Monocyte Function in Patients With Chronic Granulomatous Disease of Childhood

By Gerald R. Donowitz and Gerald L. Mandell

Adults with chronic granulomatous disease of childhood (CGD) have been described who remain relatively free of infection despite markedly abnormal neutrophil function. Monocyte function in four adults with this mild or atypical CGD syndrome was examined and compared to that of normal controls and to that of two patients with the more severe or classic CGD syndrome. Monocytes from patients with atypical CGD killed 75.7% ± 2.6% (mean ± SEM) of ingested organisms at 30 min, while monocytes from the patients with classic CGD killed only 50.3% ± 4.2% of bacteria (p < 0.001). The difference in bactericidal activity between atypical CGD monocytes and normal monocytes was relatively small (75.7% ± 2.6% versus 88.1% ± 3.7%, respectively) but was statistically significant (p = 0.007). Monocytes from both atypical and classic CGD patients showed markedly impaired oxidative metabolism. Differences in monocyte bactericidal activity may explain why atypical CGD patients have fewer infections than classic CGD patients. The presence of nonoxidative bactericidal mechanisms in atypical CGD monocytes is suggested by the demonstration of bactericidal activity despite severe oxidative metabolic defects.

CHRONIC GRANULOMATOUS DISEASE of childhood (CGD) is a syndrome of defective polymorphonuclear neutrophil (PMN) bactericidal activity, resulting in a predisposition to recurrent, severe, bacterial infections. It is usually a disease of young males who suffer infections of the skin, lung, liver, bone, and lymph nodes and often die in adolescence. An x-linked recessive pattern is the most common form of inheritance, although an autosomal recessive mode of inheritance has been invoked to explain the existence of the disease in females.

The neutrophil defect is characterized by a lack of the normal phagocytic “burst” in oxidative metabolism. There is no marked increase in oxygen consumption, little or no production of hydrogen peroxide and superoxide anion, and no augmentation of the hexose monophosphate shunt. Halogenation of bacterial protein via the hydrogen-peroxide–myeloperoxidase–halide system is consequently absent, and neutrophil bactericidal activity is markedly impaired.

Recently, patients with CGD have been described who, despite this abnormal pattern of neutrophil function, have lived to reach adulthood and subsequently have had very few infections. This observation has remained puzzling, since the extent of neutrophil abnormalities in these adults appears to be as severe as those seen in children with the more severe form of the disease.

In order to understand why these adult patients with CGD have done relatively well, we examined the function of their peripheral blood monocytes to determine if these cells played a role in keeping them free of infection. While phagocytosis by monocytes is less efficient than that of neutrophils, bactericidal activity of normal monocytes is similar to that of normal neutrophils and therefore makes them an important defense against infection. Earlier studies in young patients with the classic CGD syndrome revealed markedly abnormal monocyte bactericidal activity.

MATERIALS AND METHODS

Patients

Six patients with CGD, including two pairs of siblings, were studied. A description of neutrophil function in one pair of siblings (patients 3 and 4) has previously been published. Two patients had the classic, severe disease, and four patients had an atypically mild disease. Clinical summaries of each of the patients are presented in Table 1. Of note is the advanced age of onset of serious infectious episodes in patients 1, 2, 3, and 4 and their long infection-free intervals. These patients appear healthy despite their past infections and are considered to have an “atypical” form of CGD. In contrast, patient 5 developed his first symptoms at 3 mo of age and has continued to have recurrent serious infections requiring frequent hospitalizations and close outpatient monitoring. Patient 6 has had recurrent episodes of infection from 4 wk of age until the age of 5 mo when prophylactic trimethoprim-sulfamethoxazole therapy was instituted in hopes of preventing further infections. This patient has remained clinically stable for 14 mo. These two patients are considered to have “classic” CGD.

