Reevaluation of Cytochrome b and Flavin Adenine Dinucleotide in Neutrophils From Patients With Chronic Granulomatous Disease and Description of a Family With Probable Autosomal Recessive Inheritance of Cytochrome b Deficiency

By Yohichiroh Ohno, E. Stephen Buescher, Robert Roberts, Julia A. Metcalf, and John I. Gallin

Chronic granulomatous disease (CGD) is a genetically heterogeneous syndrome characterized by a microbial killing defect of polymorphonuclear leukocytes (PMNs) due to lack of superoxide O$_2^-$ generation. Recent studies indicate that the neutrophil O$_2^-$-generating system consists of at least two components, flavoprotein—flavin adenine dinucleotide (FAD)—and cytochrome b. We evaluate the cytochrome b and FAD content in PMN from 30 CGD patients. The method for quantitating cytochrome b was modified by using PMN sonicates incubated with azide plus hydrogen peroxide. With this approach, several absorption peaks corresponding to myeloperoxidase and eosinophil peroxidase, which overlap with peaks of cytochrome b, were obliterated from reduced-minus-oxidized spectra, whereas the peaks of cytochrome b were not and could be readily quantitated. Cytochrome b was detected in PMNs from all 24 normal adults (4.74 ± 2.9 pmol/7.5 × 10$^6$ cells), was absent in PMNs from 11 male CGD patients and one female CGD patient but was present in normal amounts in PMNs from nine male and nine female CGD patients. Stimulated nitroblue tetrazolium (NBT) tests performed on PMNs from mothers of CGD patients indicated that cytochrome b deficiency was associated with X-linked inheritance, except in one case in which probable autosomal recessive inheritance was demonstrated. The PMN NBT test of the mother of another male patient without cytochrome b deficiency suggested an X-linked form of inheritance. In related studies, the FAD content in PMN particulate fractions was reduced in 4 of 28 CGD patients studied. All four CGD patients with reduced FAD lacked cytochrome b. However, three patients with cytochrome b deficiency had normal FAD. Thus, the results indicate that PMN cytochrome b deficiency is observed in most X-linked and in some autosomal recessive CGD, that cytochrome b deficiency may be associated with FAD deficiency, and that cytochrome b and FAD are normal in most patients with non-X-linked CGD.

POLYMORPHONUCLEAR leukocytes (PMNs) release superoxide anion (O$_2^-$), hydrogen peroxide, and other oxygen radicals when stimulated with particulate stimuli such as bacteria or fungi or with soluble stimuli such as phorbol esters, chemotactic peptides, and calcium ionophore. Superoxide anion generated in the plasma membrane or phagosomal membrane by reduction of oxygen by electrons originating from neutrophil glucose-6-phosphate dehydrogenase (NADPH), dismutates or breaks down to form hydrogen peroxide.$^2$ The hydrogen peroxide contributes to the microbicidal activity of neutrophils in cooperation with myeloperoxidase and halide.$^4$ Neutrophils from patients with chronic granulomatous disease (CGD) cannot produce superoxide and hydrogen peroxide when exposed to metabolic stimuli. As a result, patients with CGD are highly susceptible to bacterial infections. The disease may be classified into several hereditary types, including X-linked,$^5$ autosomal recessive,$^6$ and, possibly, autosomal dominant types.$^5$ Deficiency of NADPH,$^7$ glutathione peroxidase,$^4$ and NAPD oxidase have been thought to be the basis for the disease in different patients.

