In the past differentiation of human neutrophils has been defined by morphology, cytochemistry, or surface markers. In our experiments we have sequenced the various events that occur during the functional differentiation of the normal human neutrophil and have also examined some of the functional properties in relationship to surface markers and biochemical events. Granulocytes were obtained from the bone marrow and blood of hematologically normal individuals. Cells were separated into different stages of maturation by their physical properties using counterflow centrifugal elutriation and density gradient separation. Three cell fractions were obtained that were enriched for either immature myeloid cells, band neutrophils, or segmented neutrophils. Since the enriched fractions were not entirely pure, methodologies for functional assays were chosen that allowed cytoclogic evaluation of the functional properties in relationship to surface markers. Thus methodologies were chosen that allowed cytologic evaluation of the functional properties in relationship to surface markers.

DIFFERENTIATION of human neutrophils is traditionally defined by the morphology of stained cells as seen by light microscopy. Schilling divided developing neutrophils into six morphologic subtypes: myeloblasts, promyelocytes, myelocytes, metamyelocytes, band neutrophils, and segmented neutrophils. Subsequent ultrastructural cytochemical studies showed the sequential acquisition of primary and secondary granules, providing additional criteria for accurately assessing the stages of neutrophilic development. Although such a classification tends to create an impression of step-like transitions rather than a continuous process, it provides useful and easily understood reference points for keying developmental events in the life history of the neutrophil.

With the development of immunologic techniques and monoclonal antibodies (MoAbs), the sequential appearance of surface markers on the developing neutrophil is now being catalogued. In addition to morphologic, enzymatic, and antigenic changes, functional changes also occur during differentiation. The neutrophil becomes more deformable, more adherent to surfaces, more actively forms pseudopods, more avidly ingests particles, and becomes more actively motile. The maturation sequence of the functional properties of the neutrophil has not yet been determined. Previous studies in our laboratory on leukemic cells suggested that the various functional properties of the neutrophil appear in a distinct order during cell development. In the present study we have sequenced the various events that occur during the functional differentiation of the normal human neutrophil. Granulocytes were isolated from the bone marrow and blood and separated into different stages of maturation by their physical properties using counterflow centrifugal elutriation and density gradient centrifugation. Three leukocyte fractions were obtained. These were enriched or purified for either segmented neutrophils, band neutrophils, or immature myeloid cells. The enriched fractions were not entirely pure. Thus methodologies were chosen that allowed cytoclogic evaluation of each stage of differentiation. Phagocytosis, bacterial killing, chemotaxis, nitroblue tetrazolium dye reduction, and opsonic receptors were evaluated. Our results indicate that phagocytosis is acquired early, followed by nonoxidative microbial killing, and that oxygen-dependent microbial killing and chemotaxis represent the terminal stages of functional differentiation.

MATERIALS AND METHODS

Bone marrow samples. Specimens were obtained from seven paid hematologically normal volunteers of the hospital staff, two hematologically normal individuals undergoing heart surgery, and one patient with Hodgkin's disease. Informed consent was obtained. Not all tests were performed on all specimens. Marrow was aspirated from the iliac crest or sternum. Particles were disrupted by successively passing the sample through 22-G and 25-G needles. Cells were resuspended in 6% hydroxyethyl starch in 0.9% sodium chloride (American McGaw, Irvine CA), allowed to settle at room temperature, and the leukocyte-rich supernatant collected. Segmented neutrophils and eosinophils were removed by Ficoll-Hypaque separation (sp. gr. 1.077) and residual erythroid cells by hypotonic lysis. Nucleated bone marrow cells were then resuspended in elutriation medium consisting of RPMI 1640 and 5% fetal calf serum.

Counterflow centrifugal elutriation. Bone marrow cells were fractioned by centrifugal elutriation using a Beckman J2-21 centrifuge, Je-6B rotor, standard chamber, and Cole-Parmer Masterflex peristaltic pump. All procedures were done at 22 °C. The rotor speed was 1740 ± 10 rpm. Fifty-milliliter samples were collected at each flow rate for a total of 16 fractions. Fractions that were similar were combined when necessary to increase the yield. The fractions were further enriched, if necessary, eliminating segmented neutrophils using Ficoll-Hypaque separation. The viability of the cells was determined in each fraction prior to testing using eosin-Y dye exclusion.

