Altered Oxidative Product Formation in Neutrophils of Patients Recovering From Therapy for Acute Leukemia

By Bayard L. Powell, Patricia Olbrantz, Daphne Bicket, and David A. Bass

During chemotherapy for acute leukemias, severe neutropenia allows acquisition of life-threatening infections that are difficult to clear with antibiotics alone. With return of myelopoiesis, even severe infections often improve dramatically. We have sequentially examined oxidative metabolic responses of polymorphonuclear leukocytes (PMNL) from 30 patients with acute leukemias before induction chemotherapy and after recovery of myelopoiesis (circulating PMNL >500/µL). Maximal oxidative metabolic responses were quantitated by flow cytometric analysis of H₂O₂-dependent oxidation of intracellular 2',7'-dichlorofluorescein (DCFH) in individual PMNL after stimulation with phorbol myristate acetate (PMA). Resting PMNL oxidized a mean of 6.8 attomoles (amol) DCFH/cell/15 min, with no difference between normal or patients' PMNL. PMA-stimulated normal PMNL oxidized 183 ± 35 amol/cell (mean ± SD, n = 120). In patients' PMNL obtained before chemotherapy, the mean DCFH oxidation was not significantly different from controls (216 ± 78 amol/cell). However, 11 of 22 samples revealed populations of granulocytes with increased (primed) oxidative responses: seven of these 11 patients had proven or suspected infection at presentation. At recovery from chemotherapy-induced neutropenia, PMNL from 19 of 21 patients possessed one or more significant subpopulations with primed oxidation in response to PMA. In these 19 patients, 61% ± 8% of PMNL comprised primed populations that oxidized 503 ± 46 amol/cell. Oxidative activity was most pronounced in patients with proven or clinically suspected infections (with 41% ± 9% of PMNL oxidizing 615 ± 79 amol/cell).

However, oxidative responses to PMA were also significantly increased in recovery PMNL from ten patients without clinical or laboratory evidence of active infection (79% ± 11% of PMNL primed to oxidize 402 ± 29 amol/cell). The peak responses of the primed subpopulations were short-lived and generally lasted three days or less, although oxidative responses remained elevated above normal for a week or more. All of the patients with increased PMNL responsiveness survived their hospitalization. In contrast, PMNL from four patients who had a significant population (18% to 82% of cells) with reduced responsiveness. Two of these four patients (with 71% and 75% subnormal cells) died during this induction attempt: the third died during a second induction attempt; only one survived to discharge. The clinical significance of these phenomena is yet to be determined. The results support the clinical observation that recovery after therapy for acute leukemia suggests the presence of granulocytes with potent functional activity. Priming apparently may occur in the absence of overt infection, possibly because of stimulatory substances released during the period of prolonged neutropenia. Only a small number of patients were observed with the opposite phenomenon of hypoactive PMNL; however, the high mortality in this group suggests that the normal response (and/or the ability to prime PMNL following neutropenia) may be important to the eventual clinical outcome.

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Acute infections are the predominant cause of death and the major source of morbidity in patients with acute leukemia.1-3 Induction chemotherapy is usually characterized by 3 or more weeks of profound granulocytopenia. In almost all patients this period is complicated by fevers, with or without culture-documented infections; an infectious cause is identified in more than half the febrile episodes.1 Patients are at greatest risk during this period of severe neutropenia.3,4 Dramatic clinical improvement, including resolution of fevers and infections, is frequently observed with the reappearance of circulating polymorphonuclear leukocytes (PMNL), even in very ill patients. However, Bodey et al found that even patients with >1,500 PMNL/µL spent 6% to 25% of hospital days with infections.4 It is unknown whether the frequent improvement and occasional worsening of clinical states might be associated with increased or decreased functional capabilities of the PMNL appearing in the blood at the time of recovery from chemotherapy (recovery PMNL).

