A New X-Linked Variant of Chronic Granulomatous Disease Characterized by the Existence of a Normal Clone of Respiratory Burst-Competent Phagocytic Cells

By Richard C. Woodman, Peter E. Newburger, Pervin Anklesaria, Richard W. Erickson, Julie Rae, Myron S. Cohen, and John T. Curnutte

Chronic granulomatous disease (CGD) is characterized by recurrent infections, and is usually associated with a complete inability of phagocytic cells to generate superoxide anion \( \text{O}_2^- \). Rarely, variant forms of CGD have been reported in which there is reduced, but detectable, \( \text{O}_2^- \) production by phagocytic cells. We describe three adult males in two kindreds with a unique form of \( \text{O}_2^- \)-deficient (CGD) CGD not previously reported. All three patients had two distinct populations of phagocytic cells, with one subset capable of normal respiratory burst activity and the other larger subset inactive, as in classic CGD (X91%). The respiratory burst activity in neutrophils purified from each patient was \( \sim 10\% \) of normal as determined by \( \text{O}_2^- \) production, \( \text{O}_2^- \) consumption, cytochrome \( b_558 \) spectroscopy, and membrane oxidase activity using a cell-free activation system. In contrast with other patients with X91%-variant CGD, the unique feature of these patients is the presence of a small but significant population (5% to 15%) of circulating neutrophils and monocytes with completely normal respiratory burst activity as assessed by nitroblue tetrazolium (NBT) reduction and flow-cytometric measurement of dihydrodichromamine oxidation. NBT reduction of peripheral blood granulocyte-macrophage progenitor cells also showed the presence of a subset of colonies derived from myeloid progenitor cells that had normal respiratory burst capabilities. A mosaic XX chromosome karyotype and an unstable oxidase complex that might occur during myeloid maturation were both excluded as possible explanations. In these families, the molecular defect in the gp91-phox gene, which is currently under investigation, appears to prevent expression of the gene in the majority of neutrophils, but not in a small subset. Our studies suggest that commitment to either a respiratory burst-competent or -incompetent phagocytic cell occurs at the level of the myeloid progenitor cell.

© 1995 by The American Society of Hematology.

Recently, it has become apparent that the active oxidase is actually a complex multicomponent enzyme system that is comprised of several membrane and cytosolic proteins. These include several recently recognized guanosine triphosphate (GTP) binding proteins, important in oxidase regulation and cytochrome \( b_{558} \), a unique membrane-associated heterodimeric cytochrome \( b \) that functions as the terminal electron transport carrier for oxidase. The cytochrome \( b_{558} \) consists of a 91-kD glycoprotein subunit (gp91-phox), a unique membrane-associated heterodimeric cytochrome \( b \) that functions as the terminal electron transport carrier for oxidase.

The vast majority of CGD patients have no detectable \( \text{O}_2^- \) production (<1% of normal) by their phagocytic cells. Occasionally (<10% of cases), patients with an X-linked variant form of CGD have been reported in which there is diminished, but measurable, \( \text{O}_2^- \) production (2% to 35% of normal). Nitroblue tetrazolium (NBT) reduction by neutrophils from these patients usually shows a homogeneous population of cells with weak staining compared with normal; i.e., each cell has only a few grains of blue formazan precipitate rather than the heavy deposits normally seen. NBT reduction by neutrophils from carriers with these variant X-linked forms of CGD are also unique in that two populations of NBT-positive cells are seen, one with weak staining identical to the patients, and one with intense reduction similar to normal. In several of the cases reported,
Family P

![Pedigree of Family P](image)

Family B

![Pedigree of Family B](image)

The reduced respiratory burst activity was associated with abnormal oxidase kinetics, specifically a reduced affinity for nicotinamide adenine dinucleotide phosphate (NADPH).16,19,22

We describe three patients from two kindreds with a gp91phox--deficient variant of CGD not previously reported. In contrast with the other reported cases of variant X-linked CGD, these patients have two distinct populations of phagocytic cells with varying respiratory burst capabilities. The majority of cells were completely unable to generate O$_2^-$, but there was a small distinct subset (5% to 15%) of cells with normal respiratory burst activity. This population of phagocytic cells appeared to be entirely responsible for the residual O$_2^-$ production that was 10% to 15% of normal.