Segmented neutrophils were prepared by collecting venous blood in heparin, adding 2 mL of a 6% hydroxyethyl starch solution to 7
ml of whole blood, harvesting the leukocyte-rich plasma, and eliminating the RBCs by hypotonic lysis. The blood leukocytes were resuspended in elutriation medium and then fractionated using centrifugal elutriation. Monocytes were effectively removed by the elutriation procedure. Conditions were similar to the separation procedure for bone marrow.

Criteria used to classify stages of neutrophil differentiation. Stages of neutrophil differentiation were evaluated by morphologic criteria from smears stained with Romanowsky dyes and by autoradiography after DNA labeling with tritiated thymidine.13 Nuclei were considered labeled if five or more grains were counted. The criteria are listed below:

1. Myeloblasts range from 15 to 20 μm in diameter and have round nuclei with stippled chromatin with one or more nucleoli, a high nuclear/cytoplasmic ratio, and nongranular cytoplasm. They show nuclear labeling with tritiated thymidine.

2. Promyelocytes differ from myeloblasts by the appearance of azurophilic granules in the cytoplasm.

3. Neutrophilic myelocytes differ from promyelocytes by the appearance of specific neutrophilic granules. The nuclei show some chromatin condensation, and nuclei are not as prominent as in the preceding stage. On Romanowsky-stained smears, the cells have round, oval, or flattened nuclei without indentation; however, this criterion was superseded when indented nuclei showed labeling with tritiated thymidine.

4. Neutrophilic metamyelocytes differ from myelocytes by the appearance of a nuclear indentation and absence of nuclear labeling with tritiated thymidine.

5. Neutrophilic bands differ from metamyelocytes in that the appearance is more than half the diameter of a hypothetical circle and the nucleus has parallel sides.

6. Segmented neutrophils differ from bands in that the nucleus is divided into lobes separated by a thin filament with no visible nuclear chromatin between the two sides.

Fc-IgG receptors. Fc-IgG receptors (FcR) were detected by rosette formation with group O type Rh,Rh erythrocytes sensitized with human anti CD (Ripley) serum (EA₁₇). Rosettes of sensitized erythrocytes attached to leukocytes were evaluated in wet preparations and on stained smears prepared with a Larc spinner (Corning Glass Works, Medfield, MA). The percentage of positive cells at each stage of differentiation was evaluated by morphology from the smears. A positive cell was considered to be a leukocyte with one or more attached erythrocytes. Anti-Leu-11b (Becton Dickinson, Mountain View, CA) was also used to study the Fc receptor.18 Immunogold was used as the detection system (Geometric Data, Wayne, PA). Twenty microliters of 10⁶ leukocytes was mixed with 20 μL of 0.5% paraformaldehyde and 5 μL of anti-Leu-11b. The suspension was incubated at 5°C for 15 minutes. The paraformaldehyde was necessary in this initial step to prevent phagocytosis of the colloidal gold by the phagocytes. After washing, 20 μL of the colloidal gold reagent was added and incubated at room temperature for 15 minutes. Cells were washed, 5 μL of autologous EDTA plasma was added, and the cells were incubated for 20 minutes at 37°C. Two microliters of 0.5% bovine albumin was then added, and push smears or cytocentrifuged slides were prepared and stained. Positive cells have dark blue–black particles visible on the cell surface.

Complement receptors. Complement receptors were assayed with MoAbs (Becton Dickinson): anti-C₅₁₉, C₃b, C₄b receptor), anti-C₅₁₉/anti-Leu-15 (C3bi receptor). The method is similar to that described for FcR using MoAbs and immunogold. The classification of the different stages of differentiation was easily evaluated from Romanowsky-stained smears.