Recently, the NAPD oxidase has been considered a multicomponent system. A nonmitochondrial cytochrome b probably is involved. Some evidence indicates that the cytochrome b functions as a terminal component of the NAPD oxidase in donating electrons to oxygen.$^3$ The cytochrome b has dual subcellular localization and has been localized to the plasma membrane and an intracellular pool that cosediments with specific granules.$^1$ Flavoprotein, flavin adenine dinucleotide (FAD), is probably another component in the O$_2^-$ generating system$^{14}$ and seems to be a proximal constituent of the electron-transport chain which accepts electrons directly from NAPD.$^8$

Segal et al studied the content of cytochrome b in PMNs from 27 patients with CGD$^9$ and suggested that neutrophil cytochrome b deficiency was related to an X-linked inheritance of the disease. In addition, several reports indicate that the FAD content in the particulate fractions of neutrophils is decreased in some CGD patients.$^6,17,20,21$ Of eight patients reported previously with PMN FAD deficiency, seven patients had PMN cytochrome b deficiency. In the current study, the cytochrome b content in neutrophils from 30 CGD patients was measured by a modified method that removes an artifact of eosinophil peroxidase. In addition, FAD content was quantitated in 28 CGD patients. Although most patients with CGD who had cytochrome b deficiency had an X-linked pattern in their disease, in one family the inheritance was best explained as autosomal recessive. In another family with X-linked inheritance, neutrophil cytochrome b was normal. Of 28 patients examined, FAD was reduced in four patients whose neutrophils also lacked cytochrome b.

MATERIALS AND METHODS

Patients. The diagnosis of CGD was determined by defective reduction of phorbol myristate acetate (PMA)-stimulated nitroblue tetrazolium (NBT) dye and impaired killing of Staphylococcus aureus by neutrophils from the patients.$^6$ Severe G6PD deficiency was excluded by demonstrating that the patients' cells responded to methylene blue with enhanced hexose monophosphate shunt activity.$^4$ Thirty patients from patients referred to the National Institutes of Health for evaluation of compromised host defenses were diagnosed as having CGD.
Preparation of cell sonicates. PMNs were collected by centrifugation on Ficoll-Hypaque and dextran sedimentation as previously described. For one study, eosinophils were collected from the blood of a patient with microfilaria infection, as described elsewhere. This method yields almost pure populations of eosinophils (>98% purity). Cells were suspended at a concentration of 7.5 x 10^6/mL in 50 mmol/L of 3-[N-morpholino] propane-sulfonic acid (MOPS), 100 mmol/L of KCl, pH 7.0, sonicated for two cycles of ten seconds at 18 W at 4°C, and solubilized by addition of sodium deoxycholate (0.1% of the final concentration).

Treatment of cell sonicates with azide plus hydrogen peroxide. Cell sonicates (7.5 x 10^6/mL) were incubated with 0.5 mmol/L of hydrogen peroxide plus 5 mmol/L of sodium azide at room temperature for 15 minutes. In some experiments, cell sonicates were incubated with 10 mmol/L of glucose plus 50 mmol/L of glucose oxidase in the presence of 5 mmol/L of azide at 37°C for 30 minutes. The glucose-glucose oxidase system is an effective hydrogen peroxide generating system. After incubation, the sonicates were cooled and kept in melting ice until spectral assay.

Reduced-minus-oxidized difference spectra. One milliliter of a sonicated sample (7.5 x 10^6 cells) was put in a cuvette. The absolute spectra of the sample were recorded from 600 nm to 400 nm with a Perkin-Elmer Lambda-3 spectrophotometer and stored in an attached computer (Perkin-Elmer 3600 data station). The sample was reduced with the addition of solid sodium dithionite (1 to 2 mg). The absolute spectra of the reduced sample was recorded from 600 nm to 400 nm and stored. Reduced-minus-oxidized difference spectra were created by subtracting the absolute spectra of the sample from the absolute spectra of the reduced sample with the computer. Cytochrome b was measured in PMN sonicates treated with azide plus hydrogen peroxide as described in the first part of the Results section, using an absorption coefficient of 106 mmol/L/cm^(-1) at the Soret peaks^23 and expressed as pmol/7.5 x 10^6 cells.