CASE REPORTS

Family P. The proband (P-II$_1$) (Fig 1), a 22-year-old man, was diagnosed with CGD at age 4 years, after presentation with a Staphylococcus aureus hepatic abscess. The postoperative course was complicated by a Serratia marcescens wound abscess. At the time of diagnosis, $\approx$3% of his neutrophils were reported to be NBT-positive. Infections since diagnosis have included S marcescens pharyngitis, submandibular lymphadenitis, recurrent pleural effusions, and bilateral pneumonias. The patient has been on daily prophylactic trimethoprim-sulfamethoxazole since age 4, and since age 18, he has also received prophylactic interferon $\gamma$ (IFNy; 0.05 mg/m²) subcutaneously three times per week. Since starting IFNy, the patient has not had any serious infections requiring hospitalization and parenteral antibiotics. Red blood cell (RBC) phenotyping showed normal expression of the Kell antigens. X-linked inheritance was established based on studies in the mother (P-I$_1$) and sister (P-II$_2$) in which a mosaic pattern of NBT-positive and NBT-negative neutrophils was found and intermediate levels of $O_2$ production and cytochrome $b_{59}$ (20% to 30% of normal) were measured. The proband’s mother (P-I$_1$) has a history of discoid lupus with weakly positive antinuclear antibodies but negative anti-DNA antibodies.

Family B. Brothers B-II$_1$ and B-II$_2$ (Fig 1), were diagnosed with CGD at ages 14 and 12, respectively. B-II$_1$, a 34-year-old man, has a life-long history of infections that includes: (1) recurrent S aureus hepatic abscesses; (2) several episodes of culture-negative pneumonia involving both lungs; (3) a myocardial abscess; (4) septic arthritis of the left knee complicated by cellulitis and septic thrombophlebitis; and (5) recurrent aphthous stomatitis. At diagnosis, $\approx$3% of his neutrophils were reported to be NBT$^-$. B-II$_2$, a 32-year-old man, has also experienced multiple infections that include: (1) right humeral osteomyelitis after a fracture that eventually required surgical debridement and open reduction; (2) two separate episodes of S marcescens bursitis, one involving the right elbow and the other the right calcaneus; (3) bacterial conjunctivitis and blepharitis; (4) multiple episodes of gram-negative and Staphylococcal soft-tissue abscesses; and (5) recurrent pneumonia. In addition, B-II$_2$ has experienced erythema multiforme, recurrent aphthous stomatitis, severe periarticular disease, and chronic interstitial pneumonitis. Because both patients are allergic to sulfonamides, they have generally used dicloxacillin or cephalaxin for daily prophylaxis. Both B-II$_1$ and B-II$_2$ have received prophylactic recombinant IFNy (0.05 mg/m²/dose) subcutaneously three times per week for the past 33 months. Neither patient has had any serious infections since starting IFNy. Both patients have normal RBC expression of the Kell antigens. Intermediate levels of neutrophil chemiluminescence and a dimorphic population of NBT$^+$ and NBT$^-$ neutrophils in the mother (P-I$_1$) established the X-linked inheritance.

MATERIALS AND METHODS

Chemicals. Cytochrome $c$ (horse heart, type VI), bovine erythrocyte superoxide dismutase (SOD), diisopropylphosphofluoridate, phorbol myristate acetate (PMA), catalase, NBT (type II), dimethyl sulfoxide (DMSO), and $\beta$-NADPH were obtained from Sigma Chemical Co. (St Louis, MO). PMA stock solutions were made in DMSO at a concentration of 2 mg/mL, stored in small aliquots, and diluted to 80 pg/mL in DMSO just before use. Sodium dodecyl sulfate (SDS) and gelatin were purchased from Bio-Rad Laboratories (Richmond, CA). Ficoll-Paque and Percoll were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Dihydrorhodamine (DHR) was purchased from Molecular Probes (Eugene, OR), dissolved in DMSO at a concentration of 1 mmol/L, stored at $-20^\circ$C in the dark under N$_2$ gas and then diluted in phosphate buffer before use. Recombinant human granulocyte colony-stimulating factor (rhG-CSF) and recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) were provided by Amgen, Inc (Thousand Oaks, CA). rhIFNy was obtained from Genentech, Inc (South San Francisco, CA). Other reagents were of the best grade commercially available and were used without further purification.
Table 1. Intact Neutrophil Respiratory Burst Activity in Patients With Variant X-Linked CGD

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>P-IIa</th>
<th>B-IIa</th>
<th>B-IIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>O$_2$ production* (nmol/min/10$^7$ cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td>119.3 ± 30.2  (n = 9)</td>
<td>14.4 ± 5.3 (n = 9)</td>
<td>9.9 ± 6.8 (n = 5)</td>
<td>7.6 ± 2.2 (n = 4)</td>
</tr>
<tr>
<td>FMLP</td>
<td>38.5 ± 15.3   (n = 5)</td>
<td>0.0 (n = 5)</td>
<td>0.0 (n = 2)</td>
<td>1.9 (n = 2)</td>
</tr>
<tr>
<td>FMLP + DHCB</td>
<td>82.1 ± 44.5   (n = 3)</td>
<td>0.0 (n = 3)</td>
<td>0.0 (n = 2)</td>
<td>0.0 (n = 2)</td>
</tr>
<tr>
<td>C$_4$ consumption† (nmol/min/10$^7$ cells) (n = 2)</td>
<td>50.1</td>
<td>6.3</td>
<td>5.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Cytochrome b$_{553}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact cells (pmol/10$^7$ cells) (n = 6)</td>
<td>6.4 ± 21.2</td>
<td>4.2 ± 1.3 (n = 6)</td>
<td>6.0 ± 1.4 (n = 3)</td>
<td>4.4 ± 1.1 (n = 3)</td>
</tr>
<tr>
<td>Membranes (pmol/10$^7$ cell equivalent) (n = 2)</td>
<td>73.9</td>
<td>6.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Flow cytometry4 (% positive)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHR (n = 2)</td>
<td>83</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>NBT Slide Test5 (% positive)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>100 (n = 15)</td>
<td>8 ± 4 (n = 12)</td>
<td>7 ± 3 (n = 3)</td>
<td>9 ± 2 (n = 3)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>100</td>
<td>7 ± 2</td>
<td>7 ± 1</td>
<td>11 ± 2</td>
</tr>
</tbody>
</table>

