The Role of Cellular Maturation in Neutrophil Heterogeneity
By Peter J. Krause, Mary B. Todd, Wayne W. Hancock, William T. Pastuszak, Eufnigio G. Maderazo, David H. Hild, and Catherine M. Koscioł

Previous studies have shown that many neutrophil (PMN) characteristics are heterogeneous but the origin of PMN heterogeneity is unknown. It is unclear if PMN functional heterogeneity is secondary to maturational differences or due to distinct subpopulations of cells that possess different functional capacities. The PMN 31D8 antigen is a useful probe for evaluation of PMN subpopulations. The majority of PMNs (approximately 85%) exhibit a high intensity fluorescence after 31D8 monoclonal antibody (MoAb) labeling (31D8 enriched or "bright" PMNs) as determined by flow cytometric analysis. These cells are more functional than cells with low intensity fluorescence (31D8 diminished or "dull" PMNs). Various immunologic, clonogenic and functional techniques were used to study the expression of the 31D8 antigen in HL-60 cells and myeloid cells in order to evaluate antigenic and functional heterogeneity during morphologic maturation. The results of this study indicate that the percentage of 31D8 antigen positive (31D8 antigen enriched and diminished) bone marrow cells increases from 20 ± 11% in myeloblast cells to 68 ± 10% in promyelocytes, 93 ± 2% in myelocytes and 99 ± 1% in bands and PMNs. 31D8 antigen enriched cells first appear at the myelocyte stage (32 ± 10%) and increase in bands (52 ± 13%), marrow PMNs (62 ± 13%) and peripheral blood PMNs (88 ± 4%). These data indicate that the heterogeneous expression of 31D8 antigen in PMNs is due, at least in part, to maturational differences within the PMN population and raise the possibility that other heterogeneously expressed PMN characteristics are also maturationally derived. They also suggest that 31D8 antigenic expression may be a more precise indicator of myeloid functional maturation than maturation as identified by cellular morphology.

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dysplastic criteria using Wright stained cytospin preparations and by functional criteria as assessed by nitroblue tetrazolium (NBT) reduction. Whole blood was obtained from healthy adult volunteers. The procedures for obtaining blood or other tissue and the use of human cells for this and other experiments were approved by the Human Investigation Committees of Hartford Hospital and Yale University School of Medicine. PMNs were purified by sedimentation over 6% HetaStat (McGaw Laboratories, Irvine, CA) for 30 minutes at 23°C followed by Ficoll-Hypaque (Sigma, St Louis, MO) gradient centrifugation. This separation procedure resulted in a population of 98% PMNs.

The HL-60 cells and PMNs were exposed to 31D8 MoAb and binding of the antibody measured by an indirect immunofluorescence technique.29 In brief, PMNs or HL 60 cells were washed once in PBS, counted and standardized to 5 million cells/mL. Cells were incubated at 4°C with antibody for 45 minutes with 31D8 MoAb or control MoAb. Non-specific antibody of the same subclass was used as the control antibody. The cells were then washed three times in PBS with 0.01% sodium azide (PBS-azide) and incubated for 30 minutes at 4°C with fluorescence isothiocyanate (FITC)-labeled goat anti-mouse IgG. The cells were washed three times in PBS-azide, fixed with 2% paraformaldehyde and stored at 4°C, in the dark, and in the absence of calcium and magnesium until use. Cells were directly observed by a Zeiss microscope using phase optics and fluorescence optics to evaluate antibody binding. In all experiments, the fluorescence intensity distribution of 10,000 cells was determined and the data were presented as the number of cells on the vertical axis and log green fluorescence on the horizontal axis divided into a three decade log scale consisting of 255 channels.

Three separate flow cytometric analyses were performed to assess the extent of 31D8 MoAb binding. First, positive fluorescence binding was assessed by flow cytometric analysis and 31D8 positive cells defined as those whose fluorescence intensity exceeded that of non-31D8 labeled control cells. Second, the mean fluorescence intensity of all 31D8 positive cells was determined by the flow cytometer and expressed as the mean fluorescence channel. Third, the percentage of 31D8 positive cells that exhibited high intensity fluorescence (31D8 enriched or "bright") cells and exhibited low intensity fluorescence (31D8 diminished or "dull") cells was determined as previously described.29 A summary of terms used to describe 31D8 MoAb binding is as follows: 31D8 antigen positive ("bright" + "dull") = cells that bind 31D8 MoAb; 31D8 antigen enriched ("bright") = cells that avidly bind MoAb; 31D8 antigen diminished ("dull") = cells that weakly bind MoAb.

