or not such heterogeneity is evident in human peripheral blood monocytes is still uncertain. Although our investigation did not reveal any differences between subpopulations with respect to PGE\(_2\), TxB\(_2\) or IL-1\(\beta\) release, numerous other monocyte functions such as F\(_c\) receptor-mediated activity, Ia expression, and phagocytosis need to be re-examined using counterflow-isolated monocytes, to assess whether these functional activities differ among subpopulations or whether previous investigations yielded artifactual differences. In addition, further studies should be carried out to compare expression of differentiation antigens on bone marrow precursors, blood monocytes and tissue and fluid phase macrophages. Hancock et al. have already shown that PHM3 labels a differentiation antigen expressed on monocytes only after entry into the circulation (29).
This antigen appears to be restricted in its distribution to circulating monocytes, as it is present only at very low antigen densities on peritoneal macrophages and Kupffer cells. Furthermore, FMC17 appears to be organ selective since its expression is confined to alveolar macrophages (29). Relationships between bone marrow precursor cells, monocytes, macrophages and other hematopoietic elements can only be elucidated by further studies which characterize the cell surface differentiation antigens of the mononuclear phagocyte system. In addition, studies which correlate functional activities that are restricted to certain tissue sites or fluids with the presence of particular cell surface antigens will enable investigators to determine whether peripheral blood monocytes are a homogeneous, multipotent population or whether discrete monocyte subsets exist with predetermined capabilities to differentiate into specific tissue or fluid macrophages.
Table 1. PGE$_2$, TxB$_2$ and IL-1$\beta$ Release from Percoll-Isolated Monocyte Fractions After DNA Correction

<table>
<thead>
<tr>
<th>Fraction #</th>
<th>PGE$_2$</th>
<th>TxB$_2$</th>
<th>IL-1$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Control)</td>
<td>0.25</td>
<td>0.12±0.11</td>
<td>0.01±0.01</td>
</tr>
<tr>
<td>1 (LPS)</td>
<td>0.32±0.07</td>
<td>0.95±0.30</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>2 (Control)</td>
<td>0.04±0.04</td>
<td>0.06±0.04</td>
<td>0</td>
</tr>
<tr>
<td>2 (LPS)</td>
<td>0.47±0.12</td>
<td>1.32±0.38</td>
<td>0.04±0.02</td>
</tr>
<tr>
<td>3 (Control)</td>
<td>0.06±0.05</td>
<td>0.12±0.08</td>
<td>0.01±0.01</td>
</tr>
<tr>
<td>3 (LPS)</td>
<td>0.60±0.22</td>
<td>1.25±0.33</td>
<td>0.05±0.04</td>
</tr>
</tbody>
</table>

All values for PGE$_2$ and TxB$_2$ represent the mean (ng eicosanoid/µg DNA) ± standard error for the 16-24 hour time interval for six determinations except PGE$_2$ for fraction 1 control, which only represents one determination, since the control release from the other culture wells was zero. All values for IL-1$\beta$ represent the mean ± standard error for three determinations.
Table 2. PGE$_2$, TxB$_2$ and IL-1$\beta$ Release from Counterflow-Isolated Monocyte Fractions After DNA Correction

<table>
<thead>
<tr>
<th>Fraction #</th>
<th>PGE$_2$</th>
<th>TxB$_2$</th>
<th>IL-1$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small (Control)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Small (LPS)</td>
<td>0.48±0.18</td>
<td>0.70±0.14</td>
<td>0.08±0.06</td>
</tr>
<tr>
<td>Medium (Control)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Medium (LPS)</td>
<td>0.45±0.15</td>
<td>0.70±0.17</td>
<td>0.07±0.07</td>
</tr>
<tr>
<td>Large (Control)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Large (LPS)</td>
<td>0.49±0.25</td>
<td>0.71±0.22</td>
<td>0.04±0.02</td>
</tr>
</tbody>
</table>

All values for PGE$_2$ and TxB$_2$ represent the mean (ng eicosanoid/µg DNA) ± standard error for the 16-24 hour time interval for six determinations. All values for IL-1$\beta$ represent the mean ± standard error for three determinations.
Table 3. Leu-M3 and Mo3e Antigen Expression on Percoll-Isolated Monocyte Subpopulations at 24 Hours