Phagocytosis. Neutrophil phagocytosis was evaluated using Staphylococcus aureus (ATCC strain 25933) as the test particle. The bacteria were opsonized by adding normal pooled human AB serum for 30 minutes at 37°C. Live bacteria were used to determine the percentage of phagocytic neutrophils, since this results in optimal phagocytosis.17 Bacteria that were heat killed at 60°C for one hour were used to evaluate differences in the number of particles ingested per phagocyte to eliminate intracellular bacterial multiplication as a variable. In the reaction mixture the concentration of the specific neutrophilic cell-type being evaluated was 1,000/μL; the bacteria to leukocyte ratio ranged from 20:1 to 50:1; the final test volume was 0.5 mL, and the suspending medium was pooled human AB serum. The leukocyte-bacterial mixtures of each of the fractions were incubated for one hour at 37°C, with constant agitation, in polystyrene test tubes. Then the leukocytes were labeled with tritiated thymidine for 60 minutes. Smears were made with a Larc spinner. All smears were stained with Giemsa solution. Five hundred cells at each stage of differentiation, except for myeloblasts and progranulocytes, were evaluated for determining the percentage of phagocytic cells, and 50 phagocytic cells at each stage were evaluated for the number of ingested particles per phagocyte.

Microbial killing. Bacterial killing was assayed by a modification of Cline's technique using S aureus.18 Mixtures of bacteria with the specified cell type were incubated at a ratio of 20:1 for 30 minutes at 37°C in a test tube with continuous agitation. After phagocytosis, the cells were washed. A 0.5-mL solution of McCoy's medium with 30% AB serum and tritiated thymidine (New England Nuclear Corp, Boston, 60 to 80 Ci/ml) was added to 1.0 mL of the cell suspension. The final mixture contained 3 μCi of tritiated thymidine. Cells were incubated for 30 minutes at 37°C for labeling, and slides were prepared for autoradiography. The total number of intracellular organisms and the number of organisms associated with grains were counted in 50 phagocytic cells of each neutrophilic cell type. The percentage of viable bacteria was calculated from the percentage of labeled cells, corrected for 100% labeling.

Autoradiography. Cells were labeled with tritiated thymidine of high specific activity (60 to 80 Ci/ml) for the labeling of both leukocytes and bacteria, as described above. Autoradiography was done as described by Durie and Salmon.19 Smears were stained through the emulsion with Giemsa solution.

Nitroblue tetrazolium dye reduction. The respiratory burst was evaluated by a modification of the cytochemical technique of Park et al.20 Cells were suspended in 0.5 mL of AB serum to which 0.05 mL of 100 μg/mL of Bacto lipopolysaccharide (Difco, Detroit) was added. A 0.1-mL aliquot of this mixture was added to an equal volume of 0.1% nitroblue tetrazolium (Sigma, St. Louis). A drop of the mixture was added to a glass slide, and the cell suspension was covered with a coverslip that was sealed and then incubated at 37°C for 30 minutes. In two experiments cells were examined with phase and light microscopy to evaluate both the stage of cellular maturation and dye reduction. At least 200 cells were counted in each fraction. In another experiment we attempted to identify the stage of cellular differentiation by vital staining with acridine orange (Fisher Scientific, Fair Lawn, NJ). Freshly prepared acridine orange (AO) solution (14.0 mg/100 mL in HBSS, pH 7.2) was added to the cells, mixed, and stained for 45 seconds at room temperature. The AO-stained cells were 98% viable. The cells were then suspended in 0.5 mL of AB serum to which 0.05 mL of endotoxin was added, and the remaining procedure was identical to that described previously. Nitroblue tetrazolium (NBT) dye reduction was evaluated by light microscopy and cell morphology by both light and fluorescent microscopy.

Locomotion. Chemotaxis was evaluated in vitro by the agarose technique.21,22 The total inoculum of the desired cell type was 10⁶. The chemotactic well was filled with thawed fresh-frozen AB serum. Interaction of the agarose with the serum generates complement-derived chemotactic factors, eg, C5a. Cells were incubated for five
hours at 37 °C in 5% CO₂ atmosphere with humidity and were stained with Giemsa solution. Chemotaxis was quantitated by counting all the leukocytes of the desired cell type that had migrated out of the well. The cells were identified by light microscopy under oil immersion (× 1000).