31D8 antigen expression in bone marrow mononuclear cells grown in suspension culture. Bone marrow cells were obtained from patients undergoing diagnostic bone marrow aspirates for purposes other than this study. Mononuclear cells were purified by Ficoll-Hypaque density gradient centrifugation. This separation resulted in a 98% pure mononuclear cell population. The cells were cultured in α-MEM media (Sigma, St Louis, MO) supplemented with glutamine (400 mg/L), 25% horse serum (Flow Laboratories, McLean, VA), 2-mercaptoethanol (10⁻⁴ mol/L) and hydrocortisone (10⁻⁴ mol/L) for eight days as previously described.33 The cells were placed in a-MEM and agar supplemented with 20% fetal calf serum and 10% conditioned medium from human peripheral blood leukocytes stimulated with pokeweed mitogen. The cells were plated in quadruplicate in 35 mm dishes. The plates were examined by a Zeiss inverted microscope to confirm that the cells were in suspension without clumping. The plates were then incubated at 37°C for seven days. Discrete aggregates of 40 or more cells were scored as colonies on day seven using a Zeiss inverted microscope. Individual colonies were harvested, washed, fixed to glass slides and labeled with 31D8 MoAb using the immunoperoxidase procedure described above with the exception of the xylene exposure. Progeny cells were examined for intensity of staining as described above.

31D8 antigen expression in myeloid cells in bone marrow biopsy specimens. Paraffin-embedded bone marrow biopsy specimens were obtained from Hartford Hospital and Yale-New Haven Hospital Pathology Departments. Only biopsies interpreted as normal by staff pathologists using conventional light microscopy were used in this study. Duplicate sections of four fixed, paraffin-embedded specimens were processed for immunoperoxidase staining by a variation of a published method.24 The slides were exposed to xylene for paraffin removal, washed in ethanol, and treated with 1% hydrogen peroxide in methanol to inactivate endogenous peroxidase activity. The specimens were rehydrated, washed in Tris-saline, and exposed to 20% normal rabbit serum for 30 minutes to decrease nonspecific antibody binding. Specimens were exposed for one hour to 1:50 dilutions of ascites containing 31D8 MoAb or nonspecific control ascites. After washing, the sections were exposed to biotinylated rabbit IgG anti-mouse immunoglobulin and then to avidin biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA). The peroxidase reaction was developed with 0.5% 3,3-diaminobenzidine (Sigma, St Louis, MO) with 0.01% H₂O₂ in PBS and the sections counterstained with hematoxylin. Bone marrow biopsy specimens were reviewed by light microscopy and the percentage of peroxidase-positive bone marrow cells was determined by three observers. Cellular morphologic identification was made by an experienced hematopathologist while the degree of peroxidase staining was graded 0 to 4+ by two observers, one of whom had no knowledge of the study.

31D8 antigen expression in bone marrow PMNs compared with peripheral blood PMNs. Bone marrow aspirate material and peripheral blood were simultaneously obtained. Only patients whose bone marrow aspirates were read as normal by staff pathologists using conventional light microscopy were included in the study. The PMNs were purified and labeled with 31D8 MoAb and secondary antibody labeled to FITC as described above. PMN 31D8 MoAb binding was analyzed by flow cytometry and the percentage of cells with fluorescein intensity above background was calculated.

The stage of maturation of peripheral blood PMNs that were enriched for or had diminished 31D8 antigen was evaluated by indirect immunofluorescence with 31D8 MoAb as described above, followed by flow cytometric cell sorting (EPICS C, Coulter, Inc, Hialeah, FL). The total PMN population, 31D8 enriched and 31D8 deficient cells were sorted onto glass slides, stained with Wright stain and then analyzed for morphologic maturation.

31D8 antigen expression in colony forming cells. In order to determine if 31D8 antigen enriched and diminished PMNs arise from separate progeny, mononuclear blood cells were assayed for colony forming units- granulocyte, macrophage (CFU-GM) 35,26 followed by 31D8 immunoperoxidase labeling as described above. Mononuclear cells were separated by Ficoll-Hypaque and washed twice in α-MEM. The mononuclear cells (1-5 x 10⁵ cells/mL) were placed in α-MEM and agar supplemented with 20% fetal calf serum and 10% conditioned medium from human peripheral blood leukocytes stimulated with pokeweed mitogen. The cells were plated in quadruplicate in 35 mm dishes. The plates were examined by a Zeiss inverted microscope to confirm that the cells were in suspension without clumping. The plates were then incubated at 37°C for seven days. Discrete aggregates of 40 or more cells were scored as colonies on day seven using a Zeiss inverted microscope. Individual colonies were harvested, washed, fixed to glass slides and labeled with 31D8 MoAb using the immunoperoxidase procedure described above with the exception of the xylene exposure. Progeny cells were examined for intensity of staining as described above.