Prior reports of granulocyte characteristics and function in patients with acute leukemia have described varying abnormalities in adherence,4 in vivo migration,6,7 in vitro chemotaxis,6,7 granule enzyme content,10-14 phagocytosis,6,11-13 oxidative metabolism,19-21 and bactericidal13,15,16,18,22-25 and candidacidal11,12,26-28 activities. Other investigators have found normal in vitro functions,29-31 and some of the aforementioned reports have revealed one or more normal functional activities in the presence of specific functional defects.12,22,23,26,28 Several of these studies have evaluated patients in remission as well as with active disease; most have shown resolution or improvement of the abnormalities during remission,6,8,10-12,14,18,22,27 but persistent defects have been demonstrated.23,25 Among studies focusing only on remission patients, both normal30 and abnormal23 PMNL function has been described. Functions of PMNL obtained immediately postrecovery from chemotherapy have not been specifically examined.

Previous studies of PMNL function have generally been limited by assay techniques that measure mean activity of bulk cell populations, frequently an admixture of mature and immature granulocytes. For example, a 50% mean decrease in functional activity of a group of cells may reflect zero activity in half of the cells and normal activity in half, a 50%
decrease in activity of all cells, zero activity in 75% of cells and twice normal in 25% of cells, or any of an infinite number of such combinations. Granulocyte heterogeneity, even among mature PMNLs, is now well recognized in the healthy as well as the diseased host.\textsuperscript{5,32,33} We used a technique that has successfully identified heterogenous subpopulations of PMNL in nonleukemic patients with acute bacterial infections.\textsuperscript{33} An assay for hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) in aqueous solutions\textsuperscript{34} was modified to quantitate H\textsubscript{2}O\textsubscript{2}-dependent oxidative product formation by flow cytometry.\textsuperscript{35,36} Flow cytometry enabled us to identify and assay only mature granulocytes and to measure metabolic activity of single PMNL obtained from blood with as few as 500 PMNL/\mu L. Therefore, PMNL subpopulations could be identified and characterized even when the absolute granulocyte count was low or immature granulocytes were present in the peripheral blood. This technique was used to define the oxidative potential of PMNL in patients with acute leukemia before and after remission induction.

**MATERIALS AND METHODS**

*Patient selection and clinical evaluation.* Between May and Nov 1984 all patients at the Bowman Gray School of Medicine receiving induction chemotherapy for acute leukemia (lymphocytic [ALL], nonlymphocytic [ANLL], chronic granulocytic leukemia in blast crisis [CGL-BC]) were asked to participate in a study of PMNL functions. In patients who gave written informed consent, blood samples were obtained prior to initiation of therapy when feasible and at least thrice weekly, often daily, from the time of initial WBC recovery (\geq500/\mu L) until normalization of results or discharge. Initially, additional samples were obtained three times weekly even during the period of leukopenia; however, this practice was discontinued since a prohibitive amount of blood would have been required to obtain an adequate number of PMNL for study. Studies became feasible when circulating PMNL were \geq500/\mu L. The patients were examined daily with special attention to clinical and laboratory evidence of infection. Patients were classified as having proven (clinical observations and positive culture or chest x-ray), suspected (fever and/or physical findings without culture positivity), or no evidence of infection. Control PMNL were obtained from noninfected normal human volunteers.

*PMNL isolation and assay techniques.* Heparinized whole blood samples were obtained from indwelling central venous catheters by standard venipuncture techniques. Isolation of PMNL by sedimentation, centrifugation through Ficoll/metrizoate, hypotonic lysis of erythrocytes, and the 2',7'-dichlorofluorescin (DCFH) oxidation assay were used as described previously.\textsuperscript{35} In brief, isolated PMNL (10\textsuperscript{6}/mL) were incubated for 15 minutes at 37°C in 5 \mu mol/L dichlorofluorescin diacetate (Eastman Kodak, Rochester, NY) in phosphate-buffered saline containing 0.1% gelatin, without Ca\textsuperscript{2+} or Mg\textsuperscript{2+}. Dichlorofluorescin diacetate readily diffuses into cells, is hydrolyzed to 2',7'-dichlorofluorescein (DCFH), a nonfluorescent fluorescein analogue, and is trapped within the cells. During the oxidative metabolic burst of PMNL, DCFH is rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF). Baseline fluorescence was measured on a Spectrum III or a Cytofluorograph 50H flow cytometer equipped with a 4-W argon laser (488-nm emission, 250-mW output) and a 2150 computer (all from Ortho Diagnostic Systems, Westwood, Mass). Immediately before addition of stimulant, EDTA (2 mmol/L final concentration) was added to prevent cellular aggregation. PMNL were then stimulated with phorbol myristate acetate (PMA) (100 ng/mL). Fluorescence of individual resting and PMA-stimulated PMNL was measured after 15 minutes of incubation with horizontal agitation in a Dubnoff water bath. Mature PMNL were distinguished from immature granulocytic precursors using a combination of low-angle, forward- and right angle-scattered laser light (Fig 1). Individual PMNL fluorescent emission (510 to 550 nm) was determined by computer gating with calibration to measure the fluorescent intensity in attomoles (1 amol = 10\textsuperscript{-18} mol) DCF per cell, as described.\textsuperscript{35}