All assays of leukocyte metabolic and bactericidal activity were carried out when patients were free of infection. Control subjects consisted of healthy adults between the ages of 20 and 25.
Neutrophil Function

Neutrophil Preparation

CGD was defined in our patient population by tests of leukocyte metabolic and bactericidal capability. Neutrophils were obtained by dextran sedimentation of peripheral venous blood. Red blood cells were lysed with hypotonic saline, washed in Hanks' balanced salt solution (HBSS) (Microbiological Associates, Walkersville, Md.), and finally resuspended in HBSS supplemented with 10% autologous serum. Cell suspensions were adjusted to yield 1-5 x 10^6 neutrophils/mL.

Bacterial Preparation

An 18-hr-old culture of *Staphylococcus aureus* 502A grown in trypticase soy broth was washed twice in chilled HBSS and adjusted with a spectrophotometer to an optical density of 1.1 at a wave length of 580 nm. This corresponded to 5 x 10^9 colony-forming units (CFU)/ml. Further dilution of the bacterial stock solution was carried out to yield a bacteria to neutrophil ratio of 1:1. Bacterial suspensions were placed in an ice bath until use in the bactericidal assay.

Neutrophil Bactericidal Assay

Four milliliters of a suspension containing 5 x 10^6 bacteria and 5 x 10^6 neutrophils were tumbled end-over-end at 37°C. In capped, 12 x 75 mm plastic tubes (Falcon, Oxnard, Calif.). Samples were removed at 0 and 60 min. The number of total CFU was determined by serial dilution 0.1-ml aliquots of the reaction mixtures in sterile distilled water and plating on agar. Samples were then centrifuged at 150 g for 5 min and supernatant fractions diluted and plated on agar to determine supernatant CFU. Ingestion of bacteria was calculated from decreases in total CFU. The number of organisms killed was calculated from decreases in total CFU. Bactericidal activity is expressed as percent organisms killed of those ingested.

Neutrophil Oxidative Metabolism

NBT reduction. Neutrophils were stimulated with endotoxin and evaluated for the reduction of nitroblue tetrazolium dye (NBT) by the method of Ochs and Igo. The percentage of NBT positive cells (blue stain present) was determined and compared to simultaneously tested control cells.

Chemiluminescence. Luminol-enhanced chemiluminescence was determined using the method of Mandell and Schadelin. Neutrophil suspensions (1 x 10^6 neutrophils/ml) were placed in glass vials with 5 x 10^-5 M luminol (Sigma, St. Louis, Mo.). Zymosan was preopsonized by incubation with autologous serum at 37°C for 30 min, washed, and resuspended in HBSS at a concentration of 1 mg/ml. Neutrophils and luminol were preincubated for 10 min at 37°C and then placed in a Chem-Glo Photometer (American Instrument Company, Silver Spring, Md.) inside a 37°C incubator. After 5 min of baseline monitoring, 0.05 ml of preopsonized zymosan was added as a phagocytic challenge, and chemiluminescence was monitored for 10-30 min until a plateau value was reached. Chemiluminescence was recorded as intensity units, and results are expressed as percent increase of chemiluminescence over unstimulated baseline values.

Neutrophil hexose monophosphate shunt activity. Hexose monophosphate shunt activity was determined by measuring the production of ^14^C02 from ^14^C-glucose by neutrophils after incubation with preopsonized zymosan. Results are expressed as percentage increase of ^14^C counts per minute (cpm) compared to baseline counts obtained with unstimulated neutrophils.

Oxygen consumption. Oxygen consumption of neutrophils was measured after incubation with preopsonized zymosan in a polarographic oxygen monitor (YSI, Yellow Springs, Ohio). Results are expressed as percent increase in oxygen consumption of stimulated cells versus that of unstimulated cells.