Statistical analysis. Statistical significance of differences between groups was determined using the Student's t-test (two-tailed). A P value of less than .05 was considered significant in all cases. Data is presented as mean ± 1 SD unless stated otherwise.
RESULTS

31D8 antigen expression in HL-60 cells compared with peripheral blood PMNs. Representative 31D8 MoAb binding patterns in DMSO differentiated HL-60 cells are compared with those of peripheral blood PMNs in Fig 1. Uninduced HL-60 cells consist almost entirely of a homogeneous subpopulation of 31D8 antigen diminished cells (31D8 dull) with a very small 31D8 antigen enriched (31D8 bright) subpopulation (5 ± 4%). HL-60 cells induced to differentiate with either DMSO or RA for seven days consisted of approximately equal percentages of a 31D8 antigen enriched cell subpopulation (59 ± 16% and 40 ± 10% respectively) and a 31D8 antigen deficient cell subpopulation (41 ± 16% and 60 ± 10% respectively). The 31D8 MoAb binding pattern of the cell subpopulations of induced HL-60 cells is more similar to that of the cell subpopulations of PMNs from the peripheral blood than that of uninduced cells (Fig 1). The amount of 31D8 MoAb binding to uninduced HL-60 cells and HL-60 cells induced with DMSO or RA is summarized in Table 1. A significant increase in 31D8 MoAb binding as measured by either mean fluorescence channel or by percentage 31D8 antigen enriched cells was noted in induced as compared with uninduced HL-60 cells.

31D8 antigen expression in bone marrow mononuclear cells grown in suspension culture. An increase in the percentage of cells binding 31D8 MoAb was noted in normal human bone marrow mononuclear cells grown in suspension as shown in Fig 2. Bone marrow myeloid cells were cultured in vitro for eight days and morphologic and functional maturation determined. On day one of culture, the majority of cells were large mononuclear cells whereas on day eight they were smaller with a percentage of cells having segmented nuclei. NBT reduction increased from 4% to 41% and corresponded to the increased expression of 31D8 antigen during the time of incubation. On day one of culture, only 5% of the cells bound 31D8 MoAb (31D8 positive) and none was 31D8 antigen enriched (31D8 bright); by day eight, 52% of the cells bound 31D8 MoAb and 20% were 31D8 antigen enriched. Subpopulations of cells as defined by 31D8 antigen expression after eight days of growth in suspension culture approximated that of PMNs from peripheral blood.

31D8 antigen expression in myeloid cells in bone marrow biopsy specimens. There was minimal 31D8 antigen expression in bone marrow cells less mature than promyelocytes as determined by immunoperoxidase staining of biopsied marrows. The intensity of staining increased with increasing morphologic maturation of the myeloid cells. There was heterogeneity of 31D8 antigen expression from the promyelocyte stage through the more mature myeloid cell stages (Fig 3). Promyelocytes labeled with an intensity ranging from 1+ to 2+. None of the promyelocytes, but all of the more mature cell types, included a percentage of cells that had intense (3+ to 4+) labeling with 31D8 MoAb. These results were similar to those of a previous study using 31D8 MoAb fluorescently labeled bone marrow cells where no promyelocytes, but a