Unless indicated results are represented as mean ± 1 SD. Each experiment is an average of two measurements.

Abbreviations: DHCB, dihydrocytochalasin B; ND, not determined; n, number of experiments.

* As indicated, cells were activated with either PMA (200 ng/mL), FMLP (10-4 mol/L), or preincubated with DHCB (10 μmol/L) at 37°C for 10 minutes followed by activation with 10-5 mol/L FMLP.

† Activated with 100 μL heat-killed S. aureus.

‡ Refers to percentage of cells with an increased mean fluorescence value after 20 minutes of activation with PMA (final concentration 100 ng/mL).

§ Activated with PMA (final concentration 200 ng/mL). NBT slide test results for unstimulated cells not shown.

Cell preparation and fractionation. Neutrophils were isolated from whole blood by dextran sedimentation, hypotonic water lysis and Histopaque density-gradient centrifugation.25 Cytoisol and solubilized membrane for O2 production in the cell-free activation system were prepared from unstimulated neutrophils collected by leukapheresis and fractionated using a previously published method.26

Cytochrome b$_{553}$. The quantity of cytochrome b$_{553}$ in neutrophil cell pellets (10$^7$ cells total) was determined by dithionite difference spectroscopy.27 Cytochrome b$_{553}$ spectroscopy was normally performed on the same day as neutrophil isolation. All measurements were done in duplicate unless otherwise indicated. In patient P-IIa, cytochrome b spectroscopy was also performed on solubilized neutrophil membranes using the same method. These measurements were expressed as picomoles per 10$^7$ cells equivalent of membrane.

O2 production and oxygen consumption. The rates of O2 production by intact neutrophils and in the cell-free activation system were measured using the SOD-inhibitable reduction of ferricytochrome c. For intact neutrophils, cells were stimulated with PMA (final concentration, 200 ng/mL).29 All measurements were done in duplicate unless otherwise indicated. The cell-free activation system consisted of membrane and cytosol fractions of neutrophils, NADPH (160 μmol/L) and was activated by the addition of SDS (40 μmol/L) without any preincubation. Cytoisol activity was determined by mixing neutrophil membranes from normal donors with partially purified cytoisol from the patients. Membrane activity was determined by mixing neutrophil cytoisol from normal donors with membranes from the patients. All measurements were done in triplicate. The lag time was calculated from the intercept of the back-extrapolated linear portion of the curve with the preactivation baseline of zero absorption change.

Neutrophil oxygen consumption was measured polarographically with an electronic oxygen monitoring system (Yellow Springs Instruments Co, Yellow Springs, OH) using a previously published method.30 Neutrophils were activated with 100 μL of heat-killed S. aureus that had been opsonized with sera from normal donors; oxygen consumption was monitored over 5 minutes.

NBT reduction. The ability of individual circulating neutrophils and monocytes to reduce NBT after stimulation with PMA (200 ng/mL) was determined using a previously published method.31 The percentage of NBT-positive cells was determined by light microscopy after counting ≥200 neutrophils (or ≥100 monocytes). The staining intensity of the patient’s NBT-positive cells compared with control cells was also determined.

Flow cytometry. Flow cytometric analysis of neutrophil respiratory burst activity was measured using a modification of a previously published method.32 Neutrophils were suspended in phosphate-buffered saline containing 5 mol/L glucose and 0.1% gelatin at a concentration of 1 × 10$^7$ cells/mL and preincubated with the DHR (1 μmol/L) for 15 minutes, with gentle mixing every 5 minutes. After incubation with DHR, catalase (275 U/mL final concentration) was added to the neutrophil suspension. Aliquots of cells were removed and incubated with either PMA (100 ng/mL final concentration) or DMSO at room temperature for 10 and 20 minutes. Samples were then analyzed on a FACSscan flow cytometer (Becton Dickenson, San Jose, CA). A total of 10,000 cells from each sample were collected, and green fluorescence emission between 530 and 550 nm was monitored. Fluorescence intensity was measured on a logarithmic scale and the mean fluorescence of the analyzed cells was calculated using FACSscan Research software (Becton Dickinson). Respiratory burst-dependent fluorescence was determined by subtracting the mean channel fluorescence of cells exposed to DMSO alone from that measured in cells stimulated with PMA.