Subcellular fractionation. Subcellular fractions were collected from normal human neutrophils and from CGD neutrophils lacking cytochrome b by whole cell assays. The method for obtaining subcellular fractions with Percoll was described previously. In brief, PMNs (1.5 x 10^8) were disrupted by nitrogen cavitation. The postnuclear cavitate was layered on a discontinuous gradient of 37.4% and 97.4% Percoll. Gradients were centrifuged for 15 minutes at 33,000 g. The postnuclear cavitate was separated into plasma membrane-rich, specific granule-rich, azurophil granule-rich, and cytotoxic fractions on the basis of well-described enzyme marker assays described below. Collected fractions were separated from the Percoll and resuspended in a MOPS-KCl buffer in a concentration of 0.2 mg/mL of protein. Alkaline phosphatase was assayed with p-nitrophenyl phosphate as a substrate. Vitamin B12 binding protein was measured with ^32-Co-vitamin B12 (Amersham, Arlington Heights, Ill.). Myeloperoxidase and cytochrome b in subcellular fractions were assayed with reduced-minus-oxidized difference spectra. An absorption coefficient of 75 mmol/L/cm^(-1) at 472 nm was used to quantitate myeloperoxidase. Cytochrome b was measured with the samples treated with azide plus hydrogen peroxide, using an absorption coefficient of 106 mmol/L/cm^(-1) at the Soret peak. The protein was determined with Coomassie blue as described previously.

Assay of flavins. FAD and flavin mononucleotide (FMN) were determined in the particulate fraction collected from activated PMNs as described previously. PMNs (10^8 cells) were activated by incubation with 100 ng/mL of PMA at 37°C for five minutes in Krebs-Ringer's phosphate buffer with 5.5 mmol/L of glucose, pH 7.4. After activation, cells were washed twice, suspended in 0.34 mol/L of sucrose, and sonicated for three cycles of ten seconds at 18 W at 4°C. The sonicates were centrifuged at 500 g for ten minutes. The postnuclear supernatant was centrifuged at 27,000 g for 20 minutes at 4°C. The pellet was resuspended in 0.34 mol/L of sucrose and centrifuged at 27,000 g at 4°C. The particulate fraction was washed once more and resuspended in 0.1 mol/L of potassium phosphate buffer with 0.1 mmol/L of EDTA, pH 7.7, to the concentration of 0.4 mg/mL of protein. FAD and FMN were quantitated by the method of Faeder and Siegel. Triton X-100 (Rohm and Haas, Philadelphia) was added to particulate fractions to a final concentration of 0.1%. The particulate fractions were immersed in boiling water for four minutes and cooled rapidly in melting ice water. Insoluble materials were removed by centrifugation in an Eppendorf microtube for ten minutes at 4°C. The fluorescence of the samples was measured with the excitation wavelength of 450 nm and the emission wavelength of 535 nm, using a Perkin-Elmer LS-5 fluorescence spectrophotometer. Therefore, the pH of the solutions was lowered to 2.6 with 1 N HCl, and the fluorescence was then determined. Standard curves for flavins at pH 7.7 and pH 2.6 were determined with commercially obtained FAD and FMN (Sigma Chemical Co, St Louis).

Neutrophil functions. The PMA-stimulated NBT test and superoxide production, were performed as described previously using 20 ng/mL of PMA or 1 mmol/L of formyl-methionyl-leucyl-phenylalanine (FMLP) as the stimulus.

RESULTS

Modification of difference spectra assay used for measurement of cytochrome b. Several peaks were observed in reduced-minus-oxidized difference spectra from normal neutrophils (Fig 1). A prominent positive peak of absorption at 472 nm and a negative peak at 420 nm were due to absorption by myeloperoxidase. A shoulder observed at 452 nm corresponded to the absorption of eosinophil peroxidase. A small positive peak at 558 nm and a prominent peak at 424 nm were the α and Soret peaks of cytochrome b, respectively. Previous workers have used the α-peak (558 nm) to quantitate cytochrome b, but the assay is difficult when PMNs contain many eosinophils, as occurs in leukocytes obtained from many patients with CGD since eosinophil peroxidase absorbs at 450 nm and 560 nm. For example, the difference spectra of eosinophils separated from a patient with hypereosinophilia (Fig 2) revealed a prominent positive peak at 495 nm (Fig 2).
Fig 2. Reduced-minus-oxidized difference spectra of eosinophil
sonicates. The difference spectra of eosinophils was recorded in
the same scale and the same number of cells as the polymorphonu-
clear leukocytes (PMN) sonicates shown in Fig 1. Tracing (left)
shows dithionite reduced-minus-oxidized difference spectra of
eosinophil sonicates; tracing (right) shows difference spectra of an
eosinophil sonicate incubated with 5 mmol/L of azide plus 0.5
mmol/L of H_{2}O_{2}.