Table 1. 31D8 MoAb Binding to Undifferentiated and Differentiated HL-60 Cells

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Mature Cells (%)</th>
<th>Mean Channel</th>
<th>31D8 Enriched (%)</th>
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<tr>
<td>RA</td>
<td>36 ± 6</td>
<td>48 ± 10</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>DMSO</td>
<td>38 ± 13</td>
<td>65 ± 12</td>
<td>59 ± 18</td>
</tr>
<tr>
<td>Control</td>
<td>1 ± 1</td>
<td>32 ± 9</td>
<td>5 ± 4</td>
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HL-60 cells were induced to differentiate with retinoic acid (RA) or DMSO and the amount of 31D8 antigen expression compared with undifferentiated cells (Control) in five consecutive experiments. There was a significant increase in the percentage of mature cells (PMNs and bands) in the cells induced with RA or DMSO compared with the control cells (P < .05 and P < .05, respectively). There was also a significant increase in 31D8 antigen expression in cells induced with RA or DMSO compared with control as measured by mean fluorescence channel (P = .012, P < .01 respectively) or by percentage 31D8 antigen enriched (bright) cells (P = .011, P = .011 respectively). There was significantly greater 31D8 antigen expression in the DMSO group compared with the RA group as measured by either mean fluorescence channel (P < .05) or percentage enriched cells (P < .05).
percentage of all the more mature myeloid cell types were 31D8 antigen enriched (bright). Thus, cells staining 3+ to 4+ with immunoperoxidase in this study were considered to be 31D8 enriched cells (31D8 bright). A summary of 31D8 MoAb binding to myeloid cells in four bone marrow biopsies is shown in Fig 4. These data confirm and extend those of previous studies because the extent of 31D8 antigen expression in each cell stage from myeloblast to PMN was examined. The data indicate that 31D8 antigen is minimally expressed on 20% of myeloblasts and 70% of promyelocytes, but none of these cells is enriched for 31D8 antigen. The antigen is increasingly expressed as myeloid maturation continues. Intense 31D8 antigen expression (3+ to 4+) first appears at the myelocyte stage of development with 30% of the cells 31D8 enriched. This enriched 31D8 subpopulation increased to 58% at the band stage and 60% at the PMN stage in the bone marrow.

31D8 antigen expression in bone marrow PMNs compared with peripheral blood PMNs. In order to compare 31D8 expression in PMNs from the bone marrow with those from the peripheral blood, PMNs were simultaneously isolated from bone marrow aspirations and from peripheral blood of four subjects. All subjects had normal bone marrow aspirates. PMNs were labeled with 31D8 MoAb and analyzed on the flow cytometer. Expression of 31D8 antigen as measured by mean fluorescence channel (117 ± 22) and the percentage of 31D8 antigen enriched cells (82 ± 4%) was significantly less in PMNs from the bone marrow than from the peripheral circulation (145 ± 19, $P = .054$ and 91 ± 3%, $P < .05$ respectively).

Indirect immunofluorescence labeling with 31D8 MoAb was performed on peripheral blood leukocytes followed by cell sorting and morphologic evaluation. The results indicate that as in bone marrow cells, increased myeloid maturation of leukocytes in the peripheral blood is associated with an increase in 31D8 expression (Table 2).

31D8 antigen expression in colony forming cells. Blood mononuclear cells were assayed for colony formation in a CFU-GM assay as described above. Individual cell colonies were harvested after seven days, fixed to glass slides, and stained by an immunoperoxidase technique. The slides were examined by light microscopy and the myeloid cells were graded 0 to 4+ for staining intensity. The results of 31D8 MoAb binding to cells from three colonies is shown in Fig 5. A heterogeneity in staining intensity from 1+ to 4+ was noted in the cells of each colony. Because a uniform staining pattern would be expected if the cells in each colony arose from separate antigen enriched or deficient stem cells, the results suggest that 31D8 antigen enriched and diminished PMNs arise from a single myeloid stem cell line.

DISCUSSION

The objective of this study was to investigate the origin of PMN heterogeneity using a monoclonal antibody directed against a novel PMN antigen (31D8) that is heterogeneously expressed in PMNs and that is associated with cellular function. The majority of PMNs bind 31D8 MoAb avidly (31D8 antigen enriched or bright). These cells have previously been shown to be more functional than PMNs that weakly bind the antibody (31D8 antigen diminished or dull). The results of this study indicate that there is an increased expression of the 31D8 antigen with myeloid maturation and suggest that heterogenous expression of the antigen is a result of maturational differences within the PMN population. The results also raise the possibility that other heterogeneously expressed PMN characteristics are maturationally derived.