**Electron microscopy.** Leukocytes were fixed in 3% glutaraldehyde made up in 0.1 mol/L phosphate buffer, postfixed in osmium tetroxide, dehydrated in alcohol, and embedded in Spurr's epoxy resin. Sections were cut on a Sorvall MT2-B ultramicrotome and mounted on uncoated 200-mesh grids. A 5% aqueous uranyl acetate solution followed by lead citrate increased contrast. The grids were lightly carbon coated and examined with the Hitachi HU-12 electron microscope. In one experiment, phagocytic myeloid cells were classified ultrastructurally using myeloperoxidase as a marker for primary granules. After phagocytosis cells were fixed in cold 3% glutaraldehyde (pH 7.2), washed, and reacted with diaminobenzidine (1 mg/mL) in 0.05 mol/L Tris buffer containing 0.03% glutaraldehyde (pH 7.2). Washed, and reacted with diaminobenzidine (1 mg/mL) in 0.05 mol/L Tris buffer containing 0.03% hydrogen peroxide for two hours in a dark room. There was no postfixation in osmium tetroxide or staining with uranyl acetate or lead citrate.

**RESULTS**

**Leukocyte fractions.** Neutrophilic leukocytes were separated from the bone marrow or blood on the basis of size and density using gradient-density separation and counterflow centrifugal elutriation, which provided sufficient numbers of cells for functional testing. Mean leukocyte viability of the fractions used for testing ranged from 95% to 100%. We were able to obtain fractions of segmented neutrophils from the blood essentially free of contamination from band neutrophils and fractions of band neutrophils from the bone marrow essentially free of segmented neutrophils. The fraction of immature cells from the bone marrow contained a mean of 60% intermediate (myelocytes and metamyelocytes) neutrophils and 3.9% promyelocytes. It also contained a moderate number of band neutrophils. The percentages of each cell type in each fraction are shown in Table 1. Since the enriched fractions were not entirely pure, techniques for the measurement of neutrophil function were selected that allowed cylogic evaluation.

**Fc-IgG receptors.** The percentage of EA rosettes in wet preparations and stained smears were in close agreement. In stained smears, sensitized erythrocytes that attached to Fc receptors on leukocytes were closely applied, frequently molded, and often showed fine hairlike projections extending from the erythrocyte to the leukocyte. The earliest cells, in which Fc receptors were detected, were promyelocytes. Fc-positive cells reached their optimal expression at the band and segmented neutrophil stages (Table 2). Results using the rosette technique differed from those obtained using anti-Leu-11b. In two experiments using the MoAb, only the most mature neutrophils were Leu-11b positive: 82% segmented neutrophils, 40% band neutrophils, 3% neutrophilic metamyelocytes, 0% neutrophilic myelocytes, 0% promyelocytes, 0% myeloblasts.

**Complement receptors.** Monoclonal antibodies to complement receptors detected CR1, and CR3 on neutrophilic leukocytes. These receptors appeared later in development than FcR. Complement receptors were occasionally detected on promyelocytes. CR3 appeared first and was the dominant receptor in later stages of development. CR3 was first detected at the promyelocyte stage. A marked increase in expression occurred in cells of the nondividing maturation pool. Thus metamyelocytes had a sixfold increase in positive cells compared with myelocytes. Greater than 90% of band and segmented neutrophils reacted with anti-CR3 (anti-Leu-15). Results are summarized in Table 3.

**Phagocytosis.** Even a rare blast had the capacity to phagocytize, and the number of phagocytic cells gradually increased as the cells matured. The phagocytic capacity of immature cells was confirmed by electron microscopy (Fig 1). There was a sharp increase in phagocytic cells as cells entered the nondividing maturation pool. Thus there were more than twice as many phagocytizing metamyelocytes as myelocytes. Peak phagocytosis was reached at the band stage. In two experiments mean values for the percentage of phagocytic cells were: myeloblasts <1%, promyelocytes 10%,