The intracellular oxidation of nonfluorescent DCFH to fluorescent DCF is mediated by H\textsubscript{2}O\textsubscript{2} generated by the PMNL oxidative burst. This reaction occurs within the cytosol\textsuperscript{37} and is in competition with other cytosolic H\textsubscript{2}O\textsubscript{2}-consuming activities including myeloperoxidase-mediated oxidation, lipid peroxidation, and H\textsubscript{2}O\textsubscript{2} destruction by catalase or the glutathione system. Therefore, increased DCF formation could reflect a decrease in these competing cytosolic reactions. To evaluate this possibility, PMNL from patients and controls were also evaluated in a similar fashion following the addition of an extracellular oxidant-generating system, xanthine oxidase (30 mU/mL) plus 10 mmol/L acetaldehyde, as described.\textsuperscript{35} We have noted significant variability among different batches of commercially available xanthine oxidase; therefore, the xanthine oxidase data are expressed as the ratios of DCF fluorescence generated by 15 minutes' exposure to xanthine oxidase for patient's PMNL divided by that for parallel control PMNL.

Oxidative activity of each patient's PMNL population was displayed in a histogram as seen in Fig 2. A mean for the total population and for each subpopulation was then calculated from the histogram. Subpopulations were defined as the mean and proportion of cells above and below the nadir of cell distribution between the two populations present. Subpopulations were generally classified as increased (primed), normal, or decreased (below normal) in comparison with the response of parallel control cells.
of all PMNL present. Eight patients, seven with suspected or proven infections, had distinct subpopulations with primed oxidative activity in response to PMA stimulation. A mean of 39% (range, 18% to 75%) of PMNL exhibited priming in these eight patients with a mean oxidation of 423 (range, 330 to 573) amol DCFH per cell in the primed population; six of eight of these patients survived their induction hospitalization.

Nine patients possessed 10% to 100% (mean 43%) of PMNL with a subnormal response to PMA (less than 113 amol/cell), with a mean DCF intensity of 66 amol (range, 36 to 106). Eight of these nine patients had suspected or proven infections at presentation, and only three of the nine survived their hospitalization. The three who survived all had a cell distribution that also included a primed subpopulation as previously described. Among patients who survived their hospitalization, no correlation between pretreatment oxidative response to PMA and response to chemotherapy was identifiable.

**Studies of PMNL at recovery from chemotherapy.** Table 2 displays patient characteristics, clinical data, and mean and subpopulation distributions of DCF intensities of PMA-stimulated recovery PMNL from the 19 patients who completed the study. The DCF data are summarized in Fig 3.

In 11 of 21 inductions clinically proven (nine) or suspected (two), infections were present at the time of recovery. The mean DCFH oxidized per cell by parallel control PMNL was 176 ± 29 amol/15 min (mean ± SD) after PMA stimulation with a unimodal distribution. The mean oxidative response to PMA stimulation in PMNL from recovering patients was elevated to 340 ± 138 amol/cell/15 min, and more importantly the distribution of cells was frequently not unimodal. Thirteen of 21 patients had two or three PMNL subpopulations, and 20 of 21 possessed populations with increased DCFH oxidation (1.5 to 6 times control). In these patients a mean of 58% (range, 9% to 100%) of the cells demonstrated priming. No clear correlations were noted between the percentage or degree of priming of PMNL and the type of leukemia or the presence of infection. However, a unimodal distribution appeared to be more common in noninfected patients, and the most dramatic oxidative bursts did occur in patients with proven severe infections (eg, patients 1 and 2). The peak oxidative activity was short-lived, often lasting only one to three days. However, PMNL continued to show priming for a week or longer (Fig 4).

