Identification and Characterization of a Unique Subpopulation (CALLA/CD10-Negative) of Human Neutrophils Manifesting a Heightened Chemotactic Response to Activated Complement

By Robert T. McCormack, Robert D. Nelson, Dennis E. Chenoweth, and Tucker W. LeBien

We have previously demonstrated that human neutrophils synthesize the common acute lymphoblastic leukemia antigen (CALLA/CD10). To determine whether CALLA/CD10-positive and -negative neutrophils have similar or distinct functional attributes, we sorted normal peripheral blood neutrophils for CALLA/CD10 expression and compared their chemotactic ability. Surprisingly, the low-frequency (~5%), CALLA/CD10-negative neutrophils displayed a dramatically heightened chemotactic response to activated complement (C') that was (a) specific for C', (b) not observed with other minor subpopulations of neutrophils, (c) not due to previous activation in vivo or in vitro, and (d) apparently not due to an increase in C5a receptors. These results underscore the concept of neutrophil heterogeneity and prompt the hypothesis that CALLA/CD10-negative neutrophils may participate in an inflammatory response to trauma involving complement activation.

© 1987 by Grune & Stratton, Inc.

The phenotypic and functional heterogeneity of peripheral blood mononuclear cells has been largely defined by using monoclonal antibodies.1,2 Functional heterogeneity of human polymorphonucleated neutrophils was initially suggested over 60 years ago,3 and more recent studies have documented heterogeneity of neutrophil function.4,13 density,14,15 and RNA/protein biosynthesis.16 Monoclonal antibodies have also been applied in further attempts to unravel heterogeneity of neutrophil origin and function.17,24

The common acute lymphoblastic leukemia antigen (CALLA/CD10) is a 95-kD cell surface molecule originally characterized on ALL cells by Greaves et al.25 CALLA/CD10 has subsequently been identified on normal and malignant cells of both hematopoietic and nonhematopoietic origin.26-34 including neutrophils.35,36 We have recently confirmed and extended the observations of others35,36 by showing that neutrophils synthesize and insert CALLA/CD10 into their membranes.37 However, blocking studies with the anti-CALLA/CD10 monoclonal antibody BA-3 failed to ascribe any role for CALLA/CD10 in neutrophil function.37

In the experiments reported herein, we have identified a rare subpopulation (~5%) of segmented peripheral blood neutrophils from normal donors characterized by (a) an absence of cell surface CALLA/CD10 and (b) the presence of a heightened chemotactic responsiveness to activated complement (C'). The potential biologic significance of our results is discussed.

MATERIALS AND METHODS

Monoclonal antibodies. The anti-CALLA/CD10 monoclonal antibody BA-3 is an IgG2b antibody that was produced and characterized in this laboratory.38 AHN-1 is an IgM monoclonal antibody that recognizes a 150/105-kD complex on neutrophils39 and was kindly provided by Dr Keith Skubitz, University of Minnesota. OKM-1, a monoclonal antibody that recognizes the C3bi receptor,40 was purchased as a fluorescein isothiocyanate conjugate (FITC-OKM-1) from Coulter Diagnostics, Hialeah, FL.

Purification of neutrophils. Peripheral blood neutrophils were purified from heparinized whole blood by using Ficoll-Hypaque centrifugation followed by hypotonic lysis of erythrocytes. Final preparations contained >95% neutrophils, with the remainder of the cells being contaminating eosinophils and basophils.

Immunofluorescence analysis and cell sorting. Neutrophils were stained with monoclonal antibodies and analyzed by indirect immunofluorescence as previously described.41 Cell sorting of peripheral blood neutrophils was accomplished by using a fluorescein-activated cell sorter (FACS IV, Becton Dickinson, Mountain View, CA) equipped with a 5-W argon ion laser. The distribution of CALLA/CD10-positive and -negative neutrophils was determined on each donor, and the fluorescent gains were adjusted to collect positive and negative cells. At a sort rate of 3 to 4 x 10^7 cells/s, we could collect enough CALLA/CD10-negative neutrophils for functional studies in four hours. When the percentage of CALLA/CD10-negative peripheral blood neutrophils was less than 5% of the total neutrophils stained, the gains were increased to 5% of the total cells to facilitate the sorting time. CALLA/CD10-positive and -negative neutrophils were collected into 15-mL conical tubes containing phosphate-buffered saline (pH 7.2) with 2.5% fetal bovine serum and 0.02% sodium azide (PBS-F). The tubes were shaken periodically to keep the neutrophils in suspension. After sorting, the cells were washed twice in PBS-F, counted, and assayed for chemotactic function. A sample from each CALLA/CD10-positive and -negative fraction was removed and examined for cell morphology by using Wright-Giemsa staining. Four populations of cells from each donor were examined for chemotaxis: (a) unstained, unsorted cells that served as a control for the effects of sorting on chemotaxis; (b) cells stained with BA-3 and FITC–goat antimouse (GAM) but not sorted to control for the effects of antibody binding on chemotaxis; (c) cells stained and sorted as CALLA/CD10-positive; and (d) cells stained and sorted as CALLA/CD10-negative. Unstained, unsorted and stained, unsorted cells were held on ice during the sort to minimize lability.
The aforementioned procedure was also followed for sorting AHN-1-positive and -negative peripheral blood neutrophils with the following exception: only two populations of cells were prepared for each donor, unstained/unsorted cells and AHN-1-negative cells (see Results).

