N-Formylmethionyl-Leucyl-[3H]Phenylalanine Binding, Superoxide Release, and Chemotactic Responses of Human Blood Monocytes That Repopulate the Circulation During Leukapheresis

By Enrica Alteri and Edward J. Leonard

Human blood monocytes comprise two subpopulations: one migrates to the chemoattractant, N-formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe), and has saturable binding sites for this peptide; the other does not migrate and exhibits little peptide binding. To determine if expression of binding sites was a function of monocyte maturation, we depleted human subjects of blood monocytes by leukapheresis so that the circulation was repopulated by monocytes released from the bone marrow. Pre- and postleukapheresis monocytes were then compared for fMet-Leu-[3H]Phe binding, superoxide generation, and chemotactic responses. No significant differences in peptide binding curves were found, suggesting that receptor expression was stable over the maturational span represented by these two groups of cells. This supports the hypothesis that there are two distinct lineages of monocytes with respect to expression of receptors for fMet-Leu-Phe. An additional finding of interest was that the number of chemotactically responsive cells immediately postleukapheresis was half the control. This was a transient state; monocyte responses were normal 3 hr after termination of leukapheresis, suggesting that they rapidly become functionally mature.

The Problem Addressed

In this work centers on our finding that there are two subpopulations of human blood monocytes: one responds to chemoattractants; the other does not. Data from desensitization studies show that approximately 80% of the responsive population has functionally distinct receptors for 3 different attractants tested: C5α, lymphocyte-derived chemotactic factor (LDCF), and fMet-Leu-Phe. Thus, we could not account for the nonmigrating population by postulating subsets of cells with receptors for one, but not another, attractant. When monocytes were separated into migrating and nonmigrating populations in a newly designed chemotaxis collection chamber, we found saturable binding of fMet-Leu-[3H]Phe to the migrating cells and negligible binding to the nonmigrating cells. The distinction between the two sets of cells was established on both a functional and biochemical basis.

The migrating and nonmigrating subpopulations could represent two distinct cell lineages, a single lineage at different maturational stages, or a single lineage of mature cells that cycle through different phenotypic expression. If there were a single lineage of monocytes, which did not acquire chemotaxin receptors until after release from the bone marrow, an increase in the proportion of recently released monocytes would result in a decreased proportion of monocytes with chemotaxin receptors. To test this single lineage hypothesis, we compared venous blood monocytes in the normal steady state with monocytes that repopulate the circulation during leukapheresis.

Over the course of a leukapheresis procedure designed to collect mononuclear cells, the donor loses about 2 × 10⁸ monocytes, equivalent to the total in the circulating blood. Despite this loss, the blood monocyte count at the end of leukapheresis is normal, implying release of monocytes from bone marrow into the circulation. Thus, study of pre- and postleukapheresis blood monocytes provides an opportunity to compare these cells at two different stages of their lifespan. We measured fMet-Leu-[3H]Phe binding and two functional responses to ligand–receptor interaction, superoxide anion release and chemotaxis.

Materials and Methods

Leukapheresis

The staff of the Blood Bank, Clinical Center, NIH, Bethesda, MD, performed the leukapheresis on 14 healthy volunteers, using an IBM 2997 Separation Channel. Antiagulant was 3,000 U of heparin in 500 ml anticoagulant citrate dextrose-NIH solution A (ACD-A), administered at 2 ml/min. Blood flow rate was 50–60 ml/min, and centrifuge speed was 920 rpm. Leukocytes, layered according to density, were located in the interface between erythrocytes and plasma. Leukocyte collection rate was set at 2 ml/min. The erythrocyte return rate was adjusted to result in maximal collection of monocytes; this occurred when the hemoglobin concentration in the plasma return line was 5 g/dl. The leukapheresis period was 2 hr, and the number of monocytes collected during this time was 1–4 × 10⁸. The differential count in the collection bag was 20%–30% monocytes and 10%–25% granulocytes, the remainder being lymphocytes. Sham leukapheresis was identical, except that the leukocyte withdrawal pump was not activated, so that all cells and plasma were returned to the donor.

Preparation of Monocytes

Blood samples of 30 ml each were drawn before leukapheresis, immediately thereafter, and 3 hr later. Monocytes were obtained by centrifugation on Ficoll-Hypaque and identified and counted as previously described.
SUBPOPULATIONS OF HUMAN BLOOD MONOCYTES

Binding of fMet-Leu-[^3H]Phe

The binding of fMet-Leu-[^3H]Phe was determined as previously described. For each concentration of fMet-Leu-[^3H]Phe, tubes containing $8 \times 10^7$ monocytes were set up in duplicate, along with a single tube containing a 100-fold excess of unlabelled peptide. After 20-min incubation at room temperature, cells were washed free of unbound peptide and counted. Specific binding refers to the total cpm bound minus counts for cells with a 100-fold excess of unlabelled peptide. Specific binding refers to the total cpm bound minus the nonspecific value.