| Table 1. Differential Counts of Hematopoietic Cells From Bone Marrow and Blood Separated by Centrifugal Elutriation |
|-------------|------------------|------------------|------------------|
| Cell Type   | Segmented Neutrophils | Band Neutrophils | Immature Neutrophils |
| Myeloblast  | 0                 | 0.9 ± 0.3        | 0.6 ± 0.3        |
| Promyelocyte| 0                 | 1.8 ± 0.6        | 3.9 ± 0.8        |
| Myelocyte   | 0.2 ± 0.2         | 9.3 ± 1.5        | 35.8 ± 2.7       |
| Metamyelocyte| 0.9 ± 0.4         | 24.3 ± 2.2       | 23.8 ± 2.6       |
| Band neutrophil | 4.6 ± 2.2          | 50.2 ± 1.9       | 18.2 ± 1.8       |
| Segmented neutrophil | 86.9 ± 2.3        | 4.3 ± 1.6        | 31.1 ± 1.4       |
| Others†   | 7.3 ± 1.8         | 9.2 ± 1.8        | 14.6 ± 2.8       |

*Mean ± SEM (n - 9). Results are expressed as a percentage.
†Others (lymphocytes, monocytes, eosinophils, basophils, plasma cells, normoblasts)
‡Contaminating segmented neutrophils were eliminated by density gradient centrifugation.

| Table 2. Expression of Fc Receptors (EA<sub>43</sub> Rosettes) on Differentiating Neutrophils |
|-----------------------------|------------------|------------------|
| Stage | Fc-positive cells %* |
| Myeloblast | -† |
| Promyelocyte | 32 |
| Myelocyte | 42 |
| Metamyelocyte | 55 |
| Band neutrophil | 78 |
| Segmented neutrophil | 82 |

*Mean values (n - 2 experiments).
†Too few cells seen for evaluation.

| Table 3. Expression of CR<sub>1</sub>, and CR<sub>3</sub> on Differentiating Neutrophils |
|-----------------------------|------------------|------------------|
| Stage | Anti-CR<sub>1</sub> % | Anti-CR<sub>3</sub> %* |
| Myeloblast | 0 | 0 |
| Promyelocyte | 0 | 3 |
| Myelocyte | 0 | 10 |
| Metamyelocyte | 0 | 58 |
| Band neutrophil | 11 | 92 |
| Segmented neutrophil | 58 | 94 |

*Mean values (n - 2 experiments).
myelocytes 34%, metamyelocytes 84%, band neutrophils 99%, and segmented neutrophils 99%. The phagocytic capacity (ie, bacteria/phagocyte) of each cell type showed the same trend as percent phagocytosis. Too few phagocytic cells were seen at the myeloblast and promyelocyte stage for a meaningful comparison with other stages of maturation. There was a marked increase of phagocytic capacity as cells entered the nondividing maturation pool. Mean values for two experiments showed that myelocytes ingested seven bacteria/cell, metamyelocytes 14/cell, band neutrophils 36/cell, and segmented neutrophils 38/cell.

Stimulated NBT dye reduction. The techniques used in these experiments allowed us to identify each morphological stage of differentiation showing nitroblue tetrazolium dye reduction. The results were striking. Only band and segmented neutrophils showed a "respiratory burst," as evaluated with the cytochemical technique. Also there was a significant difference in NBT reduction between band and segmented neutrophils (p < 0.05). Mean values in three experiments were: myeloblasts 0%, promyelocytes 0%, intermediate neutrophils (myelocytes and metamyelocytes) 1%, band neutrophils 57%, and segmented neutrophils 98%.

Microbial killing. Two normal cell donors were tested to evaluate bacterial killing. Both the percentage of bacteria killed by phagocytic cells of each cell type and the total number of bacteria killed by equivalent numbers of leukocytes of each cell type were determined. Two few phagocytic promyelocytes were seen for a statistical comparison of bacterial killing with other cell types; however, the few phagocytic promyelocytes seen showed only modest killing, and two thirds of the bacteria survived intracellularly. Since promyelocytes did not reduce nitroblue tetrazolium, all kill-
neutrophils were the most effective bactericidal cells. Only 5% of the bacteria were viable after ingestion. As shown in Table 4, differences in the bactericidal capacity of each cell type are much greater when the total number of bacteria killed by equivalent numbers of leukocytes are compared.

