PHAGOCYTES

Missense Mutations in the gp91-phox Gene Encoding Cytochrome b\(_{558}\) in Patients With Cytochrome b Positive and Negative X-Linked Chronic Granulomatous Disease

By Mizuho Kaneda, Hitoshi Sakuraba, Akira Ohtake, Akira Nishida, Chika Kiryu, and Katsuko Kakinuma

Chronic granulomatous disease (CGD) is a disorder of host defense due to genetic defects of the superoxide (O\(_2^-\)) generating NADPH oxidase in phagocytes. A membrane-bound cytochrome b\(_{558}\), a heterodimer consisting of gp91-phox and p22-phox, is a critical component of the oxidase. This enzyme system consists of multiple factors including membrane-associated cytochrome b\(_{558}\) and cytosolic components for oxidase activation. The activity of this enzyme system consists of multiple factors including membrane-associated cytochrome b\(_{558}\) and cytosolic components for oxidase activation. The oxidase consists of several proteins: gp91-phox, p22-phox, and a 22-kD light chain, p22-phox. CGD is now known to be caused by deficiency of any of these proteins: gp91-phox, p22-phox, p47-phox, and p67-phox, respectively. Several mutations of gp91-phox with X-linked recessive CGD have been reported; deletional, splicing, missense, nonsense, and duplicational mutations.

Case reports.

Three male patients, a 10-year-old (T.H.), a 19-year-old (T.M.), and a 25-year-old (J.O.), all had recurrent episodes of bacterial infection and were diagnosed as having X-linked recessive CGD by functional analysis of their neutrophils' cytochromes at the age of 6 months, 5 years, and 6 years, respectively. Patients T.H. and J.O. had received operations for liver abscesses before the diagnosis of CGD. Although they often suffered from perianal abscesses, inguinal lymphadenitis, stomatitis, and skin abscesses, they were relieved of severe infection by administration of sulfamethoxazole-trimethoprim.

Materials.

Ficoll-Paque and Dextran T-500 were purchased from Pharmacia Biotech, Uppsala, Sweden; diisopropyl fluorophosphate (DFP) was from Wako Pure Chemicals, Osaka, Japan; Sodium-p-tosyl-L-lysine chloromethyl ketone (TLCK), zymosan, and phorbol 12-myristate 13-acetate (PMA) were from Sigma, St Louis, MO; phenylmethylsulfonyl fluoride (PMSF) was from Nakarai Co, Kyoto, Japan. All other reagents were of analytical grade. Reagents for the analysis of RNA and DNA are described below. Oposazymosan (op-zy) (20 mg/mL) was prepared as described previously. PMA was dissolved in dimethyl sulfoxide (20 mg/mL) and diluted with Ca\(^2+\)-free Krebs Ringer phosphate buffer [KRP: 122 mmol/L NaCl, 4.9 mmol/L KCl, 1.2 mmol/L NaHCO\(_3\), 1.45 mmol/L NaH\(_2\)PO\(_4\), 5.6 mmol/L glucose, 25 mmol/L KCl, and 1 mmol/L CaCl\(_2\)].

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mmol/L MgCl₂, 17 mmol/L sodium phosphate buffer (pH 7.4) to 20 µg/mL before use.

Isolation of neutrophils. Neutrophils from the three CGD patients, their parents, and healthy controls were collected from 10 to 20 mL samples of heparinized venous blood by Dextran sedimentation, followed by hypotonic treatment for lysis of red blood cells, and then stepwise separation with Ficoll-Paque according to Böyum’s method, with some modifications. The cells, consisting of 98% to 99% neutrophils and less than 1% eosinophils, were suspended in ice-cold 
Ca²⁺-free KRP before use. All donors gave their informed consent.

Assay of cellular O₂⁻ formation. Neutrophils were suspended in a narrow-cuvette at 1.0 to 2.0 × 10⁶ cells/700 µL in KRP containing 5 mmol/L glucose and 0.6 mmol/L CaCl₂ and were continuously stirred with a wind-mill cell mixer for the assay of O₂⁻ release from stimulated cells. The rates of O₂⁻ formation by neutrophils from donors in the presence of PMA (0.14 µg/10⁶ cells) or op-zy (0.4 mg/10⁶ cells) were measured at 37°C by recording superoxide dismutase inhibitable-reduction of ferricytochrome c at the absorbance difference of 550 to 540 nm in a Hitachi dual wavelength spectrophotometer, model 557 (Hitachi, Tokyo, Japan).

