Monocyte Function in Children With Neutropenia and Chronic Infections


The phagocytic, bactericidal, and metabolic activities of monocytes from patients with congenital neutropenia were not significantly different than the activities of monocytes from patients with cyclic neutropenia or chronic infections. However, significant differences in metabolic, phagocytic, and bactericidal functions were found between monocytes from any source and normal neutrophils. During phagocytosis, monocytes produced more of the bactericidal product hydrogen peroxide than did neutrophils. However, monocytes in suspension had decreased phagocytic capacity compared to neutrophils and killed staphylococci less well. Moreover, monocytes had a diminished capacity to iodinate ingested bacteria. Quantitation of granular myeloperoxidase activity revealed that monocytes had a relative deficiency of this enzyme, which could explain the decreased iodination and killing of ingested bacteria by monocytes when compared to neutrophils. These findings offer an explanation for the failure of compensatory monocytosis to protect the host from bacterial infection when neutrophil reserves are diminished.

Patients with congenital neutropenia are susceptible to recurrent bacterial infections, and death frequently ensues during early childhood. The peripheral blood of these children contains normal numbers of leukocytes, but polymorphonuclear neutrophils (PMN) are markedly diminished. A paucity of neutrophilic leukocytes beyond the promyelocyte to myelocyte stage is evident in the bone marrow. The cause for this aberration is not known, but it is clear that the condition renders the affected patient susceptible to bacterial assault. Since hypergammaglobulinemia and intact cellular immunity are constant features, it has been assumed that this increased susceptibility to infection is due to diminished phagocytic clearance of bacteria. However, eosinophils and monocytes have phagocytic capacity and are usually increased in bone marrow and peripheral blood. It has recently been shown that eosinophils have less phagocytic and bactericidal capacity than PMN. Since recurrent infections continue in spite of adequate numbers of monocytes,
we have examined the metabolic and bactericidal responses during phagocytosis of peripheral blood monocytes from three unrelated children with congenital neutropenia and have compared their responses to those given by normal monocytes, by monocytes from three patients with cyclic neutropenia, and by normal PMN. Although no differences of monocyte function were found within the groups tested, we did find monocytes to be less effective than PMN in the phagocytosis and killing of bacteria.

MATERIALS AND METHODS

Subjects Studied

Two girls and one boy with congenital neutropenia were evaluated. Patient 1, 13 mo old, has had persistent bacterial infection since 3 wk of age. She had a subdiaphragmatic abscess removed surgically and has had a right lower lobectomy because of chronic infection. Patient 2, a 4½-yr-old girl, had repeated episodes of pneumonia the first 2½ yr of her life but has remained well for the past 2 yr. Patient 3, a 9-mo-old boy, has had recurrent pyogenic skin infections and pneumonia since the age of 3 mo. Patients 1 and 2 with cyclic neutropenia were 8 and 19 yr old females, respectively, and were evaluated during their neutropenic period when mouth ulcers were present. Otherwise, both of them were healthy. Patient 3 presented at age 1 yr with an ulceration of the labia majorum due to Pseudomonas aeruginosa, which cleared but recurred 1 mo later during the period of profound neutropenia. Control patients were three children with monocytosis and osteomyelitis, chronic lung disease, or subacute bacterial endocarditis; normal PMN were obtained from laboratory workers in good health.

Preparation of Cells

Peripheral blood leukocytes were prepared from heparinized venous blood samples as previously described.7 Neutropenic patients were studied when their peripheral blood contained relatively few PMN. For some bactericidal studies of controls, leukocytes were separated into monocyte-rich fractions by density gradient centrifugation in 28% bovine serum albumin (BSA) (Pentex, Div. Miles Lab., Kankakee, Ill.) according to the method of Huber and Fudenberg8 with the following modifications. The leukocyte button, obtained after the 10-min centrifugation at 200 g at 4°C, was resuspended in 0.5 ml of isologous platelet-free plasma and layered over 12.5 ml of 28% BSA (previously prepared from 10 ml 35% BSA and 2.5 ml of 0.1 M sodium phosphate-buffered saline, pH 7.4). This was centrifuged in a swinging bucket for 36 min at 2000 g at 12°C. The osmolarity of this albumin suspension was 362 milliosmols/liter. Following centrifugation the top and bottom layers of cells were washed successively in a 1:3 (v/v) dilution in water of Krebs Ringer phosphate buffer enriched with 200 mg/100 ml glucose and 1 mg/100 ml albumin (KRPGA) (104 milliosmols/liter), then a 1:2 (v/v) dilution of KRPGA (150 milliosmols/liter), and, finally, were suspended in KRPGA (278 milliosmols/liter). These modifications improved morphologic appearance of the final monocyte preparations.