**Chemotaxis.** Chemotaxis was a later manifestation of functional maturity. Essentially no chemotaxis was observed except in band and segmented neutrophils. Band neutrophils showed approximately half the activity of segmented neutrophils. In two experiments the mean chemotactic scores for the three fractions analyzed were intermediate neutrophils (myelocytes, metamyelocytes) 6, band neutrophils 424, and segmented neutrophils 900 (Fig 3).

**DISCUSSION**

The sequence of development of the functional properties of the neutrophil during differentiation has not previously been determined. Neutrophils must have the biological functions needed to eradicate microbes. In the inflammatory process a distinct sequence of events occurs. Locomotion, diapedesis, and chemotaxis precede phagocytosis. Ultimately the internal killing mechanisms of the cell destroy the microbe. During differentiation the functional properties of the neutrophil are acquired sequentially. Our data show that phagocytosis is acquired prior to the functions, followed by oxygen-independent microbial killing, whereas both oxygen-dependent microbial killing and chemotaxis are late manifestations of functional differentiation. According to our data the relative activity of each neutrophilic cell type for each function is shown in Fig 4.

Fc and complement receptors are acquired early in differentiation. Both are important in immune phagocytosis. Interaction of Fc receptors with opsonins trigger second messages, promoting both phagocytosis and superoxide generation.23 Our results show that Fc receptors appear at least as early as the promyelocyte stage. Results of the rosette technique using sensitized erythrocytes differed from those of the MoAb Leu-11b. The former must be considered the reference method for the qualitative detection of Fc receptors. There are several possible explanations for the differences. The absence of Leu-11b on immature granulocytes may indicate that further molecular modeling of Fc receptors occurs at the band neutrophil stage. It is also possible that the antigenic determinant Leu-11b is not part of the Fc receptor even though anti-Leu-11b inhibits the function of the receptor. Another explanation is that the FcR epitope detected by anti-Leu-11b is not critical for the binding of IgG-sensitized erythrocytes. In murine neutrophils it has been proposed that Fc receptors appear on the cell membrane before the cellular acquisition of phagocytosis.24 Murine myelocytes were the earliest cells to acquire Fc receptors; however, only segmented neutrophils or neutrophils with O-ring nuclei were phagocytic. Altman and Stossel studied phagocytosis in developing human neutrophils, and they did not observe phagocytosis by promyelocytes.25 In our experiments human neutrophils showed phagocytosis as early as the promyelocyte stage, and phagocytic capacity lagged slightly behind but paralleled the acquisition of Fc receptors. We used enriched fractions of bone marrow rather than a mixture of

**Table 4. Neutrophil Differentiation and Bacterial† Killing**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Bacteria Killed/100 Cells</th>
<th>Mean Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp #1</td>
<td>Exp #2</td>
</tr>
<tr>
<td>Myeloblast</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Promyelocyte</td>
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<td>0</td>
</tr>
<tr>
<td>Myelocyte</td>
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<td>54</td>
</tr>
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</tr>
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<td>3100</td>
</tr>
<tr>
<td>Segmented neutrophil</td>
<td>4004</td>
<td>5480</td>
</tr>
</tbody>
</table>

*Staphylococcus aureus.*
Fig 3. Neutrophil migration under agarose. (A) Myelocyte/metamyelocyte fraction. The well (bottom) contains immature granulocytes that show little migration. A rare myelocyte and metamyelocyte (inset) are seen migrating toward the chemotactic attractant. (B) Band neutrophil fraction. A moderate number of band neutrophils migrate toward the chemotactic attractant. (C) Segmented neutrophils. Numerous segmented neutrophils migrate toward the chemotactic attractant.