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>Leu-M3</th>
<th>Mo3e</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>324.96±71.52</td>
<td>398.39±11.10</td>
</tr>
<tr>
<td>Second</td>
<td>293.44±20.55</td>
<td>351.26±34.16</td>
</tr>
<tr>
<td>Third</td>
<td>348.48</td>
<td>289.83±45.62</td>
</tr>
</tbody>
</table>

All values represent the mean fluorescence intensity ± standard error for monocytes isolated from three different donors except for Leu-M3 expression on the third fraction which represents one determination. The mean fluorescence intensity for the irrelevant IgG2b murine monoclonal antibody (MOPC 141) was 18.43, while the mean fluorescence intensity for the irrelevant IgM murine monoclonal antibody (TEPC 183) was 15.45.
Table 4. Mo3e Antigen Expression on Counterflow-Isolated Monocyte Subpopulations

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>0 HOUR</th>
<th>8 HOUR</th>
<th>24 HOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
<td>16.26±3.18</td>
<td>136.88±10.88</td>
<td>261.21±13.42</td>
</tr>
<tr>
<td>Medium</td>
<td>16.62±3.15</td>
<td>141.69±15.62</td>
<td>275.30±22.25</td>
</tr>
<tr>
<td>Large</td>
<td>17.42±3.29</td>
<td>142.20±25.70</td>
<td>285.54±40.89</td>
</tr>
</tbody>
</table>

All values represent the mean fluorescence intensity ± standard error for monocytes isolated from three different donors. The mean fluorescence intensity for the irrelevant IgM murine monoclonal antibody (TEPC 183) was 10.19.
Table 5. Leu-M3 Antigen Expression on Counterflow-Isolated Monocyte Subpopulations

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>0 HOUR</th>
<th>8 HOUR</th>
<th>24 HOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
<td>137.07±8.76</td>
<td>100.98±12.97</td>
<td>225.75±12.14</td>
</tr>
<tr>
<td>Medium</td>
<td>133.81±5.85</td>
<td>96.74±11.79</td>
<td>232.61±17.48</td>
</tr>
<tr>
<td>Large</td>
<td>139.41±4.80</td>
<td>99.85±11.50</td>
<td>230.95±18.15</td>
</tr>
</tbody>
</table>

All values represent the mean fluorescence intensity ± standard error for monocytes isolated from three different donors. The mean fluorescence intensity for the irrelevant IgG2b murine monoclonal antibody (MOPC 141) was 22.40.
Figure 1. Fraction 1 represents the monocyte subpopulation harvested at the density interface between 10 and 40% Percoll, Fraction 2 represents the monocyte subpopulation obtained from the density interface between 40 and 45% Percoll, and Fraction 3 represents the monocyte subpopulation harvested at the density interface between 45 and 50% Percoll.
Coulter Profile for Percoll Fractions

Cell Number

Relative Fraction Size

1
2
3
Figure 2. Fraction 1 represents the first monocyte subpopulation (small monocytes) to elute from the Sanderson chamber. Fraction 2 represents the second monocyte subpopulation to elute (medium-sized monocytes). Fraction 3 represents the last monocyte subpopulation to elute (large monocytes) after turning off the rotor.
Coulter Profile for Elutriator Fractions

Cell Number

Relative Fraction Size

1 2 3
Figure 3. Time course of PGE$_2$ release from human peripheral blood monocytes that were fractionated into subpopulations by discontinuous Percoll density gradients. Monocytes were cultured with either control medium or LPS-containing medium (10 $\mu$g/ml *Salmonella typhimurium* LPS). All values represent the mean ± standard error for PGE$_2$ release from six different donors.
PGE$_2$ Release from Percoll Fractions

- First, Control
- First, LPS
- Second, Control
- Second, LPS
- Third, Control
- Third, LPS