Bactericidal Assay

The bactericidal capacity of phagocytes from patients and controls was determined using a previously described method9, modified from that of Quie et al.10 This technique provides an estimate of all factors necessary for bacterial killing by phagocytes, i.e., the rate and capacity of bacterial ingestion and the rate of activation of mechanisms necessary for killing ingested bacteria. Samples were run in duplicate with results expressed as the average. In some experiments, phagocyte-associated bacteria were separated from supernatant bacteria by centrifugation of the preparation at 200 g, followed by aspiration of the supernate and quantitation of both fractions.
Measurements of Rate and Extent of Phagocytosis

Suspension of cells: The rate of phagocytosis of heat-killed *Staphylococcus aureus* and zynosan particles was studied. Washed leukocytes were suspended in fresh normal adult serum pooled from five volunteers at 37°C, and aliquots were removed to make smears with cover slips at 1-min intervals for 5 min, then every 5 min for the next 25 min. The serum pool varied since different volunteers were used for each experiment. The ratio of particles or bacteria to phagocytes was 3:1. The smears were stained with Wright's stain; 100 monocytes or PMN were counted, and the percentage of each that contained particles, as well as the total number of particles ingested, was determined.

The phagocytic index was calculated by multiplying the percentage of ingesting phagocytes of each cell type by the average number of particles contained within each phagocyte.

Monolayer of cells: Leukocytes were prepared as monolayers adherent to plastic surfaces (Fabri-Kal Corp., Kalamazoo, Mich.), and the rate of uptake of 14C-labeled *Staphylococcus aureus* was determined. For these studies, bacteria were grown in dextrose nutrient broth with 100 μCi 14C-linoleic acid bound to albumin. An overnight culture of bacteria was pelleted by centrifugation at 10,000 rpm for 20 min and adjusted by turbidimetric analysis to a concentration of 2.0 × 10⁹/ml of Krebs Ringer phosphate (KRP) buffer. This gave a specific activity of approximately 150,000 cpm/ml of bacteria. They were then heat killed (80°C for 30 min) and frozen at −20°C in 2-ml aliquots. Monolayers were prepared in triplicate for each time point by allowing 0.5 cc of a dilute suspension of leukocytes (10⁶/ml) to preincubate in each plastic cup for 45 min at 37°C. The cups were gently rotated at intervals during this time to ensure a homogenous layer of leukocytes over the surface of the cup. The monolayers were then washed free of unattached cells five times with KRP. Just prior to use, the 14C-labeled bacteria were thawed and then opsonized with 10% (v/v) fresh human serum by incubation for 15 min at room temperature. The opsonized bacteria were centrifuged at 12,000 g at 4°C for 20 min and resuspended to twice their original volume with KRP enriched with glucose. Then 0.5 ml of the bacterial suspension was added to each cup; the incubations were terminated at intervals during 60 min by aspirating the bacterial suspension from each monolayer and then washing the monolayer three times in KRP to remove nonphagocytized bacteria. The protein of the monolayer was digested with 0.3 ml 12 N NaOH for 30 min and transferred to 10 ml of Buechler's liquid scintillation mixture. Radioactivity was determined in a Tri-Carb liquid scintillation spectrometer (Model 3735, Packard Instrument, Chicago, Ill.) Other cups were similarly prepared for protein analysis by adding 0.3 ml 0.5 N NaOH to the monolayers.

Metabolic Studies During Phagocytosis

The rate of oxygen consumption by phagocytes from patients and controls was determined with a Clark membrane-type electrode, as previously described. The production of hydrogen peroxide by phagocytes was determined by the rate of 14C-formate oxidation, and the stimulation of the hexose monophosphate shunt during phagocytosis was assessed by the rate of glucose-1-14C oxidation using previously described methods.