Assay of Superoxide Production

Superoxide production was assayed by a modification of the continuous assay procedure of Cohen and Chovaniec. Reaction mixtures containing $2 \times 10^6$ monocytes in 1.0 ml of Hanks' salt solution with 0.1 mM ferricytochrome-c were preincubated for 3-5 min at 37°C before the addition of stimuli. Reference cuvettes contained the same components plus 60 μg of superoxide dismutase. Data for phorbol myristate acetate (PMA) stimulated monocytes represent initial linear rates of superoxide generation. Since stimulation of monocytes with fMet-Leu-Phe caused a burst of superoxide production that ceased within approximately 3 min, peptide data are expressed as total superoxide generated in 3 min. The extinction coefficient of 20,000 M⁻¹ cm⁻¹ at 550 nm was used for cytochrome-c.

Chemotactic Factors

N-formylmethionyl-leucyl-phenylalanine (FMLP) was purchased from Sigma Chemical Co., St. Louis, MO. Stocks solutions of $10^{-3}$ M were prepared in either dimethyl sulfoxide (DMSO) or ethanol and stored at 20°C. Human serum-derived complement component C5a was prepared by a modified procedure of Fernandez and Hugh, as previously described. Phorbol myristate acetate (PMA) was prepared by concanavalin-A stimulation of human mononuclear nonadherent cells, as described by Altman et al. fMet-Leu-Phe, with a specific activity of 46 Ci/m mole, was purchased from New England Nuclear, Boston, MA. Absence of oxidation products was confirmed in the first of 3 lots used by silica gel thin-layer chromatography with a butanol acetic acid water solvent system (6:1:2.5). We thank Dr. Liana Harvath for performing this test. To further minimize the possibility of oxidation, we stored the vial in liquid nitrogen and removed 10-μl aliquots for experiments by piercing the vial cap with a Hamilton syringe.

Chemotaxis Assay

Chemotaxis was assayed in a multiwell chamber as described previously. The number of migrated cells per square millimeter was counted with an image analyzer (Optomax Inc., Hollis, NH). Assay points were in triplicate, and the standard error of the mean did not exceed 15%. Experiments that distinguished between chemotaxis and chemokinesis can be found in ref. 1.

RESULTS

Repopulation of Circulating Blood by Monocytes During Leukapheresis

Leukapheresis of normal volunteers was carried out for a period of 2 hr, with operating parameters chosen for optimal delivery of blood monocytes. The mean number of monocytes collected per donor was $2.8 \times 10^8$ (SEM for 10 donors, $0.4 \times 10^8$). This is more than the total number of circulating monocytes, the mean of which was $1.3 \pm 0.2 \times 10^9$ for 10 donors (calculated from monocyte count and an average whole blood volume). To determine the response of the donors to this leukocyte depletion, we obtained venous blood samples before leukapheresis, immediately after the procedure, and 3 hr later. An aliquot was used for monocyte counts, and the remainder was processed on Ficoll-Hypaque for monocyte isolation. The separated monocytes were 99% viable by trypan blue dye exclusion, and over 95% were peroxidase positive. No difference between pre- and postleukapheresis monocytes could be seen by these criteria. The mean for 10 donors of the preleukapheresis venous blood monocyte count was $2.8 \pm 0.3 \times 10^7$/ml. The postleukapheresis count was $2.6 \pm 0.4 \times 10^7$/ml. Since the postleukapheresis blood monocyte count was normal, we conclude that the donors responded to monocyte depletion by releasing new cells into the circulation. It is probable that the new cells were liberated from the bone marrow, which has a sufficient supply of preformed monocytes, approximately $7 \times 10^9$, to replace the lost cells.

Binding of fMet-Leu-[^3H]Phe to Pre- and Postleukapheresis Monocytes

To compare expression of receptors for chemotactic peptide in pre- and postleukapheresis monocytes, we measured binding of fMet-Leu-[^3H]Phe. In previous experiments with normal monocytes, uptake reached a plateau value within 20 min and was specific and saturable. This was confirmed in the present study. Figure 1 shows binding curves for pre- and postleukapheresis monocytes based on data from 11 donors. There was no difference between the two curves. Thus, expression of receptors for fMet-Leu-Phe appeared to be stable over the maturational span represented by these two groups of cells. We also studied two different cellular responses to fMet-Leu-Phe binding: generation of superoxide anion and chemotaxis.