**Interval**
- 0-2 h
- 2-8 h
- 8-16 h
- 16-24 h

**ng/ml**
- 0
- 1
- 2
- 3
- 4
Figure 4. Time course of TxB$_2$ release from human peripheral blood monocytes that were fractionated into subpopulations by discontinuous Percoll density gradients. Monocytes were cultured with either control medium or LPS-containing medium (10 µg/ml Salmonella typhimurium LPS). All values represent the mean ± standard error for TxB$_2$ release from six different donors.
TxB₂ Release from Percoll Fractions

Interval

ng/ml

First, Control
First, LPS
Second, Control
Second, LPS
Third, Control
Third, LPS
Figure 5. Time course of IL-1β release from human peripheral blood monocytes that were fractionated into subpopulations by discontinuous Percoll density gradients. Monocytes were cultured with either control medium or LPS-containing medium (10 μg/ml Salmonella typhimurium LPS). All values represent the mean ± standard error for IL-1β release from three different donors.
IL-1β Release From Percoll Fractions

- First, Control
- First, LPS
- Second, Control
- Second, LPS
- Third, Control
- Third, LPS

Interval

ng/ml

0-2 h 2-8 h 8-16 h 16-24 h
Figure 6. Time course of PGE2 release from human peripheral blood monocytes that were fractionated into subpopulations by counterflow centrifugal elutriation. Monocytes were cultured with either control medium or LPS-containing medium (10 μg/ml Salmonella typhimurium LPS). All values represent the mean ± standard error for PGE2 release from six different donors.
PGE$_2$ Release from Elutriator Fractions

- Small, Control
- Small, LPS
- Medium, Control
- Medium, LPS
- Large, Control
- Large, LPS

*ng/ml*

Interval

- 0-2 h
- 2-8 h
- 8-16 h
- 16-24 h
Figure 7. Time course of TxB$_2$ release from human peripheral blood monocytes that were fractionated into subpopulations by counterflow centrifugal elutriation. Monocytes were cultured with either control medium or LPS-containing medium (10 $\mu$g/ml *Salmonella typhimurium* LPS). All values represent the mean $\pm$ standard error for TxB$_2$ release from six different donors.
TxB$_2$ Release from Elutriator Fractions

<table>
<thead>
<tr>
<th>Interval</th>
<th>Small, Control</th>
<th>Small, LPS</th>
<th>Medium, Control</th>
<th>Medium, LPS</th>
<th>Large, Control</th>
<th>Large, LPS</th>
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</thead>
<tbody>
<tr>
<td>0-2 h</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>2-8 h</td>
<td>20</td>
<td>25</td>
<td>30</td>
<td>35</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>8-16 h</td>
<td>15</td>
<td>20</td>
<td>25</td>
<td>30</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>16-24 h</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>25</td>
<td>15</td>
<td>20</td>
</tr>
</tbody>
</table>

ng/ml
Figure 8. Time course of IL-1β release from human peripheral blood monocytes that were fractionated into subpopulations by counterflow centrifugal elutriation. Monocytes were cultured with either control medium or LPS-containing medium (10 μg/ml Salmonella typhimurium LPS). All values represent the mean ± standard error for IL-1β release from three different donors.
IL-1β Release from Elutriator Fractions

- Small, Control
- Small, LPS
- Medium, Control
- Medium, LPS
- Large, Control
- Large, LPS

Interval:
- 0-2
- 2-8
- 8-16
- 16-24

ng/ml
Figure 9. Time course of $[^3\text{H}]$PGE$_2$ release from LPS-treated human monocytes fractionated into subpopulations by countercurrent centrifugal elutriation. Monocytes were cultured with medium containing 0.1 $\mu$Ci/ml $[^3\text{H}]$arachidonic acid for two hours during the period of cell adherence after which the cells were washed twice with unlabeled medium. Monocytes were then cultured in LPS-containing medium (10 $\mu$g/ml Salmonella typhimurium LPS) for 24 hours without label, with media exchanges at 2, 8, 16 and 24 hours. Each determination represents labeled eicosanoid extracted from replicate (four) pooled culture supernatants. Histogram bars represent the mean ± standard error for $[^3\text{H}]$PGE$_2$ release from three different donors.
Specific Activities of PGE<sub>2</sub> from Pulse-Labeled Elutriator Fractions