Peroxidase Content of Monocytes and PMN

Estimation of granule peroxidase content was performed on cover slip preparations of blood. The smears were stained by the method of Kaplow, and 100 monocytes or PMN were scored between 0 to 4, based on the intensity of the peroxidase-stained granules. In addition, spectrophotometric determination of peroxidase activity was done by the method of Maehly.

Iodination of Phagocytized Bacteria

Iodination of phagocytized, heat-killed 14C-*Staphylococcus aureus* by monocytes and PMN was performed with 125I using the monolayer technique described above. For these studies, 0.010 ml of 125I (100 μCi/cc) (New England Nuclear, Boston, Mass.) was added to each monolayer plate prior to addition of the 14C-*Staphylococcus aureus*, and the same
The rate of iodination was determined by counting samples in a gamma scintillation spectrometer. (Packard Gamma Scintillation Spectrometer, model 578, Chicago, Ill.). The rate of uptake of $^{14}$C-$S$. aureus was determined by counting the digested monolayer in the liquid scintillation spectrometer and then subtracting the $^{125}$I counts that contributed to the counts obtained for each sample in this spectrometer. This contribution was previously determined for $^{125}$I by measuring the equivalent cpm/μCi in both the gamma and liquid scintillation spectrometers.

RESULTS

The total and differential leukocyte counts of the congenitally neutropenic patients were as follows: patient 1, 2850/cu mm, 0% PMN, 20% lymphs, 10% eosinophils, 70% monocytes; patient 2, 4000/cu mm, 8% PMN, 14% lymphs, 15% eosinophils, 63% monocytes; patient 3, 2250/cu mm, 2% PMN, 18% lymphs, 3% eosinophils, 77% monocytes. The patients with cyclic neutropenia had the following leukocyte counts at the time of their neutropenia: patient 1, 3150/cu mm, 20% PMN, 10% lymphs, 10% eosinophils, 60% monocytes; patient 2, 5400/cu mm, 0% PMN, 25% lymphs, 5% eosinophils, 70% monocytes; patient 3, 6000/cu mm, 0% PMN, 18% lymphs, 18% eosinophils, 64% monocytes. Those with chronic infection had the following values: patient 1, 12,500/cu mm, 48% PMN, 22% lymphs, 6% eosinophils, 24% monocytes; patient 2, 14,700/cu mm, 42% PMN, 26% lymphs, 7% eosinophils, 25% monocytes.

Preparation of Cells

Phagocyte preparations used for study were predominantly monocytes, as indicated in Table 1. Repeated attempts were made to separate monocytes from PMN with density gradient centrifugation on 28% albumin, using leukocytes from children with monocytosis and chronic infection. Results of two such separations are also given in Table 1.

Bactericidal Studies

Monocytes from two patients with congenital neutropenia were studied for bactericidal capacity in an in vitro system that measures the combined effect of phagocytosis and intracellular killing of live Staphylococcus aureus. As indicated in Fig. 1A, at a ratio of two bacteria to one phagocyte (2.5 $\times$ 10$^6$ phagocytes), control PMN killed about 95% of the inoculum of $S$. aureus during a 2-hr incubation. However, phagocytes from patients 1 and 3 with congenital

<table>
<thead>
<tr>
<th>Table 1. Differential Leukocyte Counts of Phagocyte-rich Plasma</th>
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<td><strong>PMN</strong></td>
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<tr>
<td>Congenital neutropenia 1</td>
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<tr>
<td>Congenital neutropenia 2</td>
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<tr>
<td>Congenital neutropenia 3</td>
</tr>
<tr>
<td>Cyclic neutropenia 1</td>
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<tr>
<td>Cyclic neutropenia 2</td>
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<td>Cyclic neutropenia 3</td>
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<td>Chronic infection 1</td>
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<td>Chronic infection 2</td>
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Fig. 1. Bactericidal capacity of monocytes compared to PMN at 37°C. Number of viable bacteria in reaction mixture is plotted as a function of time. Panel A shows pattern for monocytes from patients 1 and 3 with congenital neutropenia. Ratio of Staphylococcus aureus to phagocytes in suspension was 2:1. Panel B shows pattern for monocytes from patients 2 and 3 with cyclic neutropenia. Ratio of Staphylococcus aureus to phagocytes was 3:1.