Granulocyte-macrophage colony assays. Human peripheral blood mononuclear cells were prepared by Histopaque (Sigma Chemical Co, St Louis, MO) centrifugation,33 frozen in 10% DMSO in a programmable cell freezer, then later thawed, counted, and used for colony assays as follows. Cells were resuspended in culture media supplemented with 30% fetal calf serum (FCS), then plated at 10$^5$/mL in 1.1% methylcellulose containing 30% FCS, 1.2% bovine serum albumin, 5 × 10$^{-6}$ mol/L β-mercaptoethanol, 10 ng/mL human recombinant interleukin-3 (IL-3; Immunex Corp, Seattle, WA), and 1,000 U/mL of G-CSF (Amgen). Cultures were incubated in humidified chambers at 37°C and 5% CO2 for 14 days. The functional capacity of mature granulocytes in the colonies was then evaluated.
histochemically by NBT dye reduction in response to PMA stimulation, as previously described. \textsuperscript{34,35} Colonies of \(>25\) cells were counted and scored as positive (\(>90\%\) of cells within the colony stained blue with reduced NBT), mixed (\(10\%\) to \(90\%\) of cells reducing NBT), or negative (\(<10\%\) of cells reducing NBT). In addition, colonies were plucked, cytocentrifuged onto glass slides, stained with Wright-Giemsa, and examined microscopically for cell morphology.

\textit{Southern blot analysis.} High molecular-weight genomic DNA was extracted from whole peripheral blood, digested with restriction endonuclease \textit{Taq} \(I\), electrophoresed in 0.8% agarose, and transferred to nitrocellulose filters by standard methods. \textsuperscript{36} Filters were hybridized with labeled \textsuperscript{75} probe 754, a 2.3-kb \textit{HindIII} fragment closely linked to the \(X\) chromosome CGD locus. \textsuperscript{38} In addition, DNA was analyzed (by Collaborative Research, Inc, Waltham, MA) for restriction fragment-length polymorphisms (RFLPs) detected by the highly polymorphic, non-CGD–linked probe S-232, which maps near the \(X\) chromosome centromere. \textsuperscript{39}

\section*{RESULTS}

\textit{NBT reduction by circulating leukocytes.} Although the large majority of cells remained negative, all three patients showed a small subset (5\% to 10\%) of neutrophils and monocytes that were capable of reducing NBT after stimulation with PMA (Table 1). Not only did the percentage of NBT-positive cells approximate the patients’ intact cell \(O_2^\cdot\) production, but they stained as intensely as cells from normal donors, unlike the uniformly weakly-staining cells from patients with the more common form of variant \(X\)-linked CGD (Fig 2).

\textit{Neutrophil respiratory burst activity.} Intact neutrophil respiratory burst activity from patients P-II\(_1\), B-II\(_2\), and B-II\(_1\) is summarized in Table 1. Neutrophils from all three patients had \(O_2^\cdot\) production, as determined by PMA-induced cytochrome \(c\) reduction, that was consistently 5\% to 15\% of normal (P-II\(_1\), 12\%; B-II\(_2\), 8\%; B-II\(_1\), 6\%). Lag times after PMA activation were similar for patient (P-II\(_1\), 48 sec; B-II\(_2\), 54 sec; B-II\(_1\), 53 sec; \(n = 2\)) and control neutrophils (54 \(\pm\) 8 sec; \(n = 8\)).

In two of the patients (P-II\(_1\) and B-II\(_1\)), neutrophil oxidase activity in response to a particulate agonist (opsonized \textit{S aureus}) was also examined. Neutrophil \(O_2^\cdot\) consumption in P-II\(_1\) (12\%) and B-II\(_1\) (11\%) closely approximated the neutrophil PMA-induced \(O_2^\cdot\) rates and the proportion of NBT-positive cells, when these are expressed as a percentage of normal. Although neutrophils from P-II\(_1\) and B-II\(_1\) responded to soluble (PMA) and particulate agonists (opsonized \textit{S aureus}), for reasons that remain unclear, we were unable to detect \(O_2^\cdot\) production after FMLP stimulation (with or without dihydrocytochalasin B pretreatment). Studies on neutrophils from P-II\(_1\) and other members of his family (P-I\(_1\), P-I\(_2\), P-II\(_1\), P-II\(_2\)) showed normal surface expression of FMLP receptors (data not shown). One possible explanation is that
with oxidase activity of only 5% to 15% of normal, FMLP stimulation that is typically 20% to 25% of maximal PMA rates may be below the level of detection of ferricytochrome c reduction. Together the O₂ production and O₂ consumption results show that the neutrophil oxidase from these patients respond comparably to both soluble (PMA) and particulate stimuli (opsonized S. aureus).