peak at 450 nm, a negative peak at 414 nm, and a small
positive peak at 560 nm; these correspond to the maxima of the
spectra of eosinophil peroxidase. Difference spectra of PMN
sonicates from one CGD patient (E.W.) showed a
prominent shoulder at 452 nm due to a high content of
eosinophil peroxidase (Fig 1). A small peak observed at
560-nm absorption, corresponding to eosinophil peroxidase,
makes assay of cytochrome b at 558 nm impossible.

Therefore, to improve quantitation of cytochrome b, we
developed a new technique to delete peaks of myeloperoxi-
dase and eosinophil peroxidase. As shown in Fig 1 and Fig 2,
the peaks of myeloperoxidase and eosinophil peroxidase were
completely obliterated by treating the cell sonicates with
azide plus hydrogen peroxide, and an \( \alpha \) peak and a Soret
peak of cytochrome b were clearly observed. Difference
spectra of PMN sonicates from two male CGD patients
suspected of X-linked CGD (R.K. and E.W.) are shown in
Fig 1. Treatment of the sonicated sample with azide plus
hydrogen peroxide obliterated the absorption peaks of myelo-
peroxidase and eosinophil peroxidase, clearly delineating the
absence of the \( \alpha \) and Soret peaks of cytochrome b. In a
female patient with non-X-linked CGD (L.O.), absorption
peaks for myeloperoxidase and cytochrome b were observed
before treatment with azide plus hydrogen peroxide (Fig 1).
After treatment with azide plus hydrogen peroxide, the \( \alpha \)
and Soret peaks of cytochrome b were seen.

To determine whether treatment with hydrogen peroxide
plus azide affects the absorption of cytochrome b, we studied
the effect of azide plus hydrogen peroxide on PMN subcellu-
lar fractions. Marker proteins in each fraction are shown in
Table 1. The membrane-rich fraction was enriched in alkaline
phosphatase. The specific granule-rich fraction contained
a large amount of vitamin B_{12} binding protein.
Myeloperoxidase was mainly enriched in the azurophil gran-
ule-rich fraction, but the specific granule-rich fraction also
included a small amount of myeloperoxidase. Cytochrome b
was located on both the plasma membrane-rich and specific
granule-rich fractions as reported previously. In Fig 3,
the difference spectra of each fraction before and after
treatment with azide plus hydrogen peroxide are shown.
Following treatment with azide plus hydrogen peroxide, both
the positive peak at 472 nm and the negative peak at 435 nm
were eliminated, indicating that myeloperoxidase was suc-
cessfully deleted from the azurophil granule-rich and specific
granule-rich fractions. The height of a Soret peak (424 nm)
of cytochrome b observed in the membrane-rich fraction,
which lacked myeloperoxidase, was not decreased by the
treatment. Therefore, this peak was used for all measure-

| Table 1. Marker Proteins in Subcellular Fractions From Neutrophils |
|-------------------------|----------------------|-------------------|----------------------|----------------------|
| Fraction                | Alkaline Phosphatase (mU/mg of Protein) | Vitamin B_{12} Binding Protein (ng Vit B_{12} Bound/mg of Protein) | Myeloperoxidase (nmol/mg of Protein) | Cytochrome b (mmol/mg of protein) |
| Normal                  |                                    |                  |                      |                      |
| Membrane                | 149 ± 32                           | 0.80 ± 0.24      | 0.02 ± 0.01          | 1.38 ± 0.04          |
| Specific granule        | 21 ± 3                             | 3.83 ± 1.04      | 0.40 ± 0.02          | 0.73 ± 0.08          |
| Azurophil granule       | 24 ± 4                             | 0.47 ± 0.16      | 1.47 ± 0.15          | 0.01 ± 0.01          |
| Cytosol                 | 8 ± 1                              | 0.21 ± 0.08      | 0 ± 0                | 0 ± 0                |
| CGD (R.K.)              |                                    |                  |                      |                      |
| Membrane                | 186                                | 0.73             | 0                    | Trace                |
| Specific granule        | 36                                 | 3.47             | 0.28                 | Trace                |
| Azurophil granule       | 40                                 | 0.38             | 1.07                 | 0                    |
| Cytosol                 | 7                                  | 0.42             | 0                    | 0                    |