Subnormal oxidative activity was observed in a significant proportion (>10%) of PMNL from four patients whose leukemia was not completely eradicated by their induction chemotherapy. Two of these patients subsequently died from overwhelming infections during their hospitalization; in each of these, the defective PMNL constituted a majority of the PMNL population, and little or no successful priming of circulating PMNL occurred. In both these patients the defective responses were observed on two or more occasions and persisted more than 1 week. The other patients with defective cells recovered from the induction regimen; however, one of these died after a subsequent induction attempt. Only one of the patients with defective PMNL responses was

**RESULTS**

*Clinical characteristics of the patients.* Thirty adults with acute leukemia (25 ANLL, 4 ALL, 1 CGL-BC) participated in this study; one patient elected not to participate. Nineteen patients met the criteria for evaluation including PMNL recovery. Eleven patients failed to complete the study; nine (eight ANLL, one CGL-BC) died during induction, and two patients (one ANLL, one ALL) were discharged prior to PMNL recovery. Two patients with ANLL completed two separate inductions for a total of 21 evaluable induction cycles with granulocyte recovery.

*Studies of PMNL prechemotherapy.* Pretreatment samples were obtained from 22 of 30 patients (Table 1) and from 14 of 19 patients who subsequently had recovery samples evaluated. Eight of the 22 patients with pretreatment samples had proven infection prior to therapy; seven had suspected and seven had no evidence of infection. In the DCFH oxidation assay, normal PMA-stimulated PMNL oxidized 183 ± 35 amol DCFH/cell/15 min (mean ± SD, N = 120); the normal range was therefore defined as 113 to 253 amol/cell/15 min. The oxidative responses of pretreatment PMNL were variable and, on the average, oxidized 213 ± 77 amol DCFH/cell (range, 101 to 369), but 11 patients had granulocytes with oxidative activity increased above the normal range. In three patients, the oxidative population distribution was unimodal, with a modest increase (range, 265 to 279 amole/cell) in oxidative activity...
discharged from the hospital. No patients who cleared their leukemia (CR) had subpopulations with defective oxidation.

Studies regarding altered efficiency of patients’ cells in the DCFH oxidation assay. Oxidation of DCFH by xanthine oxidase/acetaldehyde, an extracellular oxidant, was unimodal in subjects and controls alike. This suggests uniformity in the efficiency of DCF oxidation within the cytosol. In contrast, bimodal (and in two patients trimodal) responses of patients’ PMNL after PMA stimulation suggest that the subpopulation responses were due to differential endogenous oxidant generation by the respiratory burst. The amount of DCFH oxidized by xanthine oxidase did vary; however, there was no significant correlation between the effect of xanthine oxidase (to monitor assay efficiency in patients’ cells) and endogenous oxidation after PMA stimulation (r = .23, N = 21). Normalized patient DCF were calculated using the product of the raw DCF and the ratio of control/patient response of xanthine oxidase. Although the absolute DCF values were changed by this calculation, patterns of response and the overall results were the same (results not shown).

DISCUSSION

The results of this investigation support a time-proven clinical observation—recovery PMNL possess potent functional activity. Understanding of granulocyte function has been limited by bulk cell analyses in prior studies. The flow cytometric quantitation of H2O2-dependent DCFH oxidation provided several advantages over standard assays that measure mean oxidative metabolic responses of bulk cell populations. Scattered laser light could readily distinguish mature PMNL from blasts, allowing accurate analysis of only the mature circulating PMNL. Small numbers of cells were required; accurate studies could be obtained with 20 mL of blood from patients with fewer than 500 PMNL/μL. Subpopulations of PMNL with reduced ( deactivated) or increased (primed) responsiveness could be detected, and the specific metabolic activity of each subpopulation was quantitated.