neutropenia (about 90% monocytes) failed to diminish the number of bacteria during the incubation, although there was a slight decrease noted for the first 60 min. When the ratio of bacteria to phagocytes was made 1:10, leukocytes from patient 1 could still not effectively reduce the number of viable bacteria after 2 hr incubation (data not shown). Phagocyte preparations from patients 2 and 3 with cyclic neutropenia and a predominance of monocytes were also less effective in killing S. aureus than normal PMN (Fig. 1B). At a bacteria to phagocyte ratio of 3:1, monocytes killed only 20% of the original inoculum, whereas PMN again killed greater than 90% of the inoculum. Mixed phago-

Fig. 2. Rate of clearance of Staphylococcus aureus from suspension and rate of pellet-associated bacterial killing by monocytes compared to PMN are represented. Number of viable bacteria in supernate (super) or pellet after centrifugation is plotted as function of time. Black circles indicate monocyte function from patient 1 with congenital neutropenia, and open circles indicate monocyte function from patient 2 with cyclic neutropenia. See text for additional details.
Fig. 3. Rate and extent of uptake of *Staphylococcus aureus* (A) and zymosan (B) particles by monocytes from patient 1 with congenital neutropenia compared to control PMN at 37°C. Phagocytic index is product of percentage of phagocytizing cells and the number of particles or bacteria ingested by monocytes.

Phagocyte preparations that contained predominantly monocytes obtained from patient 1 with cyclic neutropenia and patients 1 and 2 with chronic infection (Table 1) reduced the inoculum between 30 and 40% (data not shown). The two components involved in bacterial killing by phagocytes, i.e., the rate and extent of bacterial uptake by phagocytes and the rate of intracellular killing of ingested bacteria, were examined separately by isolating phagocyte-associated bacteria from the bacterial suspension by differential centrifugation. As noted in Fig. 2, bacteria associated with the monocyte pellets from patient 1 with congenital neutropenia and patient 2 with cyclic neutropenia were not killed as effectively as were the bacteria associated with the PMN pellet. In addition, bacteria were cleared from the suspension at a slower rate by monocytes than PMN.

*Rate and Extent of Phagocytosis*

The rate and extent of uptake of *S. aureus* and zymosan particles by PMN from a normal adult and monocytes from patient 1 with congenital neutropenia are shown in Fig. 3. PMN initiated ingestion sooner, took up staphylococci and zymosan faster, and ingested more bacteria and particles than did patients’ monocytes. Identical results were obtained using monocytes from patient 2 with cyclic neutropenia. To be certain that these differences were due to the type of phagocytic cell rather than to a defect specific for the monocytes of patients with neutropenia, the rate of uptake was compared for PMN and monocytes from a child with subacute bacterial endocarditis. The ratio
of his PMN to monocytes was 3:2. In a pattern similar (almost identical) to that in Fig. 3, PMN ingested more particles faster than did monocytes from the same individual in the same cell preparation.

**Metabolic Studies**

Monocytes from patient 1 with congenital neutropenia and patient 2 with cyclic neutropenia displayed a greater increase in oxygen consumption during phagocytosis of latex particles than did control PMN, as indicated in Table 2. The product of this respiratory burst is hydrogen peroxide, which was measured by $^{14}$C-formate oxidation, and this was also increased in both patients' monocytes compared to control PMN during phagocytosis (Table 2). There was a brisk stimulation of the hexose monophosphate shunt in both patients' monocytes (72 and 86 mmole glucose $1^{14}$C $\rightarrow 1^{14}$CO$_2$ hr$^{-1}$ mg$^{-1}$ protein) compared to four control PMN (range, 46–70 mmole $1^{14}$CO$_2$ hr$^{-1}$mg$^{-1}$ protein). These data coupled with the uptake indicate that patients' monocytes have intact oxidative metabolism that responds to a larger extent during phagocytosis than does that of PMN, even though fewer particles may be ingested.