Iodination of protein. An adaptation of the method of Root and Stossel was utilized. 5 x 10^7 neutrophils in 0.3 ml calcium-free Krebs-Ringer phosphate solution was incubated with 0.1 μCi of Na 125I. Preopsonized zymosan was used as the phagocytic challenge. The reaction was stopped after 30 min by the addition of 0.1 ml of a 0.01 M solution of sodium thiosulfate and 1 ml of a 10% solution of trichloracetic acid (TCA). TCA precipitates were counted in a Beckman-300 Gamma counter. Results are expressed as percent increase of precipitated 125I produced by stimulated cells versus unstimulated cells.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Relationship of Other Patients</th>
<th>Age of Onset of Infections</th>
<th>Present Age</th>
<th>Infections/Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M</td>
<td></td>
<td>Brother of no. 2</td>
<td>11</td>
<td>50</td>
<td>Recurrent cellulitis of nose and lip/11-17; furuncles/11; liver abscess/11; intraperitoneal abscess/37; periappendiceal abscess/40; pneumonia/47,50</td>
</tr>
<tr>
<td>2 F</td>
<td></td>
<td>Sister of no. 1</td>
<td>10</td>
<td>51</td>
<td>Lung abscess/10; adenitis/27; furuncles/27; cellitis/39; pneumonia/48</td>
</tr>
<tr>
<td>3 M</td>
<td></td>
<td>Brother of no. 4</td>
<td>8</td>
<td>46</td>
<td>Pneumonia/8; pneumonia: 1-yr/B-14; adenitis/38</td>
</tr>
<tr>
<td>4 M</td>
<td></td>
<td>Brother of no. 3</td>
<td>6</td>
<td>34</td>
<td>Pneumonia/6; pneumonia: 1-2-yr/B-17; adenitis/6</td>
</tr>
<tr>
<td>5 M</td>
<td></td>
<td>Unrelated to the others</td>
<td>3 mo</td>
<td>30</td>
<td>Recurrent FUO/3 mo–7 yr; adenitis/7; periappendiceal abscess/7; adenitis/11; pneumonia: 1-4-yr/14-30; hepatic abscess/26; abdominal wall abscess/26; recurrent skin abscess/25-30</td>
</tr>
<tr>
<td>6 M</td>
<td></td>
<td>Unrelated to the others</td>
<td>4 wk</td>
<td>2</td>
<td>FUO/4 wk; pneumonia/3 mo; adenitis/4 mo; pneumonia/5 mo; bacteremia/5 mo</td>
</tr>
</tbody>
</table>

Table 1. Clinical Characteristics of Patients With CGD
Monocyte Function

Monocyte Preparation

Peripheral blood monocytes were obtained via density gradient separation utilizing a diatrizoate salt-sucrose polymer solution (LSM, Bionetics Laboratories, Kensington, Md.) using a modification of the technique of Boyum.24 Mononuclear cells were harvested with a sterile Pasteur pipette, washed twice in heparinized (10 U/ml) HBSS, and finally resuspended in Medium 199 (Microbiological Associates, Walkersville, Md.) supplemented with 10% autologous serum. The cell suspension obtained consisted of monocytes, lymphocytes, and less than 5% neutrophils. The percentage of monocytes was determined by cytochemical staining for nonspecific esterase, using the method of Yam et al.25 Cell suspensions were adjusted to yield a final concentration of 0.5-1 x 10^6 monocytes/ml.

Monocytes monolayers were prepared by incubating 1 ml of monocyte cell suspension in 15 mm x 45 mm glass vials (General Scientific, Richmond, Va.) at 37°C in 5% CO₂ for 2 hr. Nonadherent cells were removed by gently washing monolayers with warm Medium 199. Monolayers were then overlaid with 1 ml of Medium 199 supplemented with 10% autologous serum. This procedure yielded a monolayer consisting of 95%-100% monocytes.

Monocyte Bactericidal Activity

Bacteria were prepared as noted in the previous section to yield bacteria:monocyte ratios of 3-5:1. Using a modification of the procedure of Territo and Cline,36 bacteria were “force-fed” onto monocyte monolayers by centrifugation at 200 x g for 15 min. To minimize phagocytosis during the actual force-feeding procedure, centrifugation was carried out at 0-4°C. Monolayers were then incubated at 37°C in 5% CO₂ for 2 hr. Nonadherent cells were removed by gently washing monolayers with warm Medium 199. Monolayers were then overlaid with 1 ml of Medium 199 supplemented with 10% autologous serum. This procedure yielded a monolayer consisting of 95%-100% monocytes.