Separation of subcellular fractions and the assay of various proteins are described in Materials and Methods section. Data for the normal PMNs are mean ± SEM of nine preparations whereas CGD data are from a typical experiment.
Table 2. Cytochrome b and FAD in PMN From Patients With CGD

<table>
<thead>
<tr>
<th>Case</th>
<th>Cytochrome b (pmol/7.5 × 10^6 Cells)</th>
<th>FAD (pmol/mg of Protein)</th>
<th>NBT in Mother's PMNs (% Positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male CGD without cytochrome b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M.J.</td>
<td>0*</td>
<td>24*</td>
<td>21</td>
</tr>
<tr>
<td>D.H.</td>
<td>0*</td>
<td>90</td>
<td>45</td>
</tr>
<tr>
<td>E.W.</td>
<td>0*</td>
<td>85</td>
<td>21</td>
</tr>
<tr>
<td>R.W.</td>
<td>0*</td>
<td>34*</td>
<td>61</td>
</tr>
<tr>
<td>S.S.</td>
<td>0*</td>
<td>128</td>
<td>60</td>
</tr>
<tr>
<td>P.K.†</td>
<td>0*</td>
<td>68</td>
<td>70 Mother, 47</td>
</tr>
<tr>
<td>R.K.†</td>
<td>0*</td>
<td>55</td>
<td>Same as above</td>
</tr>
<tr>
<td>D.H.</td>
<td>0*</td>
<td>120</td>
<td>50</td>
</tr>
<tr>
<td>E.H.‡</td>
<td>0*</td>
<td>24*</td>
<td>67</td>
</tr>
<tr>
<td>S.H.‡</td>
<td>0*</td>
<td>25*</td>
<td>Same as above</td>
</tr>
<tr>
<td>T.H.</td>
<td>0*</td>
<td>68</td>
<td>NT</td>
</tr>
<tr>
<td>Male CGD with cytochrome b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H.P.</td>
<td>40.1</td>
<td>122</td>
<td>100</td>
</tr>
<tr>
<td>T.A.</td>
<td>51.9</td>
<td>144</td>
<td>100</td>
</tr>
<tr>
<td>C.T.</td>
<td>35.4</td>
<td>73</td>
<td>NT</td>
</tr>
<tr>
<td>R.G.</td>
<td>23.0</td>
<td>145</td>
<td>98.0</td>
</tr>
<tr>
<td>B.J.§</td>
<td>32.4</td>
<td>153</td>
<td>NT (deceased)</td>
</tr>
<tr>
<td>M.B.§†</td>
<td>40.1</td>
<td>54</td>
<td>NT (deceased)</td>
</tr>
<tr>
<td>J.H.</td>
<td>20.0</td>
<td>48</td>
<td>100</td>
</tr>
<tr>
<td>P.R.</td>
<td>55.0</td>
<td>135</td>
<td>99</td>
</tr>
<tr>
<td>R.D.</td>
<td>74.6</td>
<td>59</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>41.4 ± 5.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female CGD with cytochrome b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.A.</td>
<td>31.3</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>L.O.</td>
<td>32.4</td>
<td>NT</td>
<td>100</td>
</tr>
<tr>
<td>D.J.§</td>
<td>45.4</td>
<td>143</td>
<td>NT (deceased)</td>
</tr>
<tr>
<td>S.J.§</td>
<td>35.4</td>
<td>155</td>
<td>NT (deceased)</td>
</tr>
<tr>
<td>K.L.§</td>
<td>70.7</td>
<td>113</td>
<td>NT (deceased)</td>
</tr>
<tr>
<td>J.M.**</td>
<td>53.1</td>
<td>143</td>
<td>100</td>
</tr>
<tr>
<td>M.M.**</td>
<td>46.0</td>
<td>143</td>
<td>Same as above</td>
</tr>
<tr>
<td>L.H.††</td>
<td>73.7</td>
<td>105</td>
<td>100</td>
</tr>
<tr>
<td>C.H.††</td>
<td>49.7</td>
<td>105</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>48.6 ± 5.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female CGD without cytochrome b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.V.</td>
<td>0*</td>
<td>101</td>
<td>98</td>
</tr>
</tbody>
</table>