Superoxide Anion Production by Pre- and Postleukapheresis Monocytes

No superoxide release could be detected in unstimulated monocytes. Addition of phorbol myristate acetate (PMA) caused an immediate production of $O_2^-$, which was linear for at least 15 min. Table 1 shows that there was no difference in PMA-induced $O_2^-$ production between pre- and postleukapheresis monocytes. The response to $10^{-7}$ M and $10^{-6}$ M fMet-Leu-Phe was likewise not different for the two populations of cells.

Chemotactic Responses of Pre- and Postleukapheresis Monocytes

Three attractants, C5a, FMLP, and LDCF, were used at four different concentrations to test chemotac-
tic responses of pre- and postleukapheresis venous blood monocytes. A typical result for one donor is shown in Fig. 2. Compared to the preleukapheresis control, the number of postleukapheresis venous blood monocytes that responded to each attractant sufficiently to move to the bottom of the filter was decreased by about 50%. The shapes of the dose–response curves were not grossly altered. In view of the similarity of fMet-Leu-[3H]Phe binding curves for pre- and postleukapheresis monocytes, we conclude that approximately half of the receptor-bearing postleukapheresis monocytes failed to migrate to fMet-Leu-Phe. This conclusion was supported by detection of fMet-Leu-[3H]Phe binding to nonmigrating postleukapheresis monocytes (data not shown).

Mean values for chemotaxis responses of monocytes from the whole group of donors are shown in Table 2, where it can be seen that the postleukapheresis response to each attractant was about 50% of the control. Furthermore, random migration (Gey-BSA column) was comparably diminished.

In contrast to the immediately postleukapheresis sample, chemotactic responses of cells obtained 3 hr after leukapheresis were normal; persistent depression of the C5a response in Fig. 2 was not typical. Mean values in Table 2 show that the 3-hr postleukapheresis chemotaxis responses were comparable to the preleukapheresis values. fMet-Leu-Phe binding to monocytes from 6 donors 3 hr postleukapheresis was slightly, but not significantly, higher than preleukapheresis values. The results suggest that the monocytes released into the circulation in response to leukocyte depletion change rapidly, and with respect to chemotactic responses, they soon acquire the characteristics of the steady-state circulating population.

To determine whether the observed differences were caused by monocyte depletion or by an incidental effect (anticoagulant, mechanical stress, plastic tubing), we tested cells from four individuals who underwent the identical leukapheresis procedure, except that the leukocyte collection pump was not activated. In the absence of any cell loss, no significant changes in monocyte chemotactic responses were observed. An example is shown in Fig. 3; mean values for the group are presented in Table 2.

DISCUSSION

Over the course of the 2-hr leukapheresis described here, the number of monocytes removed was more than the total in the circulating blood. The human subjects responded to this loss by releasing a sufficient number of new cells into the circulation to maintain the blood monocyte count at normal levels. In estimating the proportion of new monocytes in the circulation, we postulate that, as monocytes were removed, bone marrow monocytes were released pari passu into the circulation. This model leads to the conclusion that, in the immediately postleukapheresis venous blood, approximately 88% of the monocytes were released.
from the bone marrow during the 2-hr procedure.* Since the fMet-Leu-Phe binding curve for these recently released postleukapheresis monocytes was not different from the preleukapheresis curve, it is likely that the proportion of monocytes with fMet-Leu-Phe receptors is not diminished postleukapheresis. The result does not favor the hypothesis of a single lineage at different maturational stages and suggests that the monocyte populations with and without receptors represent two distinct lineages.

Since the evidence against a margined monocyte pool15 has not convinced all workers in the field, it is not certain that postleukapheresis monocytes are young cells released from the bone marrow. However, the chemotactic and random migration responses of postleukapheresis monocytes are half normal, and they rise to the level of preleukapheresis monocytes within 3 hr. This suggests that they are a young population, which rapidly acquires characteristics of mature circulating monocytes. The normal responses of post-sham-leukapheresis monocytes show that the decreased capacity for translational movement is not due to the centrifugation procedure.

The two lineage hypotheses should be tested with binding studies on both bone marrow cells and tissue macrophages to determine if chemotaxin receptor expression is stable over the whole life span of the mononuclear phagocyte. Data from in vitro systems provide no answer to this question, since the results are quite diverse. Our unpublished results of fMet-Leu-Phe binding by blood monocytes after 1–3 days in culture show no increase in binding, which is consistent with stability of receptor expression. Measurement of the number of mouse bone marrow cells that migrated to the complement-derived attractant in endotoxin-activated mouse serum showed a progressive increase to 100% over the first 13 days in culture, followed by a decrease in the ensuing 9 days.16 We found one line, among several macrophage-like cell lines tested, in which 100% of the cells responded to chemotactivating monocytes from venous blood pre- and postleukapheresis. The graphs illustrate dose–response curves for monocytes from venous blood of one donor. Squares: preleukapheresis. Triangles: immediately postleukapheresis. Open circles: 3 hr later. The cells from each blood sample were separated on Ficoll-Hypaque and tested immediately. Input monocyte number was 50,000/ well. The data are mean number of migrated monocytes per well for triplicate wells. The SEM was less than 10%.