Myeloid differentiation of HL-60 cells resulted in increased expression of the 31D8 antigen in cells that were previously antigen diminished. The binding patterns of the differentiated cells were more similar to those of mature PMNs than those of the undifferentiated HL-60 cells. Similarly, nonattached bone marrow mononuclear cells had increased 31D8 antigen expression after eight days in suspension culture (0% to 52% 31D8 antigen enriched), at which time the cells were morphologically and functionally more mature. Examination of bone marrow biopsy specimens indicated that 31D8 antigen is absent in early myeloid progenitor cells. The 31D8 antigen first appears in small
Fig 3. 31D8 antigen expression in bone marrow cells. Bone marrow cells were processed with a monoclonal antibody immunoperoxidase technique. (A) Marrow exposed to 31D8 MoAb. PMNs (P) have the greatest 31D8 MoAb binding as evidenced by the highest intensity of staining. Band forms (B) stain less intensely, and promyelocytes (Pr) least. (B) A different section of the same marrow processed in the same way except that an IgG control MoAb was used rather than 31D8 MoAb. The absence of staining of all cells including PMNs indicates that the increased staining of PMNs with 31D8 MoAb is not due to an increased amount of endogenous peroxidase.

amounts (31D8 antigen diminished cells) at the myeloblast stage, but no myeloblasts or promyelocytes are enriched for 31D8 antigen. By the myelocyte stage most cells are 31D8 positive and 30% are 31D8 antigen enriched. The percentage of 31D8 antigen enriched cells continues to increase through the metamyelocyte, band and PMN stages with an increase from 82% to 91%. Comparison of PMNs in the bone marrow and in the peripheral circulation indicate that 31D8 antigen expression continues to increase even at the "mature" PMN stage. These results are consistent with previous studies demonstrating an increase in cell function during myeloid maturation because 31D8 antigen diminished cells are less functional than 31D8 antigen enriched cells. They are also consistent with a recent study by Brown et al that concluded that 31D8 dull PMNs reside primarily in the bone marrow and are released by agents that enhance bone marrow release of PMNs. The present study supports the possibility that 31D8 antigen heterogeneity in PMNs is at least in part due to differences in maturation between 31D8 subpopulations,
I and graded 0 to 4. Four bone marrow biopsies are shown.

**Table 2. Percentages of PMNs and Bands in 31D8 Antigen Enriched and Deficient Cells**

<table>
<thead>
<tr>
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<th>Unsorted 31D8 Positive Cells</th>
<th>31D8 Enriched Cells</th>
<th>31D8 Deficient Cells</th>
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<tr>
<td>PMNs (% ± 1SD)</td>
<td>88 ± 3</td>
<td>88 ± 5</td>
<td>71 ± 15</td>
</tr>
<tr>
<td>Bands (% ± 15D)</td>
<td>12 ± 3</td>
<td>12 ± 5</td>
<td>29 ± 15</td>
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Peripheral blood leukocytes from five adults were labeled with 31D8 MoAb and evaluated morphologically for the percentage of PMNs and bands before (unsorted 31D8 positive cells) and after (31D8 enriched or bright and 31D8 diminished or dull cells) flow cytometric cell sorting. The difference between the percentage of bands in the 31D8 antigen deficient subpopulation and the 31D8 antigen enriched subpopulation was significant (p < .03).

expression when PMNs are incubated in vitro with a number of chemoattractants and monokines that alter other cell surface antigens. Although more recent studies in our laboratory have shown a decrease in 31D8 antigen expression with high concentrations of PMA (EG Maderazo, personal communication), these conditions are very unlikely to be present in the marrow or peripheral blood of healthy individuals. Previous studies have shown that neither C5a, fMLP, or opsonized zymosan cause changes in PMN 31D8 antigen expression.

There are several important practical implications of the observations made in this study in addition to the obvious utility of 31D8 MoAb for studying PMN heterogeneity. The results of this study suggest that there may be a disparity between PMN nuclear morphologic maturation and functional maturation, and that changes in functional maturation among cells may be better detected by antigenic differences. Previous studies have demonstrated that as myeloid maturation proceeds in the marrow, changes in nuclear morphology are accompanied by increased cell function. The fact that some band forms are 31D8 antigen enriched and some mature-appearing PMNs are 31D8 diminished suggests that PMN nuclear morphology and membrane functional maturation are not always linked. Other studies support this observation. For example, Boner et al and Krause et al, using direct observation of cellular chemotaxis with an underagarose technique, found that although PMNs were generally more motile than the less mature band forms, some bands were more motile than some PMNs. Nuclear morphology may not always be concordant with cellular function, but release of PMNs, bands and metamyelocytes from the marrow are thought to be closely related to cellular function. Therefore, a more accurate assessment of increased marrow release during inflammation may be obtained with the use of PMN membrane maturation markers such as...
31D8 MoAb binding than assessment of nuclear morphology as an indication of maturation. Consequently, 31D8 MoAb antibody analysis of PMNs in patients with sepsis may more accurately assess release of immature marrow PMNs and indicate the presence of infection than does the peripheral band count.

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