Unsorted, purified neutrophils were subjected to two-color flow cytometric analysis to determine the relationship between expression of CALLA/CD10 and C5a receptors. Neutrophils at $1 \times 10^5$/mL were first stained with FITC-C5a for 25 minutes at room temperature at a final C5a concentration of 5 or 50 nmol/L.40 The cells were washed once with PBS-F and sequentially stained with BA-3 or control ascitic fluid (both 1:100), GAM-biotin (Boeringer Mannheim Biochemicals, Indianapolis) at 7.5 μg/mL, and 5 μL stock phycoerythrin-avidin (Becton Dickinson), all for 30 minutes at 4°C in a final volume of 100 μL interspersed with two washes in PBS-F. Two-color analysis was conducted with a FACS 440 analyzer (Becton Dickinson) with a 488-nm excitation wavelength from an argon laser. FITC and phycoerythrin emission wavelengths were detected by using 535-20 and 575-15 dichroic interference filters, respectively. Phycoerythrin emission detection was corrected for stray FITC emission, and the results were analyzed on a Consort 40 computer (Becton Dickinson) using a DISP2D program.

To examine the relationship between CALLA/CD10 and C5bi receptor expression, neutrophils were sorted into CALLA/CD10-positive and -negative fractions by using BA-3, GAM-biotin and phycoerythrin-avidin. Sorted cells were washed twice with PBS-F, and CALLA/CD10-positive and -negative and unstained cells were then stained with FITC-MO-1 according to the manufacturer’s instructions. The cells were then examined for green fluorescence.

Chemotaxis. Chemotaxis was performed by the agarose method of Nelson et al41 with gelatin as a protein source. Serum collected from the same donor whose neutrophils were being assayed served as an eventual source of C'. N-Formyl-methionyl-leucyl-phenylalanine (F-mlp, Pierce Biochemicals, Rockford, IL) was used at a final concentration of $5 \times 10^{-5}$ mg/mL.

Statistics. The two-tailed Student's t-test was used as a test for statistical significance.

RESULTS

Chemotaxis and random migration of CALLA/CD10-positive and CALLA/CD10-negative peripheral blood neutrophils. We37 and others35,36 have previously reported that ~95% of normal peripheral blood neutrophils express CALLA/CD10. To determine the relationship between CALLA/CD10 expression and neutrophil function, we sorted peripheral blood neutrophils from six normal donors into CALLA/CD10-positive and -negative populations and tested for chemotactic response to C' and F-mlp. The results in Table 1 show that there was no significant difference in the chemotactic response to C' between unstained/unsorted, stained/unsorted, and sorted CALLA/CD10-positive cells. The slight inhibition observed in the stained/unsorted and sorted CALLA/CD10-positive cells is consistent with other data from this laboratory indicating that BA-3 has a slight inhibitory effect on chemotaxis.37 In striking contrast, the chemotactic response of CALLA/CD10-negative cells toward C' (5.52 ± 0.99) was significantly greater than that of either CALLA/CD10-positive cells (3.19 ± 1.48, $P < .001$) or unstained/unsorted cells (3.68 ± 1.51, $P < .001$). Differences between CALLA/CD10-negative neutrophils and unstained/unsorted neutrophils are most meaningful because the latter population represents the true migratory capacity of neutrophils in vitro. CALLA/CD10-negative neutrophils from male and female donors were equally responsive to C' (data not shown). The heightened chemotactic response of CALLA/CD10-negative cells was only observed for C'. There were no significant differences in the chemotactic response to F-mlp or in random migration among the cell populations tested. The similarity in chemotaxis to C' of unstained/unsorted, stained/unsorted, and CALLA/CD10-positive cells in addition to the absence of differences in chemotaxis to F-mlp of all populations tested indicates that the cell sorting procedure was not unduly detrimental to chemotaxis.