Levels of neutrophil cytochrome b₅₅₈ from each patient also corresponded (P-II₃, 6%; B-II₃, 9%; B-II₅, 7%) to the amount of intact cell respiratory burst activity. Spectroscopy of an enriched membrane preparation from neutrophils of P-II₃ confirmed the presence of a small amount of cytochrome b₅₅₈ (9% of control).

The cytosolic and membrane oxidase activity for neutrophils from patients P-II₃ and B-II₃ is shown in Fig 3. Cytosol from both patients, when reconstituted with normal membranes, was capable of O₂ production comparable with control cytosol. In contrast, oxidase activity of neutrophil membranes from both patients, when reconstituted with normal cytosol, was detectable, but significantly reduced compared with normal membranes. The membrane oxidase activity that was present closely approximated the percentage of oxidase activity observed with intact cells from each patient. Overall, these results are consistent with the diagnosis of cytochrome b-deficient variant CGD and suggest that the residual respiratory burst (5% to 15% of normal) in P-II₃, B-II₃, and B-II₅ was caused by a small subset of circulating neutrophils (and monocytes) that appeared to have a normal oxidase activity.

Flow cytometry. Flow-cytometric analysis of DHR-loaded neutrophils also confirmed that 5% to 6% of the neutrophils underwent a normal respiratory burst after PMA. Representative histograms illustrating the subset of DHR-positive cells from P-II₃ and B-II₃ are shown by the arrows in Fig 4, A and B, respectively.

To confirm these results, experiments (n = 3) were performed in which purified neutrophils from a normal donor were mixed (0% to 6%) with neutrophils (94% to 100%) from an X91⁺ CGD patient with undetectable oxidase activity. This cell suspension was then activated with PMA under identical conditions as described in Materials and Methods.
The mean fluorescence value of the oxidant-producing cells from these mixing experiments equaled the results that were obtained with neutrophils from P-II3, B-I12 and B-II3 (data not shown). More importantly, the calculated percentage of DHR-positive cells was within 1.0% of the number of normal neutrophils that were added to the cell suspension for flow cytometry.

Even in the presence of excess catalase (1,100 U/mL), a small increase in mean fluorescence intensity was also observed in the population of cells that corresponded to the patient’s NBT-negative cells. It was determined that this was secondary to released extracellular oxidants produced by the respiratory burst-competent cells, probably permeating the membranes of the respiratory burst-incompetent cells and causing oxidation of the DHR label present in these cells. This change in mean fluorescence was not observed when X91° CGD neutrophils were incubated alone with PMA.

**NBT reduction after methylprednisolone.** One possible explanation for the presence of NBT-negative cells could be the presence of an unstable oxidase complex that decayed during the final stage of neutrophil maturation in the bone marrow storage pool. Although such a process has not been previously reported, it could theoretically result in a progressive loss of respiratory burst activity. To exclude this possibility, each patient had serial NBT tests performed at selected times up to 4 hours after the intravenous administration of 20 mg methylprednisolone, which was given to accelerate premature release of neutrophils from bone marrow reserves. Despite a twofold or more increase in absolute neutrophil counts, the percentage of NBT-positive neutrophils remained stable for each patient at ~5% (Table 2). This finding suggests that the proportion of cells capable of oxidase activity remains unchanged during myeloid storage pool maturation and that the subset of NBT-positive cells is in fact a distinct clone of cells.

**NBT reduction by progenitor cells.** To examine the level of clonal origin of the subsets, intact colonies grown from peripheral blood CFU-GM were also evaluated for respiratory burst activity by NBT staining in their methylcellulose semisolid culture medium. This technique has previously been used to identify a CGD carrier state. As illustrated in Fig 5 and quantitated in Fig 6, colonies from patient P-II1 showed striking clonal expression of residual oxidase activity. A minority of the colonies were virtually all NBT-positive cells (Fig 5A), similar to colonies from normal controls (E); whereas the remaining colonies resembled classic CGD cells in their complete absence of NBT reduction (B). However, cultures from both patients in family B (B-II3 and B-II3) showed a heterogeneous pattern, in which most colonies contained both NBT-positive and NBT-negative cells in varying proportions (C and D), in addition to negative colonies, but no completely positive ones. Microscopic examination of cells plucked from parallel cultures showed similar cell maturation, morphology, and granularity-macrophage ratios in colonies derived from patient and normal peripheral blood progenitors. The patterns of distribution of NBT reduction in both of these kindreds differed from the homogeneous pattern previously observed in other patients with variant CGD in which cells are uniformly, faintly NBT-positive in all colonies.

**Effects of cytokines on respiratory burst activity.** Superoxide production by neutrophils from P-II3 was also measured after prolonged in vitro incubation (1 and 2 hours) with each of the following cytokines: recombinant G-CSF (1,000 U/mL) alone, GM-CSF (1,000 U/mL) alone, and rhIFNγ (250 U/mL) alone, using SOD-inhibitable reduction of ferricytochrome c. None of these cytokines had any appreciable effect on neutrophil O2 production after activation with PMA (200 ng/mL), FMLP (10−5 mol/L), or PMA alone (Fig 5A).