Phagocytosis was calculated from changes in CFU in the supernatant over time as compared to 0 time values:

\[
\text{Percent bacteria phagocytized (time } t) = \frac{\text{CFU supernatant (time 0)} - \text{CFU supernatant (time } t) \times 100}{\text{CFU supernatant (time 0)} - \text{CFU supernatant (time } t)}
\]

The number of organisms killed over time was calculated from changes in total CFU (CFU in the sediment + CFU in the supernatant) for each time period compared to total CFU at 0 time. Bactericidal activity was expressed as percent of organisms killed of those ingested for each time period.

\[
\text{Percent of organisms killed of those ingested (time } t) = \frac{\text{CFU total (time 0)} - \text{CFU total (time } t) \times 100}{\text{CFU supernatant (time 0)} - \text{CFU supernatant (time } t)}
\]

Growth of Staphylococcus aureus incubated in monocyte-free Medium 199 and serum was also measured.

To check the accuracy of our determinations for bacterial ingestion, values for phagocytosis derived as described above were compared to values obtained by the determination of cell-associated ¹⁴C-labeled organisms.27 In this latter method, Staphylococcus aureus grown in the presence of 10 µCi of ¹⁴C-labeled amino acids were heat-killed, washed, and adjusted as described above for bactericidal assays. The bacteria were centrifuged onto monolayers as described above. At timed intervals, 2 ml of chilled 0.1 M NaF solution was added to stop phagocytosis. After washing with EDTA as described above, cell pellets were dried overnight, digested with NaOH, and 1.0 ml aliquots counted in a Beckman Scintillation Counter. Results were expressed as: percent uptake (time 0) = [cpm (time 0) - cpm (time 0)]/cpm (time 0).

To distinguish between humoral and cell-related factors in monocyte bactericidal activity, experiments were carried out using pooled human serum from five normal controls instead of autologous serum. Monocytes from a patient with classic CGD and a patient with atypical CGD were tested.

Oxidative Metabolism of Monocytes

Monocyte oxidative metabolism was evaluated by examining NBT reduction, chemiluminescence, hexose-monophosphate shunt activity, and oxygen consumption utilizing methods previously described for neutrophils.

Statistical Analysis

The unpaired Student’s t test was used to compare metabolic and functional parameters of the four patients with atypical CGD to those of normal controls. Metabolic and functional parameters of patients with classic CGD were compared to those of normals and to those of the atypical CGD patients by constructing normal distribution curves around the means of the latter two groups. Values around the mean were calculated from the standard t tables as boundaries for p values of 0.05-0.001. Observed values for the patients with classic CGD were fit into the distributions for determinations of p values.

RESULTS

Neutrophil Bactericidal and Metabolic Function

The results of neutrophil bactericidal and metabolic tests in patients with classic and atypical CGD are shown in Table 2. Each of the metabolic parameters studied was significantly depressed when compared to those of normal controls, consistent with the diagnosis of CGD. No significant differences in metabolic function were noted when classic CGD neutrophils were compared to atypical CGD neutrophils. Similarly, bactericidal activity of CGD neutrophils was markedly depressed compared to that of normal cells. No significant difference in bactericidal activity was noted when classic CGD neutrophils were compared to atypical CGD neutrophils.

Monocyte Bactericidal Activity

Ingestion of Bacteria

Uptake of bacteria as determined by quantitating changes in supernatant CFU was similar to that determined by measuring cell-associated ¹⁴C-labeled bacteria. A representative comparison is shown in Fig. 1. Maximal rates of phagocytosis occurred in the first
Table 2. Neutrophil Oxidative Metabolism and Bactericidal Activity in Patients With CGD