all bone marrow leukocytes. This enabled us to standardize the ratios for each cell type and to diminish the competitive effect of more actively phagocytic cells, thereby allowing leukocytes such as promyelocytes to express their full phagocytic potential. Enriched fractions may simply allow more promyelocytes to be evaluated experimentally. We also used a different particle, live bacteria rather than opsonized albumin or lipopolysaccharide-coated paraffin oil droplets. The type of particle and the method of preparation of the particle are critical. For example, in our experiments we noted that promyelocytes phagocytized opsonized live bacteria more readily than opsonized dead bacteria. This is consistent with the observation that human segmented neutrophils distinguish between viable and nonviable bacteria and preferentially live organisms.17 The best evidence in humans that Fc receptors are acquired by granulocytes prior to the capacity for phagocytosis comes from studies with human myeloid leukemic cell strains.9 Whereas some leukemic strains show prominent EA<sub>43</sub> rosettes and little or no phagocytosis, no Fc receptor-negative myeloid-leukemic strains have shown phagocytosis. Thus human leukemic cells clearly demonstrate that the acquisition of Fc receptors precedes phagocytosis.

In addition to FcR, complement receptors also promote phagocytosis. Ligands derived from the third component of complement (C3) are the principal opsonic forms of complement. There are two complement receptors on neutrophils that react with C3 opsonins: CR<sub>1</sub> and CR<sub>3</sub>. These receptors react synergistically with Fc receptors to promote phagocytosis.23,24 They appear at a later stage of development than Fc receptors and phagocytosis and are not overtly expressed until the myelocyte/metamyelocyte stage. Once complement receptors are acquired, phagocytosis is enhanced. It is teleologically satisfying that the receptor for the predominant opsonic species C3bi appears prior to other complement receptors.

The next function acquired by differentiating neutrophils was microbial killing. The labeling technique coupled with the NBT test can be used to assess the contribution of both oxygen-independent and oxygen-dependent bactericidal mechanisms. It is well established that the biochemical basis of NBT reduction in normal human neutrophils is due to superoxide generation and that cytochemical NBT reduction is an expression of the respiratory burst.27 Phagocytic promyelocytes killed few bacteria. Promyelocytes have no oxidative killing; however, it also appears that their oxygen-independent bactericidal mechanisms are inoperative. Promyelocytes have primary granules that contain lysosomal enzymes, lysozyme, and cationic bactericidal proteins. Thus the armamentarium for oxygen-independent killing is present. It is possible that suboptimal phagolysosome formation

Fig 4. Schematic representation of the relative activity of neutrophil functions at various stages of neutrophil differentiation.
or absence of secondary granules, which contain bactericidal substances such as lactoferrin, accounts for impaired nonoxidative killing. Like promyelocytes, neither myelocytes nor metamyelocytes showed a respiratory burst; however, both cell types killed bacteria. The importance of neutrophilic nonoxidative microbial killing has been thoroughly reviewed by Spitznagel.28

Oxygen-dependent bacterial killing is a late manifestation of functional differentiation. Only band and segmented neutrophils showed a substantial respiratory burst. Segmented neutrophils had significantly more activity than band neutrophils. These results are similar to the observations of Zakhireh and Root.29 Since oxygen-independent and oxygen-dependent antimicrobial mechanisms are synergistic and overlapping, it may not be possible to assess their relative roles in band and segmented neutrophils.

Chemotaxis is also a late manifestation of functional differentiation. Previous studies on immature myeloid leukemic cells indicated that random motility was expressed in the absence of chemotaxis.9 Further modulation of the cell is necessary for chemotaxis. This may relate to the molecular modeling of C5a receptors, the translation of chemotactic stimuli, or the cytoskeleton.30’31 Studies using the human promyelocytic leukemia HL-60 cell line as a model of myeloid differentiation show that the binding of chemotactic factors to receptor sites alone is not sufficient for chemotaxis.32 In our study chemotaxis was rarely observed except in band and segmented neutrophils. The latter had twice the activity of band neutrophils. Thus chemotaxis is expressed late and represents a terminal stage of functional differentiation.

We have studied a fundamental aspect of leukocyte differentiation, neutrophil functional differentiation. According to these studies we propose the following sequence for the functional differentiation of the neutrophil: Fc receptors → immune phagocytosis → complement receptors → oxygen-independent microbial killing → oxygen-dependent microbial killing → chemotaxis.

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Functional differentiation of normal human neutrophils

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