**Table 2. Effect of Corticosteroid Challenge on % of NBT-Positive Neutrophils**

<table>
<thead>
<tr>
<th>Time After IV Corticosteroid Administered (hr)</th>
<th>Neutrophils (x10^9/mm³)</th>
<th>NBT⁺ Neutrophils (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-II1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.0</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>2.9</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>8.1</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>7.4</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>2.4</td>
<td>4</td>
</tr>
<tr>
<td>B-I12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4.0</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>5.7</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>7.3</td>
<td>5</td>
</tr>
<tr>
<td>B-II3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.8</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>3.4</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>6.4</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>5.4</td>
<td>5</td>
</tr>
</tbody>
</table>

Patients were administered 20 mg of methylprednisolone intravenously over 10 minutes. Complete blood count and NBT slide tests were performed serially at the time points indicated. Abbreviation: IV, intravenous.

† Neutrophils were activated with PMA 200 ng/mL.
Fig 5. NBT reduction by CFU-GM grown in semisolid medium from peripheral blood progenitor cells from the indicated donors: patient P-I13 (A and B); patient B-I2 (C and D); and normal (E). Colonies were cultured, NBT tested as described in Materials and Methods, and photographed through a 6.3× objective on a Zeiss inverted microscope (Zeiss, New York, NY). NBT reduction in granulocyte-macrophage colonies grown in vitro from peripheral blood progenitor cells of a normal donor (E) and P-I13 patient (A, B) are shown. Colonies from normal progenitor cells show uniform heavy deposits of reduced NBT dye (E). Colonies from P-I13 progenitors show either similar, uniformly active NBT reduction (A) or complete absence of activity (B). Colonies from B-I2 showed a mixed pattern of NBT-positive and NBT-negative cells (C, D).

FMLP (10⁻⁸ mol/L) plus DHCB (10 μmol/L) pretreatment (data not shown). P-II₁, B-II₂, and B-II₃ have also received prolonged IFNγ therapy, and although they have had a clinical response, the percentage of NBT-positive neutrophils, O₂ production, and cytochrome b₅₅₈ levels have not changed. Although some selected patients with X-linked variant CGD may have enhanced O₂ production after IFNγ administration, the results support other studies suggesting that this is probably not the mechanism of action of IFNγ in most CGD patients.

Cytogenetic studies. Another potential explanation for the subset of NBT-positive phagocytic cells in these patients is the inheritance of an X chromosome mosaicism. For example, a variant Klinefelter’s syndrome (46, XY/47, XXY) or an unexpected 46, XX karyotype could give rise to a normal male phenotype and the inheritance of either two maternal X chromosomes or one maternal and one paternal X chromosome. Through random inactivation of X chromosomes, clonal expression of X-linked genes could occur in the patient’s neutrophils, leading to two populations of cells with different respiratory burst capabilities.

Cytogenetic analysis of peripheral blood lymphocytes (P-II₁, B-II₂, and B-II₃) and cultured fibroblasts (P-II₁) showed normal male karyotypes (46, XY) in each case. Because cytogenetic analysis was limited to nonmyeloid cells, we also examined two informative X chromosome RFLPs in family P. This procedure was performed to exclude pseudodiploidy of a mosaic state limited to myeloid cells that could result in random inactivation of X chromosomes in the patient’s (P-II₁) neutrophils. Southern blots of genomic DNA from peripheral blood (ie, primarily granulocytes) showed only one allele for each polymorphism in the P-II₁. The distribution of alleles for the X-CGD-linked probe 754, for members of family P is shown in Fig 7. Longer exposure films of this filter, as well as Southern blots hybridized to the non-CGD-linked probe S-232, also showed no evidence of any additional chromosome-derived DNA other than that from the single maternal chromosome. Thus, neutrophils from patient P-II₁ contained X chromosome DNA derived only from the maternal X chromosome bearing the CGD mutation, with no detectable DNA from either the paternal or other maternal X chromosome.

DISCUSSION

In this study, we describe the respiratory burst characteristics of three patients from two kindreds with a unique phenotype of variant CGD. Phagocytic cells from all three patients showed neutrophil O₂ production, O₂ consumption, and cytochrome b₅₅₈ levels that were all ≈5% to 15% of normal. Studies with the cell-free activation system confirmed the presence of a respiratory burst defect in the membrane fraction of the patients’ neutrophils. Furthermore, the cell-free membrane activity was comparable with the spectral levels of cytochrome b and the intact neutrophil respiratory burst activity. An X-linked inheritance was established in both kindreds based on the identification of female relatives who were carriers for cytochrome b-deficient CGD.