**Peroxidase Content of Monocytes**

Monocytes were noted to contain less peroxidase than PMN (Table 3). The histochemical peroxidase scores of monocytes from patients with neutropenia and three children with monocytosis due to osteomyelitis, chronic lung disease, or subacute bacterial endocarditis were similar. These values were less than the values obtained with PMN. Spectrophotometric determination of peroxidase activity of pure preparations of monocytes revealed that PMN had threefold more enzyme activity than did monocytes (Table 3).

**Table 2. Oxygen Consumption and Hydrogen Peroxide Production**

<table>
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<tr>
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<th>Rest</th>
<th>Phagocytosis</th>
<th>P–R*</th>
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<tr>
<td><strong>O$_2$ consumption</strong></td>
<td>(μl O$_2$/hr/mg protein)</td>
<td></td>
<td></td>
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<tr>
<td>Monocytes</td>
<td>1.48</td>
<td>7.95</td>
<td>6.47</td>
</tr>
<tr>
<td>Monocytes</td>
<td>1.20</td>
<td>8.00</td>
<td>6.80</td>
</tr>
<tr>
<td>PMN</td>
<td>0.58</td>
<td>3.94</td>
<td>3.36</td>
</tr>
<tr>
<td>PMN</td>
<td>0.20</td>
<td>3.67</td>
<td>3.47</td>
</tr>
<tr>
<td>PMN</td>
<td>1.32</td>
<td>3.00</td>
<td>1.68</td>
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| **Hydrogen peroxide production** | (mmOLE $14$C-formate $\rightarrow 14$CO$_2$/30 min/10$^7$ WBC) |              |        |
|---------------------------------|---------------------------------------------------------------|--------------|
| Monocytes                       | 0.81                                          | 3.70         | 2.89   |
| Monocytes                       | 0.50                                          | 3.50         | 3.00   |
| PMN                             | 0.43                                          | 1.70         | 1.27   |
| PMN                             | 0.31                                          | 2.19         | 1.88   |
| PMN                             | 0.71                                          | 1.26         | 0.55   |

* Value represents difference between resting and phagocytizing values. Monocyte preparations contained 88–90% monocytes; PMN preparations contained 92–95% polymorphonuclear leukocytes.
Fig. 4. Rate and extent of iodination of ingested bacteria by monocytes from patients with neutropenia compared to PMN from controls. Results expressed as percentage of PMN $^{125}$I/$^{14}$C-Staphylococcus aureus ratio at 60 min. Points indicate the mean and range of four patients and four controls.

**Rate of Iodination of Ingested Bacteria by Monocytes and PMN**

The rate that ingested $^{14}$C-Staphylococcus aureus were iodinated with $^{125}$I was determined for monocytes from patients 1 and 3 with congenital neutropenia and patients 2 and 3 with cyclic neutropenia and for PMN obtained from four controls. As indicated in Fig. 4, monocytes were limited in their capacity to iodinate ingested bacteria compared to PMN, since the $^{125}$I/$^{14}$C ratio obtained by monocytes was lower at each time point compared to PMN.

**DISCUSSION**

Despite the fact that eosinophils and monocytes regularly comprise at least 50% of their circulating leukocytes, children with congenital neutropenia are susceptible to serious and often fatal bacterial infection. Eosinophils have been shown to be less bactericidal than PMN due, at least in part, to their slower uptake of bacteria. Since monocytes predominated in the peripheral blood of our patients, we were able to study relatively pure preparations of these cells without contaminating PMN. We compared monocytes from three children with congenital neutropenia, monocytes from three patients with cyclic neutropenia, and PMN from four controls.