Cytochrome b in PMNs from CGD patients was measured from difference spectra of PMN sonicate treated with azide plus hydrogen peroxide as shown in Fig 1.

*P < 0.05 v normal, Student's t test.

No significant difference as compared to normal.

†Brothers; ‡Brothers; §siblings (D.J. and S.J.: nonhomozygous twins); ¶siblings; **homozygous twins. ††C.H. is a fraternal aunt of L.H., suspected of autosomal dominant inheritance. §Parents of the patients (H.P., M.B., K.L., and C.T.) are first cousins. Cytochrome b content in PMNs from 24 normal adults was 47.4 ± 2.9 pmol/7.5 × 10^6 cells. FAD content of PMN particulate fraction from 14 normal adults was 104.7 ± 9.2.

NT, not tested.
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A subcellular deficiency of FAD has been suggested as the basis of a defect in some patients. To assess this possibility, neutrophils from three patients with low normal FAD, (M.B., J.H., and R.D., Table 2), were fractionated into cytosol-, membrane-, specific granule-, and azurophil granule-enriched fractions as described in Table 1. All fractions from the CGD patients contained FAD; the only possible deficiency noted was in the specific granule-enriched fraction of M.B., which contained 101 pmol/mg protein ± 44 µg/mg protein for the three normal subjects. Thus, although a subcellular deficiency of FAD may exist in one of these patients, the magnitude of the defect is not dramatic.

A family with probable autosomal recessive cytochrome b deficiency (Table 3). Although most of our patients with cytochrome b deficiency had an X-linked recessive pattern of inheritance, the disease appeared to be inherited in an autosomal recessive pattern in one female patient (A.V.). The PMA-stimulated NBT test confirmed that the patient PMNs could not reduce NBT after stimulation. The NBT scores of PMNs from the mother and father and three male siblings were normal. Superoxide generation was totally absent in the patient’s PMNs. PMNs from the mother, father, and two siblings showed a common pattern of superoxide production; normal response to PMA but reduced response to fMet-Leu-Phe.

The cytochrome b of the patient’s PMNs was not detected in three separate examinations. The cytochrome b content of PMNs from the mother and the father were significantly reduced to about half-normal (Table 3) although PMNs from two of the patient’s siblings with somewhat reduced O₂⁻ production to fMet-Leu-Phe contained normal amounts of cytochrome b. FAD of PMN particulate fractions were normal in every member of the family, including the patient.

DISCUSSION

The most commonly used method for quantitating cytochrome b is the method of Segal et al who used the α peak of cytochrome b (558 nm) absorbance to measure neutrophil

\[
\text{Cytochrome b FADFMLP PMA}^{2.4} 2.9 (22) 105
\]