Figure 1 is a composite of micrographs showing CALLA/CD10-negative (Fig 1A, C) and CALLA/CD10-positive (Fig 1B, D) neutrophils migrating in response to C' in the agarose assay. These micrographs provide direct evidence that the predominant CALLA/CD10-negative cells exhibiting a heightened chemotactic response to C' were segmented neutrophils that were morphologically indistinguishable from CALLA/CD10-positive neutrophils. Furthermore, there were no CALLA/CD10-positive neutrophils contaminating the CALLA/CD10-negative population when checked by immunofluorescence microscopy. Contaminating CALLA/CD10-negative eosinophils and basophils constituted less than 6% of the CALLA/CD10-negative cell population and did not contribute to the leading migratory front. CALLA/CD10-positive cells were composed entirely of mature neutrophils. There were no consistent differences observed in the number of cells constituting the leading front (as shown in the micrograph) in the CALLA/CD10-positive or -negative populations.

Specificity of the heightened chemotactic response. To determine whether the heightened chemotactic response to C' was unique to CALLA/CD10-negative neutrophils, neutrophils were sorted according to reactivity with the monoclonal antibody AHN-1. AHN-1 is a neutrophil-specific antibody recognizing an antigen that, like CALLA/CD10, is expressed on 95% of peripheral blood neutrophils. Because AHN-1 has been shown to inhibit neutrophil chemotaxis,32 only two populations of neutrophils were tested: unstained/unsorted and AHN-1-negative. Results in Table 2 show that, unlike CALLA/CD10-negative neutrophils, AHN-
I-negative neutrophils did not exhibit a heightened chemotactic response to C' (2.02 ± 0.05) when compared with unstained/unsorted neutrophils (2.72 ± 0.48). Indeed, AHN-I-negative neutrophils were less responsive to both C' and F-mlp.

**Relationship between CALLA/CD10 and C5a receptor expression on peripheral blood neutrophils.** To determine whether the heightened chemotactic response to C' was due to increased C5a receptors on CALLA/CD10-negative cells, we performed two-color flow cytometric analysis on purified neutrophils by using FITC-C5a and BA-3 plus GAM-biotin and phycoerythrin-avidin as described in Materials and Methods. Figure 2 is a representative contour plot from one of three normal donors tested by using 50 nmol/L FITC-C5a. The vast majority of neutrophils (95%) coexpressed CALLA/CD10 and C5a receptors as shown in the upper right quadrant. CALLA/CD10-negative, C5a receptor-positive and CALLA/CD10-negative, C5a receptor-negative cells each represented 2% to 3% of the total cells analyzed as shown in the lower right and lower left quadrants, respectively. It is apparent that no distinct population of CALLA/CD10-negative neutrophils express C5a receptor levels greater than CALLA/CD10-positive neutrophils, although there is some heterogeneity in C5a receptor levels in CALLA/CD10-negative neutrophils. Similar results were obtained from two other donors when using 5 and 50 nmol/L FITC-C5a. We conclude that the heightened chemotactic response of CALLA/CD10-negative neutrophils to C' is not attributable to increased numbers of C5a receptors on CALLA/CD10-negative neutrophils.
CALLA-CD10 NEGATIVE NEUTROPHILS

Relationship between CALLA/CD10 and C3bi receptor expression on peripheral blood neutrophils. One possible explanation for the increased chemotactic response displayed by CALLA/CD10-negative neutrophils is that these cells were preactivated, either in vivo or in vitro (as a consequence of sorting). We therefore sorted neutrophils into CALLA/CD10-positive and -negative populations and examined each for C3bi receptor expression, a receptor previously shown to dramatically increase after neutrophil activation. Ninety-eight percent of sorted, CALLA/CD10-positive neutrophils were C3bi receptor-positive with a mean channel of 620. Eighty-seven percent of sorted, CALLA/CD10-negative neutrophils were C3bi receptor-positive with a mean channel of 607. Unstained, unsorted neutrophils from the same donor were 89% C3bi receptor-positive with a mean channel of 619. These results confirmed data obtained from three donors when using two-color analysis to compare CALLA/CD10-positive and -negative neutrophils.

Collectively, these results document the existence of a low-frequency, CALLA/CD10-negative neutrophil population with subtle differences in receptor affinity between chemotactic analysis.

Table 2. Chemotactic Response of AHN-1–Positive and AHN-1–Negative Peripheral Blood Neutrophils

<table>
<thead>
<tr>
<th>Cells</th>
<th>Stimulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorted</td>
<td>C' F-mlp RM</td>
</tr>
<tr>
<td>Stained</td>
<td>2.72 ± 0.48*</td>
</tr>
<tr>
<td>+ AHN-1</td>
<td>2.02 ± 0.51</td>
</tr>
</tbody>
</table>

Neutrophils were separated into AHN-1–positive or -negative fractions by using a FACS IV, and the AHN-1–negative cells were tested for chemotaxis.