- 0-2 h
- 2-8 h
- 8-16 h
- 16-24 h

Interval

- Small, LPS
- Medium, LPS
- Large, LPS

cpm/ng
Figure 10. Time course of $[^3\text{H}]$TxB$_2$ release from LPS-treated human monocytes fractionated into subpopulations by countercurrent centrifugal elutriation. Monocytes were cultured with medium containing 0.1 µCi/ml $[^3\text{H}]$arachidonic acid for two hours during the period of cell adherence after which the cells were washed twice with unlabeled medium. Monocytes were then cultured in LPS-containing medium (10 µg/ml Salmonella typhimurium LPS) for 24 hours without label, with media exchanges at 2, 8, 16 and 24 hours. Each determination represents labeled eicosanoid extracted from replicate (four) pooled culture supernatants. Histogram bars represent the mean ± standard error for $[^3\text{H}]$TxB$_2$ release from three different donors.
Specific Activities of TxB$_2$ from Pulse-Labeled Elutriator Fractions

- Small, LPS
- Medium, LPS
- Large, LPS

Interval:
- 0-2 h
- 2-8 h
- 8-16 h
- 16-24 h

cpm/ng
Figure 11. Time course of $[^{3}\text{H}]$PGE$_{2}$ release from LPS-treated human monocytes fractionated into subpopulations by countercurrent centrifugal elutriation. Monocytes were cultured with medium containing 0.1 $\mu$Ci/ml $[^{3}\text{H}]$arachidonic acid during the two hour cell adherence period. Monocytes were washed twice with unlabeled medium and then treated with 10 $\mu$g/ml *Salmonella typhimurium* LPS plus 0.1 $\mu$Ci/ml $[^{3}\text{H}]$arachidonic acid. Medium was exchanged at 2, 8, 16, and 24 hours and replaced with identical medium. Each determination represents labeled eicosanoid extracted from replicate (four) pooled culture supernatants. Histogram bars represent the mean $\pm$ standard error for $[^{3}\text{H}]$PGE$_{2}$ release from three different donors.
Specific Activities of PGE$_2$ from Continuously-Labeled Elutriator Fractions

- Small, LPS
- Medium, LPS
- Large, LPS

Interval:
- 0-2 h
- 2-8 h
- 8-16 h
- 16-24 h

Units: cpm/ng
Figure 12. Time course of $[^3\text{H}]\text{TxB}_2$ release from LPS-treated human monocytes fractionated into subpopulations by countercurrent centrifugal elutriation. Monocytes were cultured with medium containing 0.1 µCi/ml $[^3\text{H}]$arachidonic acid during the two hour cell adherence period. Monocytes were washed twice with unlabeled medium and then treated with 10 µg/ml *Salmonella typhimurium* LPS plus 0.1 µCi/ml $[^3\text{H}]$arachidonic acid. Medium was exchanged at 2, 8, 16, and 24 hours and replaced with identical medium. Each determination represents labeled eicosanoid extracted from replicate (four) pooled culture supernatants. Histogram bars represent the mean ± standard error for $[^3\text{H}]\text{TxB}_2$ release from three different donors.
Specific Activities of TxB₂ from Continuously-Labeled Elutriator Fractions

- Small, LPS
- Medium, LPS
- Large, LPS

Interval:
- 0-2 h
- 2-8 h
- 8-16 h
- 16-24 h

cpm/ng

Graph showing the specific activities of TxB₂ at different intervals with varying sizes of LPS.
Figure 13. Light-Scattering and FACS Profiles of Monocyte Preparations (a, b, c)

(a) Representative light-scattering profile of a monocyte subpopulation isolated by Percoll density centrifugation.

(b) Representative FACS profile of a Percoll-isolated monocyte subpopulation stained with a negative control IgM monoclonal antibody by indirect immunofluorescence after a 24 hour incubation with Salmonella typhimurium LPS (10 μg/ml).
(c) Representative FACS profile of a Percoll-isolated monocyte subpopulation stained with anti-Mo3e by indirect immunofluorescence after a 24 hour incubation with *Salmonella typhimurium* LPS (10 µg/ml).
LITERATURE CITED