**Table 3. Study of a Family of a Female CGD Patient With Cytochrome b Deficiency**

<table>
<thead>
<tr>
<th>(%)</th>
<th>FAD (pmol/mg Protein)</th>
<th>PMA (20 ng/mL)</th>
<th>F Dwarf PMN (1 µmol/L)</th>
<th>NBT Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&gt;90.0</td>
<td>3.56 ± 0.70 (8)</td>
<td>8.75 ± 1.16 (8)</td>
<td>45.8 ± 2.9 (22)</td>
</tr>
<tr>
<td>CGD (A.V.)</td>
<td>0</td>
<td>0 ± 0 (3)</td>
<td>0 ± 0 (3)</td>
<td>0 ± 0 (3)</td>
</tr>
<tr>
<td>Mother</td>
<td>98.0</td>
<td>1.46 ± 0.57 (3)</td>
<td>8.00 ± 3.81 (3)</td>
<td>18.2 ± 2.4 (3)</td>
</tr>
<tr>
<td>Father</td>
<td>98.0</td>
<td>1.44 ± 0.13 (3)</td>
<td>7.66 ± 2.06 (3)</td>
<td>15.8 ± 0.7 (3)</td>
</tr>
<tr>
<td>Brother</td>
<td>99.0</td>
<td>1.05 ± 0.54 (3)</td>
<td>8.94 ± 4.79 (3)</td>
<td>30.7</td>
</tr>
<tr>
<td>Brother</td>
<td>97.5</td>
<td>1.68 ± 0.30 (3)</td>
<td>8.94 ± 2.44 (3)</td>
<td>31.4</td>
</tr>
<tr>
<td>Brother</td>
<td>99.0</td>
<td>4.61 ± 0.91 (2)</td>
<td>7.91 ± 0.67 (2)</td>
<td>31.4</td>
</tr>
</tbody>
</table>

Data show the mean ± SEM of the number of experiments in parentheses.

*Maximal rate nmol/10⁶ cells/1 min.

†NBT reduction was examined on PMN stimulated with PMA (20 ng/mL). P < 0.01.

§P < 0.02; §§P < 0.001; §§P < 0.01. v normal.
cytochrome b. With this approach, a peak of eosinophil peroxidase (560 nm) overlaps the \( \alpha \) peak of cytochrome b, making it difficult to measure cytochrome b in PMNs containing eosinophils, which in our experience are frequently mildly elevated in CGD. We overcame this problem by treating PMN sonicates with azide and hydrogen peroxide. This method obliterated the absorption peaks of myeloperoxidase and eosinophil peroxidase without significant alteration of cytochrome b absorption. We have shown previously that azide plus hydrogen peroxide irreversibly inactivates the activity of myeloperoxidase and eosinophil peroxidase.

PMN cytochrome b of 24 normal adults and 30 CGD patients was measured. Cytochrome b was detected in cells from each normal adult (47.4 ± 2.9 pmol/7.5 × 10⁶ cells, n = 24). This value was slightly lower than those described by Segal et al., probably because the absorption at 558 nm they measured represented the \( \alpha \) peak of cytochrome b plus the 560 nm absorption peak of eosinophil peroxidase. In 11 of 20 male CGD patients, PMN sonicates lacked cytochrome b (Table 2). Neutrophils from mothers of the patient group were examined for the stimulated NBT test. A mosaic pattern of normal and abnormal neutrophils was found in all patients, indicating heterozygous carriers and an X-linked trait of the disease (Table 2). On the other hand, cytochrome b was present in neutrophils from nine other males with CGD. Neutrophils from five mothers had a normal NBT score and one, R.D., had a mosaic pattern (Table 2). Cytochrome b content was normal in PMNs from nine female patients with CGD. The neutrophil NBT test from each mother in this group of female CGD patients was normal (Table 2).