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Controls (Mean ± SEM)</th>
<th>p CGD vs Normals</th>
<th>Patients</th>
<th>p Classic vs Atypical CGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulated NBT reduction (% of cells with reduced dye)</td>
<td>93.6 ± 2.8 (n = 10)</td>
<td>&lt;0.001</td>
<td>1 2 3 4 5 6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Postphagocytic O2 consumption (% increase)</td>
<td>1.598 ± 354 (n = 6)</td>
<td>0.002</td>
<td>133 259 25 0 —*</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Postphagocytic hexose monophosphate shunt activity (% increase)</td>
<td>1.588 ± 348 (n = 6)</td>
<td>0.002</td>
<td>33.9 0 5.1 23 0 —*</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Postphagocytic protein iodination (% increase)</td>
<td>4.496 ± 1.640 (n = 6)</td>
<td>0.02</td>
<td>109 47 — 33 0 —*</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Postphagocytic chemiluminescence (% increase)</td>
<td>5.900 ± 1.603 (n = 4)</td>
<td>0.002</td>
<td>0 0 0 — 0 0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Bactericidal activity (% killed of those phagocytized)</td>
<td>116 ± 4.3† (n = 5)</td>
<td>0.002</td>
<td>75.0 35.6 92 57 32</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Patients 1-4 have atypical CGD. Patients 5 and 6 are patients with classic CGD. Both atypical and classic CGD patients demonstrated abnormal neutrophil metabolic and functional activity. No consistent differences between atypical and classic CGD patients were noted in any of the parameters tested.

*Because of patient's age, a limited amount of blood was obtained, preventing evaluation of the full battery of metabolic tests.
†Numbers of organisms killed were measured independently of numbers of organisms ingested. In several cases, values for the former were greater than those for the latter, yielding values greater than 100%.

25–30 min, with 63.2% ± 11.8% of bacteria ingested by 30 min (CFU determination). Between 30 and 60 min, rates of phagocytosis decreased, with 70.3% ± 4.5% of bacteria ingested by 60 min (an increase of only 7% in 30 min).

Ingestion of bacteria by CGD monocytes was compared to that of normal controls (Fig. 2). Comparisons were made at 15 and 30 min. Phagocytosis of bacteria by classic CGD and atypical CGD monocytes was similar to that of normal monocytes at 15 and 30 min (p = 0.18 and p = 0.19, respectively, for atypical CGD monocytes and p > 0.05 for classic CGD monocytes at both time periods).

Monocyte Killing of Staphylococcus aureus (Fig. 3)

The bactericidal ability of CGD monocytes were not all similar and appeared to fall into two distinct groups. Killing of *Staphylococcus aureus* by monocytes from the patients with the classic form of CGD was markedly impaired compared to that of normal cells at 15 min (27.5% ± 13.7% versus 86% ± 3.0%; p < 0.001) and at 30 min (50.3% ± 5.2% versus 88.1% ± 3.7%; p < 0.001). In contrast, monocytes from the patients with atypical CGD killed bacteria as effectively as did normal monocytes at 15 min (78.8% ± 2.8% versus 86% ± 3%; p = 0.07), though less efficient killing was noted at 30 min (75.7% ± 2.6% versus 88.1% ± 3.7%; p = 0.007). When atypical and classic CGD monocytes were compared, significantly greater bactericidal activity was noted in atypical CGD monocytes at both 15 and 30 min (p < 0.001 and p < 0.005, respectively).

Using monocytes from patient no. 1 with atypical CGD in the presence of pooled human serum instead of autologous serum, no difference in monocyte bactericidal activity was noted. By 30 min, 71% of ingested organisms were killed in the presence of pooled human serum versus 76% of ingested organisms in the presence of autologous serum. Similarly, when pooled human serum was used with monocytes from patient no. 5 with classic CGD, 50% of ingested organisms were killed compared to 50.3% of ingested organisms killed in the presence of autologous serum.
Fig. 2. Uptake of Staphylococcus aureus (as delineated by quantitation of supernatant CFU) in monocytes of classic and atypical CGD patients compared to that of normals. Graph points here and in Fig. 3 represent mean ± SEM for 2-3 separate determinations for each of the 6 patients studied and for 8 normal controls.

Monocyte Oxidative Metabolism

The results of metabolic function tests of monocytes from patients with classic and atypical CGD are shown in Table 3. Parameters of oxidative metabolism were markedly decreased in all six CGD patients. In comparing the metabolic activity of classic CGD monocytes to those of atypical CGD cells, no persistent trend was noted.