![Graph](image)

Table 2. Chemotaxis of Postleukapheresis Monocytes, Expressed as the Percentage of the Preleukapheresis Response

<table>
<thead>
<tr>
<th>Time</th>
<th>fMet-Leu-Phe</th>
<th>LDCF</th>
<th>C5a</th>
<th>Gey-BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postleukapheresis</td>
<td>52 ± 7</td>
<td>59 ± 5</td>
<td>53 ± 7</td>
<td>61 ± 8</td>
</tr>
<tr>
<td>3 hr later</td>
<td>80 ± 13</td>
<td>101 ± 15</td>
<td>102 ± 20</td>
<td>96 ± 16</td>
</tr>
<tr>
<td>Post-sham-leukapheresis</td>
<td>94 ± 19</td>
<td>114 ± 26</td>
<td>109 ± 26</td>
<td>212 ± 78</td>
</tr>
<tr>
<td>3 hr later</td>
<td>89 ± 4</td>
<td>87 ± 30</td>
<td>78 ± 26</td>
<td>187 ± 64</td>
</tr>
</tbody>
</table>

Input number of monocytes: 50,000. Incubation time: 90 min. For each donor, we determined the mean value for the chemotactic responses to the two optimal concentrations of each attractant (10⁻⁸ and 10⁻⁷ M fmet-leu-phe and relative concentrations of 4 and 8, as noted in Fig. 2 for LDCF and C5a). The responses of preleukapheresis monocytes, expressed as the percentage of the input number migrating ± SEM for 10 donors, were 28 ± 3, 18 ± 1, 20 ± 2, and 5 ± 1 for fmet-leu-phe, LDCF, C5a, and Gey-BSA, respectively. The postleukapheresis values in the table are percentages of these preleukapheresis responses.

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*Let T = the total number of circulating monocytes. Let P = the total number of monocytes removed from the circulation in time t. Let M₁, . . . n = the number of old monocytes remaining in the circulation after time (1 . . . n). After the first very short time interval, t, of leukapheresis, the number of old monocytes remaining, Mₜ, is T = P. After the next interval, the number Mₜ₊₁ is Mₜ₊₁ = (Mₜ/T)P, where Mₜ/T is the proportion of old monocytes in the circulation for that time interval. The series is continued until the time interval total = 2 hr. The error in Mₜ/T decreases as t decreases and was negligible for values of t of 1 min or less.
important. The human monocyte cell line, U937, exhibits chemotaxis only after a surface receptor is induced by lymphokines. Thus, there are in vitro examples of all cells responding or changing numbers responding. These results suggest that expression of receptors may be under subtle control and that stability of receptor expression in the migrating and nonmigrating blood monocyte subpopulations can best be evaluated by obtaining cells after their in vivo maturation into tissue macrophages.

The pattern of normal superoxide release and decreased chemotaxis by postleukapheresis monocytes raises the possibility that these monocytes have a lower proportion of high-affinity fMet-Leu-Phe receptors than preleukapheresis monocytes. The concentration of fMet-Leu-Phe required to induce superoxide release by human neutrophils is higher than that required for chemotactic response; thus, the chemotactic response may be induced by interaction of ligand with high-affinity receptors or by lower receptor occupancy than is required for superoxide generation. Two sets of binding sites with different affinities for fMet-Leu-Phe have been detected on neutrophils; the $K_m$ for fMet-Leu-Phe binding to neutrophils can be altered by prior stimulation with the ligand or by agents that alter membrane fluidity. However, since the shapes of the fMet-Leu-Phe binding curves of pre- and postleukapheresis monocytes are not significantly different, we have no evidence for altered receptor affinity. Furthermore, the decreased random migration of postleukapheresis monocytes (Table 2) suggests that, independently of receptor–ligand interaction, mechanisms for translational movement are not fully developed.

The decreased number of chemotactically responsive postleukapheresis monocytes may have a parallel in clinically observed chemotaxis defects, in which diminished responses may be secondary to elevated turnover rates and an increased percentage of recently released monocytes in the circulation. We are currently studying surface markers and several functional aspects of postleukapheresis monocytes. Preliminary data show that the number of chemotactically responsive cells increases to normal levels after 3 hr in tissue culture; thus, functional characteristics of these cells may be changing very rapidly.

ACKNOWLEDGMENT

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N-formylmethionyl-leucyl-[3H]phenylalanine binding, superoxide release, and chemotactic responses of human blood monocytes that repopulate the circulation during leukapheresis

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