**Table 3. Peroxidase Content of Monocytes**

<table>
<thead>
<tr>
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<th>Histochemical (Score/100 Phagocytes)</th>
<th>Spectrophotometric (OD$_{470}$ U/min/10$^7$ Phagocytes)</th>
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<tbody>
<tr>
<td>Congenital neutropenia 1</td>
<td>130</td>
<td>3.050</td>
</tr>
<tr>
<td>Congenital neutropenia 2</td>
<td>222</td>
<td>---</td>
</tr>
<tr>
<td>Cyclic neutropenia 1</td>
<td>150</td>
<td>---</td>
</tr>
<tr>
<td>Cyclic neutropenia 2</td>
<td>160</td>
<td>3.580</td>
</tr>
<tr>
<td>Osteomyelitis</td>
<td>113</td>
<td>---</td>
</tr>
<tr>
<td>Chronic lung disease</td>
<td>218</td>
<td>---</td>
</tr>
<tr>
<td>Subacute bacterial endocarditis</td>
<td>132</td>
<td>---</td>
</tr>
<tr>
<td>Control PMN (4)</td>
<td>380–400</td>
<td>10.030–13.690</td>
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penia, and monocytes from children with chronic infection to each other and to normal PMN in an attempt to elucidate phagocytic properties that might characterize all monocytes and to determine if a specific aberration in monocyte function exists in congenital neutropenia. Although no functional differences were found for monocytes from any source, significant differences in phagocytic metabolic, and bactericidal functions were found between monocytes of all three groups and PMN.

The bactericidal capacity of monocytes has been reported to be equal to that of PMN using an in vitro bactericidal method in which antibiotics were added to kill extracellular bacteria. The remainder of bacterial killing was then ascribed to intracellular mechanisms. Our in vitro method measured overall killing of staphylococci in a reaction mixture of bacteria, 10% serum, and cell preparations of predominantly PMN or predominantly monocytes. In this assay, monocyte preparations from all three groups tested failed to effectively kill S. aureus. Moreover, bactericidal studies designed to distinguish cell-associated bactericidal activity from the removal of S. aureus from the suspension suggested that monocytes removed S. aureus more slowly than PMN but also killed ingested S. aureus at a slower rate and to a lesser extent than did PMN.

Kinetic analysis of the rates of uptake of bacteria and particles revealed that monocytes showed a slower rate of uptake of zymosan and S. aureus and a diminished capacity for ingestion of these particles than PMN. The reason for this difference in rate and capacity of particle uptake by different types of phagocytes is not known. Glycolysis maintains ATP levels in PMN and monocytes, and metabolic glycolytic inhibitors that depress ATP formation also depress uptake of bacteria by both phagocytes. Since the rates of lactate production by monocytes and PMN were similar, the difference in rates of ingestion is most likely due to factors other than depressed glycolysis in monocytes. Receptor sites for opsonized bacteria on the membrane surface of monocytes and PMN may be important in this regard.

The precise bactericidal mechanisms that operate in monocytes are not well defined at present. Monocytes from children with chronic granulomatous disease fail to reduce the redox dye NBT and fail to kill nonperoxide-forming bacteria. This suggests that the bactericidal mechanism proposed by Klebanoff for PMN, i.e., hydrogen peroxide-peroxidase-halide, may also apply to monocytes. This does not exclude intravacuolar lactic acidosis, granule hydrolases, and cationic proteins as contributors to the overall bacterial killing process.

Monocytes from a patient with congenital neutropenia and from a patient with cyclic neutropenia consumed more oxygen, oxidized more glucose, and produced more hydrogen peroxide during phagocytosis than did normal PMN. Oren et al. showed that guinea pig monocytes also displayed greater oxidative metabolism during phagocytosis than did guinea pig PMN. In spite of the greater phagocytic production of hydrogen peroxide by monocytes, their capacity for intracellular killing of S. aureus was less than that of PMN. Since monocytes were found to contain one-third as much granule peroxidase as
PMN, the diminished rate of iodination of ingested *S. aureus* that we observed suggests that peroxidase, not hydrogen peroxide, may be the limiting factor for intracellular iodination and killing of *S. aureus* by monocytes. It cannot be said with certainty that the difference in myeloperoxidase levels accounts for the difference in killing of *S. aureus* between monocytes and PMN. However, PMN that lack granule myeloperoxidase have diminished capacity to kill bacteria and *Monilia*. Moreover, chemical inhibition of myeloperoxidase by cyanide or azide reduces the bactericidal capacity of the PMN.

Our studies indicate that the monocytes associated with congenital neutropenia are metabolically and bactericidally similar to monocytes from infected patients and from patients with cyclic neutropenia. The severity and extent of infection in this disease is most likely due to the chronic deficiency of circulating PMN, the superior phagocyte of the peripheral blood.

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REFERENCES

MONOCYTE FUNCTION

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