Pretreatment studies were notable for two important findings. First, eight of 22 patients, seven of whom had proven or suspected infections, had a subpopulation of primed PMNL. This pattern of response is similar to that described in earlier studies of infected nonleukemic patients3 and provides supportive evidence for the presence of a population of granulocytes with normal oxidative function and with the ability to respond appropriately to infection compared with nonleukemic patients. In contrast, a subpopulation of relatively inactive cells was also identified in nine patients. In three of the patients the deactivated PMNL population was relatively large (range, 26% to 71% of cells) and the oxidative responses markedly below normal (44 to 56 amol/cell/15 min). An equivalent population has not been
Table 2. Clinical Characteristics and DCFH Oxidation of PMNL Obtained at Time of Granulocyte Recovery

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Infection</th>
<th>DCFH, amol/Cell (Percentage of total PMNL*)</th>
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<tr>
<td></td>
<td>Mean</td>
<td>Low Pop</td>
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<tr>
<td>Survived and obtained CR</td>
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<tr>
<td>1 47/M ANLL LoDAC</td>
<td>CR P P</td>
<td>575</td>
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<tr>
<td>Died</td>
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<tr>
<td>13 65/M ANLL ArC/DNR</td>
<td>PL P P</td>
<td>173</td>
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<tr>
<td>Died after quantitative granulocyte recovery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 47/F ANLL HiDAC/ASP</td>
<td>ID P P</td>
<td>133</td>
</tr>
<tr>
<td>Died after subsequent induction attempt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 60/F ANLL HiDAC/ASP</td>
<td>ID P N</td>
<td>102</td>
</tr>
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*DCF responses; PMNL populations, diagnosis, and presence of infection, defined as in Table 1.
†Therapy: HiDAC, high-dose cytosine arabinoside; ASP, L-asparaginase; ArC, standard cytosine arabinoside; DNR, daunorubicin; VCR, vincristine; PR, prednisone; DHAD, mitoxantrone; LoDAC, low-dose cytosine arabinoside; MTX, methotrexate; AMSA, amsacline.
‡Response: CR, complete remission; PL, partial remission; ID, induction death.
§Patients who were evaluated during two separate inductions.
 Died after subsequent induction attempt.

identified in either control cells or PMNL from nonleukemic patients with infections. This might suggest that these granulocytes either originate from a leukemia clone or are depressed by a leukemia-related inhibitor of PMNL function. In further support of this concept, five of nine patients who failed to obtain a complete remission had a depressed PMNL population at recovery from chemotheraphy. Such a depressed PMNL population was not identified in any patient who achieved complete remission.

Responses to PMA stimulation in recovery granulocytes were unique and consistent. All patients but one had a population of PMNL with increased DCFH oxidation. This population was evident independently of the presence, type or site of infection, type of leukemia, type of therapy, or response to therapy. However, the most dramatic oxidative responses did occur in patients with severe proven infection.

The mechanism(s) of priming of PMNL have not been defined. The presence of distinct PMNL subpopulations appears to be unique to situations of leukopoietic stress as demonstrated in prior studies of patients with bacterial infections and the current data on patients after chemotherapy for acute leukemia. The stimulant in the recovering leukemia patients, especially the noninfected patients, is unknown. Perhaps clinical or subclinical infections provided the stimulus. All patients did have evidence of infection at some period during their hospitalization, but ten of 21 were...
free of evidence of infection at the time of granulocyte recovery. As previously noted, similar primed PMNL subpopulations have been observed in bacteremic nonleukemia patients; however, the degree of PMA-stimulated DCFH oxidation, even in severely infected patients, has generally been less than that seen with recovery in patients with acute leukemia. It is also possible that a leukemia-associated inhibitor of PMNL maturation and/or function is eliminated by therapy and therefore allows for full functional activity in recovering normal PMNL; this seems unlikely since very active populations were noted in some patients (eg, no. 16) with incomplete eradication of leukemia cells. A more likely possibility is a granulocyte-stimulating factor produced during the prolonged and profound granulocytopenia. One is tempted to surmise that the duration and/or degree of granulocytopenia plays a major role in the priming phenomena and may be further potentiated by infections.

The significance of these primed recovery PMNL is difficult to assert at present. The importance of their role is suggested, though by no means proven, by the infectious deaths of both patients 20 and 21 who had a definite paucity of primed cells and a predominance of relatively inactive PMNL. Although the clinical correlates, and indeed the relevance, of the primed PMNL oxidative response are yet to be determined, these and future studies using single-cell techniques should help to elucidate the functional characteristics of pretreatment and recovery granulocytes in patients with acute leukemia.

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