*Means ± SD of the chemotactic response (measured in centimeters) from three donors.

DISCUSSION

CALLA/CD10 is a 95 kD cell surface molecule originally identified on leukemic cells from patients with acute lymphoblastic leukemia and subsequently shown to be expressed on human neutrophils. We recently demonstrated that neutrophils actively synthesize CALLA/CD10 and also observed that leukemic cell and neutrophil CALLA/CD10 have similar if not identical protein backbones. We and others have shown that myelopoietic CALLA/CD10 expression occurs at the level of the segmented neutrophil, 90% to 95% of segmented neutrophils being CALLA/CD10–positive. Because only 5% to 10% of segmented neutrophils are CALLA/CD10–negative, we hypothesized that these cells were possible precursors of CALLA/CD10–positive neutrophils and may be functionally immature. The experiments in Table 1 were initiated to test this hypothesis and showed, quite surprisingly, that the low-frequency, CALLA/CD10–negative neutrophils manifest a heightened chemotactic response to C'. Additionally, the isolation and analysis of a population of peripheral blood neutrophils that lack another cell surface antigen (AHN-1) did not result in a similar increased chemotactic response to C' (Table 2). Collectively, these results document the existence of a low-frequency subpopulation of neutrophils manifesting an increase in chemotaxis toward C' that is not demonstrable with other chemotactants (eg, F-mlp) or minor neutrophil subpopulations.

Several explanations for the heterogeneous chemotactic responses of neutrophils have been suggested, including differences in ligand binding and cell age. The subject of ligand binding to individual cells and its relation to chemotaxis has been controversial. Harvath and Leonard divided peripheral blood neutrophils into migrating and nonmigrating populations but showed that both populations bind F-mlp equally. Conversely, Seligmann et al have shown that neutrophils bind fluoresceinlabeled F-mlp in a heterogeneous manner, which offers a possible explanation for the heterogeneous chemotactic response of individual neutrophils to this ligand. Using two-color flow cytometric analysis we found no CALLA/CD10–negative neutrophil population with C5a receptor levels clearly above CALLA/CD10–positive neutrophils. These results agree with those of Van Epps and Chenoweth who found no identifiable neutrophil subpopulations with greatly increased numbers of C5a receptors. Our results imply that the heightened chemotactic response to C' of CALLA/CD10–negative cells is best explained by a postligand binding event, but we cannot exclude the possibility of subtle differences in receptor affinity between CALLA/CD10–positive and -negative neutrophils.

The concept of neutrophil heterogeneity has been reviewed, but its biologic significance remains uncertain. It seems likely that the phenotypic/functional differences detected in this study and others reflect differences in gene regulation and expression between subpopulations. Unfortunately, little is known concerning the regulation of C5a receptor expression and the signal transduction that follows binding of C5a. Because the difference in responsiveness is quantitative rather than qualitative, we must assume...
that CALLA/CD10-negative and -positive neutrophils are equally endowed with the necessary machinery (ie, receptors and second messengers) to manifest an effective response. However, preliminary evidence indicates that although distinct subpopulations are not identifiable by quantification of C5a receptors, not all C5a receptors are associated with the cytoskeleton (D.E. Chenoweth, unpublished observations). The relationship between cytoskeleton-associated/nonassociated C5a receptors and CALLA/CD10 expression requires further investigation.

The relationship between the heightened chemotactic response to C and the absence of surface CALLA/CD10 is unclear. The presence or absence of CALLA/CD10 may be fortuitous, or the acquisition of CALLA/CD10 may be functionally significant. The possible role of CALLA/CD10-negative neutrophils in an inflammatory response in vivo should be pursued.

ACKNOWLEDGMENT

The authors would like to thank Brad Anderson and Mike Hupke for conducting the flow cytometry cell sorting, Neil Kay for making the Veterans Administration Hospital flow system available, and Keith Shubitz for generous provision of AHN-1. They thank Anne Goldman for assistance with the statistical analyses and Carol El-Ghandour for typing the manuscript.

REFERENCES


44. Gallin JI: Neutrophil heterogeneity exists, but is it biologically meaningful? Blood 63:977, 1984
Identification and characterization of a unique subpopulation (CALLA/CD10/negative) of human neutrophils manifesting a heightened chemotactic response to activated complement

RT McCormack, RD Nelson, DE Chenoweth and TW LeBien