The most novel aspect of these CGD patients is the presence of a small subset of circulating neutrophils and monocytes (5% to 10%) that are comparable with normal cells in their respiratory burst activity as shown by NBT reduction and flow cytometric analysis. The proportion of cells capable
of NBT reduction (6% to 12%) closely corresponds to the percentage of total normal O$_2$ production achieved by intact cells. Studies involving myeloid progenitor cells (peripheral blood CFU-GM) also showed a discrete population of cells with normal respiratory burst capabilities as determined by NBT reduction. The inheritance of a mosaic diploid X chromosome state as an explanation for the NBT-positive cells or the occurrence of an unstable oxidase complex as an explanation for the NBT-negative cells (P-I13, B-II2, B-I13) were both excluded. Together these results suggested the presence of two distinct clones of phagocytic cells, one of which is respiratory burst-competent.

Phagocytic cells from the majority of patients with X-linked CGD are unable to generate O$_2$ because of a complete deficiency of the gp91-phox subunit of cytochrome b.$^1$ Occasionally, patients with diminished but detectable, levels of O$_2$ production and cytochrome b have been reported and are referred to as variant X-linked CGD (X91$^-$$^1$).$^{1,18-24,43,46-48}$ The characteristics of the previously reported cases of variant X91$^-$ CGD, as well as the three patients described in this study, are summarized in Table 3. In almost all cases of X-linked variant CGD described so far, NBT staining has shown a homogeneous population of weakly positive neutrophils and monocytes. Thus, all of the phagocytic cells in these patients collectively contributed to the residual respiratory burst activity. In a subset of patients with variant X91$^-$ CGD, abnormal oxidase kinetics have been described: the Michaelis-Menten constant (Km) for NADPH has been reported several-fold higher (10 to 100) than normal with the Vmax varying between 5% and 70% of normal.$^{19,21,22}$ Although the exact relationship between the oxidase kinetics and levels of cytochrome b remains uncertain, studies from these variant patients do suggest that minimal amounts of cytochrome b may be adequate for some electron transport.$^3$

In contrast with the typical X91$^-$ CGD variants described above, the patients in this report each have a subset of NBT-positive phagocytic cells, the percentage of which is directly proportional to the measured respiratory burst activity (5% to 15%). Only one other patient has been reported with a similar, albeit less completely characterized, X-linked variant of CGD that resembles the three patients described in this study.$^{21}$ This patient also had a small subset of NBT-positive cells (16%) that corresponded to the measured intact cell respiratory burst activity (12% to 30%). Although a population of phagocytic cells with near-normal respiratory burst activity was suspected, more detailed studies to confirm

---

**Fig 6.** Distribution of NBT reduction in granulocyte-macrophage colonies from the indicated donors. Colonies of greater than 25 cells were counted and scored as positive (>90% of cells within the colony stained blue with reduced NBT; III), mixed (10% to 90% of cells reducing NBT; IV), or negative (<10% of cells reducing NBT; V). Each graph represents pooled results from the following number of experiments for each donor: P-II12, n = 4; B-II2, n = 4; B-III13, n = 2; normal, n = 6.

---

**Fig 7.** Southern blot of genomic DNA isolated from neutrophils of patient P-II12 and other members of family P. Hybridization was done with the RFLP probe 754, which is closely linked to the X chromosome CGD locus.$^{30}$ Each lane contains DNA from the individual indicated in the overlying pedigree. The lanes show (from left to right) patient's hemizygous father (P-I1); heterozygous CGD carriers, mother (P-I1) and sister (P-II1); unaffected hemizygous brother (P-II13); and the proband (P-II12), hemizygous for the CGD-associated RFLP allele. No trace of the higher molecular-weight band is evident in the proband. Blotting and hybridization were performed as described in Materials and Methods. (W), unaffected male; (G), carrier female; (M), CGD
NEW X-LINKED VARIANT OF CYTO-B-DEFICIENT CGD