DISCUSSION

Since the original clinical description of CGD, a wide range of clinical presentation have been associated with the syndrome. Classically, symptoms of CGD occur in early childhood, with two-thirds of patients developing infections before 1 yr of age and approximately one-third of patients developing infections before age 3 mo. Recurrent infections are unusual, with death often occurring before the third decade. Atypical cases of CGD have been described in patients in whom the diagnosis was not suspected until adulthood, although recurrent infections were noted at earlier ages. CGD has been noted in patients in whom serious infections occurred only in late adolescence or early adulthood with a relatively benign childhood history. The four patients with atypical CGD described here, patients 1, 2, 3, and 4, are unusual in that not only was the onset of infection relatively late, occurring between ages 6 and 11, but long infection-free intervals of from 12 to 26 yr were observed. In marked contrast, the two patients with the more classic form of CGD, patients 5 and 6, had the typical early onset of infectious complications, with

### Table 3. Monocyte Oxidative Metabolism in Patients With CGD

<table>
<thead>
<tr>
<th>Metabolic parameter</th>
<th>Normal Controls</th>
<th>CGD vs Normals</th>
<th>Patients</th>
<th>p</th>
<th>CGD vs Atypical CGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulated NBT reduction (% of 52.8 ± 9.4 cells with reduced NBT)</td>
<td>52.8 ± 9.4 (n = 5)</td>
<td>0.003</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Postphagocytic chemiluminescence (% increase)</td>
<td>3.850 ± 1.062 (n = 6)</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Postphagocytic hexose monophosphate shunt activity (% increase)</td>
<td>304 ± 46 (n = 7)</td>
<td>0.00001</td>
<td>30.8 ± 11.2</td>
<td>36.7 ± 36.7</td>
<td>32.7*</td>
</tr>
<tr>
<td>Postphagocytic oxygen consumption (% increase)</td>
<td>280 ± 43 (n = 6)</td>
<td>0.03</td>
<td>0</td>
<td>99.5 ± 1.4</td>
<td>0*</td>
</tr>
</tbody>
</table>

Metabolic function of monocytes from patients with atypical CGD (patients 1-4) and from patients with classic CGD (patients 5 and 6) is compared to that of monocytes from normal controls. Patient values represent the mean ± SEM of at least two separate determinations.

*Values represent single determinations.
The correlate with that of monocytes. Despite abnormal degree of oxidative metabolism. It is conceivable that nonoxidative bactericidal mechanisms in classic CGD monocytes was altered when pooled serum was substituted for autologous serum. This suggests that the bactericidal activity demonstrated by these cells was not a function of humoral factors peculiar to CGD patients.

The difference in bactericidal activity noted in monocytes from atypical CGD patients versus monocytes from the classic CGD patients is puzzling. Phagocytic cells from both types of CGD patients have abnormal oxidative metabolism. Dual defects of oxidative and nonoxidative bactericidal mechanisms in classic CGD patients and only oxidative defects in the atypical CGD patients are possible, but the genetics of the disease suggest that only a single gene is involved. Such a genetic defect could involve the activation of oxidative and bactericidal mechanisms rather than the actual mechanisms. The presence of different “triggers” for phagocyte oxidative metabolism has been suggested.

Further, Harvath and Anderson have described a patient with recurrent infections whose neutrophils demonstrated decreased values for chemiluminescence and oxygen consumption with particulate stimuli, but normal values after stimulation with soluble stimuli. This again suggests that there exists more than one mechanism for triggering cellular oxidative metabolism and that triggering defects may play a role in causing leukocyte bactericidal defects. Common pathways may exist for the activation of oxidative and nonoxidative bactericidal activity. A defect in an early triggering mechanism could affect both oxidative and nonoxidative killing, leading to the markedly impaired killing seen in the patients with classic CGD. A defect in a later step of the activation mechanism could affect only oxidative killing, leading to the situation hypothesized in patients with atypical CGD. Further work will be necessary to evaluate both the nature of mononuclear cell nonoxidative killing systems and the mechanisms involved in triggering their activity.

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