As reported previously, X-linked transmission and autosomal recessive transmission of CGD are commonly observed, and one family with autosomal dominant inheritance has been reported. A recent study suggested that the autosomal recessive form of CGD has less severe symptoms than does the X-linked form of the disease. We saw no difference in symptoms among our patient groups. However, this may be because the use of prophylactic bactrim therapy makes comparison of disease severity among the patient groups difficult to interpret. Segal et al suggested that the cytochrome b content in neutrophils from CGD patients is valuable for determining the type of genetic transmission. Our study supports Segal's conclusion except for two patients. One patient (A.V.) is a female with cytochrome b deficiency. Extreme lyonization of a heterozygote is not a likely explanation for the inheritance in A.V. because the NBT score of the mother's PMNs was not compatible with X-linked CGD. Furthermore, the cytochrome b content in PMNs of both her mother and father was reduced to half-normal, strongly suggesting an autosomal recessive type of cytochrome b deficiency. In addition, the superoxide production of the mother and father's PMNs was reduced when FMLP, but not PMA, was used as a stimulant (Table 3). Abnormal superoxide production was also observed in PMNs from two of her siblings, although PMN cytochrome b of the siblings was not sufficiently reduced to indicate that they were heterozygotes (Table 3). Another description of a family with an autosomal form of cytochrome b deficiency appeared in a recent paper. Thus, a phenotype of neutrophil cytochrome b deficiency can be separated into two genetic types, X-linked or autosomal recessive. The synthesis of mature cytochrome b must require several processes; putative gene activator proteins, transcription, RNA processing, nuclear to cytoplasmic transport of mRNA, translation, and posttranslational modification including incorporation of the heme moiety. If any step is defective or lacking, cytochrome b will be undetectable by spectroscopy. The identification of families with autosomal recessive cytochrome b inheritance furthers our understanding of the genetic mechanism of neutrophil cytochrome b production. Probably a structural gene for cytochrome b will be located on an autosomal chromosome. Thus, a mutation of one of the homologous autosomal chromosomes (heterozygote) would lead to a half-normal cytochrome b. The X chromosome may have a gene encoding a putative cytochrome b gene activator protein that may positively switch on the transcription of the cytochrome b gene. Alternatively, the X chromosome may have an "enhancer element" that ensures accurate and efficient initiation of transcription. Further understanding of the molecular defect of CGD patients with cytochrome b deficiency will require purification of cytochrome b and cloning of the cytochrome b gene.

The other patient who did not fit the generalization that all X-linked CGD patients have cytochrome b deficiency is a male CGD patient (R.D.) strongly suspected of X-linked CGD from the NBT score of his mother's PMNs; however, the patient's PMNs contained normal amounts of cytochrome b (Table 2). Moreover, the mother's PMNs also contained normal amounts of cytochrome b (49.7 pmol/7.5 × 10⁶ cells). This is important because heterozygotes of X-linked cytochrome b deficiency should show reduced PMN cytochrome b according to the NBT score. The patient's PMNs also contained normal FAD. Therefore, in patient R.D., deficiency of another substance controlled by the X-chromosome is suspected. In this respect, a recent report suggested a requirement of a cytosolic factor for \( \alpha \) generation, which may be missing in patient R.D.

Several publications reported reduced FAD in CGD neutrophils. Cross et al reported three cases of X-linked CGD, in whom neutrophil membrane fractions contained 50% of the normal level of FAD; these patient's neutrophils also lacked cytochrome b. In contrast, in three cases with autosomal recessive CGD, FAD and cytochrome b were normal. Cross et al suggested that there is a close association between FAD and cytochrome b. Gabig and Lefker reported three male CGD patients with FAD deficiency of the neutrophil particulate fraction; two of the patients also had neutrophil cytochrome b deficiency. Borregaard and Tauber reported two CGD patients whose neutrophils showed a deficiency of FAD and absent cytochrome b in neutrophil fractions rich in specific granules although these patients' PMNs had normal quantities of FAD in fractions containing plasma membrane and cytosol. We determined the FAD content in the particulate fractions of PMNs from 28 patients with CGD. FAD was normal in 14 patients whose PMNs contained normal levels of cytochrome b (Fig 4). CGD patients with PMN cytochrome b deficiency were separated into two groups; significantly reduced FAD (~25% of normal) was observed in four patients, whereas FAD was normal in eight patients (Table 2, Fig 4). The results support a close relation-
ship between cytochrome b deficiency and reduced FAD content but indicate that cytochrome b deficiency with normal FAD also exists.

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Reevaluation of cytochrome b and flavin adenine dinucleotide in neutrophils from patients with chronic granulomatous disease and description of a family with probable autosomal recessive inheritance of cytochrome b deficiency

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