Table 3. Reported Cases of X-Linked Variant CGD

<table>
<thead>
<tr>
<th>No. of Patients</th>
<th>Age of Onset (yrs)</th>
<th>Visceral Infections</th>
<th>NBT Score (% positive)</th>
<th>Intact Cell O2 Production (% normal)</th>
<th>Cytochrome b Spectrum (% normal)</th>
<th>Oxidase Activation in Cell-Free System</th>
<th>Oxidase Kinetics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>4, 12, 14</td>
<td>3 Cases</td>
<td>5-10 (strong)*</td>
<td>5-15</td>
<td>5-15</td>
<td>Membrane defect</td>
<td>ND</td>
<td>This report</td>
</tr>
<tr>
<td>1</td>
<td>19</td>
<td>None</td>
<td>100 (weak)</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>22</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>None</td>
<td>100 (weak)</td>
<td>2</td>
<td>88</td>
<td>ND</td>
<td>ND</td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>None</td>
<td>16</td>
<td>12-30</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>&lt;1, 9, 15</td>
<td>None</td>
<td>100 (weak)</td>
<td>9-30</td>
<td>0-10</td>
<td>ND</td>
<td>ND</td>
<td>18, 43</td>
</tr>
<tr>
<td>3</td>
<td>3, 3, 18</td>
<td>1 Case</td>
<td>80 (weak)</td>
<td>13-35</td>
<td>8-50</td>
<td>ND</td>
<td>ND</td>
<td>23</td>
</tr>
<tr>
<td>1</td>
<td>89</td>
<td>None</td>
<td>100 (weak)</td>
<td>6</td>
<td>1</td>
<td>Membrane defect</td>
<td>ND</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>&lt;1, &lt;1, 2, 8, 12</td>
<td>1 Case</td>
<td>&gt;90 (weak)</td>
<td>0-24</td>
<td>7-65</td>
<td>Membrane defect</td>
<td>ND</td>
<td>24, 47</td>
</tr>
<tr>
<td>2</td>
<td>2, 3</td>
<td>1 Case</td>
<td>&gt;90 (weak)</td>
<td>5-10</td>
<td>14-17</td>
<td>Membrane defect</td>
<td>ND</td>
<td>48</td>
</tr>
<tr>
<td>Total 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

* Refers to intensity of NBT staining compared with control.

this (such as NBT reduction by CFU-GM colonies or flowcytometric analysis of circulating neutrophils) were not performed.

Many of the patients with variant X91- CGD have had a delayed presentation and a milder clinical course with infections limited to the skin, lungs, and lymph nodes.2,19,22 Presumably, this amelioration is caused by the residual respiratory burst activity in their phagocytic cells. Although the patients in this report have residual respiratory burst activity of 5% to 10% of normal, they have had visceral infections similar to that seen in patients with classical X91- CGD. It had previously been suggested that patients with variant X-linked CGD, characterized by a subset of phagocytic cells with normal respiratory burst activity, would have fewer and less severe infections.21

Rarely, X-linked carriers have been reported with only a very small population of NBT-positive cells (<10% of normal) because of extreme unbalanced X chromosome inactivation (Lyonization).23,24 Although the proportion of NBT-positive cells is similar to that seen in variant X91- CGD, these carriers are usually free of infections. This is consistent with our experience of more than 70 CGD patients and kindreds; from these individuals, only two X-linked carriers with extreme Lyonization (8% of cells NBT-positive) were identified and both were asymptomatic (unpublished observations, J.T. Curnutte, April 1994). To our knowledge there have been only two other X-linked carriers reported with extreme Lyonization (approximately 5% of their cells positive after NBT reduction) and both had mild infectious complications as a result of their reduced respiratory burst activity.25,26

The frequency and severity of infections amongst the X91- variant patients, the extremely Lyonized X-linked carriers, and the three patients described in this report vary considerably, even though these individuals collectively all have comparable levels of respiratory burst activity (5% to 15% of normal). Reasons for the discrepancies between the infectious complications and the respiratory burst capabilities in these patients are unclear. One potential explanation is that a critical threshold exists in which infectious complications are more likely to occur when respiratory burst activity falls below 5% of normal. It is also possible that other variables of host defense influence the clinical phenotype in CGD patients and are not predicted by the percentage of NBT-positive cells. In support of this, we have seen several families where more than one sibling has inherited CGD, each with identical amounts of respiratory burst activity and NBT-positive cells, yet they have an entirely different spectrum of infectious complications (unpublished observations). This latter explanation, combined with our earlier studies regarding the lack of effect of IFNγ on the oxidase in the majority of CGD patients, suggests that other aspects of immune function warrant further investigation in CGD patients.31

The precise molecular defect responsible for this unique variant of X91- CGD is currently under investigation. It is likely that new information regarding the structure and function of the gp91-phox gene will be uncovered as a result of these studies. Although variant forms of CGD are rare, they continue to provide important clues to our understanding of the oxidase complex of phagocytic cells.

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of Dr O. Jones and the University of California San Diego Cytogenetics Laboratory for performing the karyotypes; the San Diego Blood Bank for the Kell antigen testing; Sally Teo for preparation of the manuscript; and the General Clinical Research Center staff of Scripps Clinic and Research Foundation for their assistance.

REFERENCES


24. Roos D, de Bor M, Borregaard N, Bjerrum OW, Valerius NH, Seger RA, Muhlebach T, Belobravsky BH, Weening RS: Chronic granulomatous disease with partial deficiency of cytochrome b\textsubscript{558} and incomplete respiratory burst: Variants of the X-linked, cytochrome b\textsuperscript{-}negaive form of the disease. J Leuk Biol 51:164, 1992


A new X-linked variant of chronic granulomatous disease characterized by the existence of a normal clone of respiratory burst-competent phagocytic cells

RC Woodman, PE Newburger, P Anklesaria, RW Erickson, J Rae, MS Cohen and JT Curnutte