Preparation of membrane and cytosol fractions. Neutrophils from each donor were treated with 2 mmol/L DFP on ice and were suspended in sonication buffer consisting of 1 mmol/L PMSF, 1 mmol/L TLCK, and 20 mmol/L sodium phosphate buffer (pH 7.4) at a final concentration of 2 × 10⁶ cells/mL. Aliquots of the cell suspensions were placed in microtubes and sonicated in a Bioruptor, model UCD-200TM (Cosmo-Bio, Tokyo, Japan) at 0°C, setting its controller at an optimal sonication time of 1.5 seconds with intervals of 1 second. After sedimentation of cell debris and nuclei at 4000 x g for 10 minutes, the supernatants were centrifuged at 2.3 × 10⁵ g for 15 minutes at 2°C in a table top ultracentrifuge (Beckman model TL-100; Beckman, Fullerton, CA) to separate the membrane and cytosol fractions as precipitates and supernatants.

Immunoblot analysis. Rabbit polyclonal antibodies were raised against synthetic polypeptides corresponding to the COOH-terminal peptides (17 residues) of p22-phox and gp91-phox of cytochrome b₅₅₈ of human neutrophils. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli with 4% stacking gel and 10% or 11% separating gel. Membrane electrophoresis (SDS-PAGE) was performed as described by Laemmli of human neutrophils. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli with some modifications. The cells, consisting of 98% to 99% neutrophils and less than 1% eosinophils, were suspended in ice-cold 
Ca²⁺-free KRP before use. All donors gave their informed consent.

RESULTS

Neutrophils from the two patients (T.M. and J.O.) had no O₂⁻ generating activity, whereas those from the other patient (T.H.) exhibited slight O₂⁻ generation (6% to 7% and 8% to 9% of the control activities on stimulation with PMA and op-zy, respectively).

Figure 1 shows traces of O₂⁻ generation by phagocytosing cells from the patient (T.H.) and his mother and father. The rates were 3.9 ± 0.7, 25 ± 1 and 44 ± 3 mmol O₂⁻/minute/10⁷ cells (n = 3), which were 8.8%, 55%, and 100% of the control value, respectively.

Figure 2 shows immunoblots of the membrane fractions from neutrophils of the CGD patients. Two patients (T.M. and J.O.) had no p22-phox or gp91-phox (Fig 2A and C), characteristic of typical X-linked recessive CGD, whereas the membranes from the variant CGD patient (T.H.) had small amounts of both p22-phox and gp91-phox (Fig 2B). The glycosylated heavy chain, gp91-phox, showed broad electrophoretic migration, and so the stained band appeared faint, but detectable in the gel, in contrast to its complete absence in the two typical cases of CGD. Densitometric analysis of the stained bands of the variant CGD showed the presence of p22-phox and gp91-phox at 14% and 13% of the control, respectively (Table 1).

Figure 3 shows the reduced minus oxidized absorption difference spectra of neutrophils from a typical CGD patient; J.O. (Fig 3A), the variant CGD patient; T.H. (Fig 3B), and a healthy donor (Fig 3C). The spectrum of the variant CGD (Fig 3B) showed a small peak at 558 nm of cytochrome b, in contrast to the complete absence of the peak in the typical CGD (Fig 3A). The large absorption band of myeloperoxidase with a peak at 475 nm gave no evidence for the presence of eosinophils, because eosinophil peroxidase gives a distinct peak at 453 nm.
causing a shoulder even if the preparation is contaminated with only a few percent of this cell fraction. Indeed, the cell fraction from the variant CGD patient contained 99% neutrophils, but undetectable eosinophils. Figure 4 shows the reduced minus oxidized difference spectra of neutrophils from the variant CGD family: father (F), mother (M), and the patient (P). The spectra in the α-band region were five times enlarged (inset of Fig 4). The spectra obtained (Fig 4) were differentiated as \( \frac{d(\Delta A)}{d\lambda} \), where \( \Delta A \) is the absorption difference and \( \lambda \) is the wavelength, as shown in Fig 5. The differential spectrum of the variant CGD (T.H.) shows a small peak in the range of 550 to 560 nm in contrast to that of typical cases of X-CGD (J.O. or T.M.), which show only a trough, but no peak, as shown in the inset. The differential spectra gave further evidence for the indication of the presence of heme in the spectrum of the variant CGD. The heme contents of samples of the variant P, and the M and F were about 12%, 48%, and 100% of the control value, respectively.

The protein coding regions of overlapping fragments of gp91-phox cDNA from CGD patients, carriers, and healthy controls were amplified by PCR and the products were subjected to sequence analysis. All three patients showed normal sized fragments on an agarose gel (data not shown). The sequence of patient T.M. showed a point mutation at bp 1558 of thymine to cytosine, predicting an amino acid substitution at residue 516 of Trp to Arg. In the sequence of patient T.H. with variant CGD, a point mutation at bp 937 of guanine to adenine was found, predicting an amino acid substitution at residue 309 of Glu to Lys. The sequence of patient J.O. showed a point mutation at bp 1271 of thymine to cytosine, predicting a Leu to Pro conversion at amino acid position 420.

To confirm the mutations found, genomic DNA fragments containing these mutated sites were amplified by PCR and then analyzed by means of restriction digestion or direct sequencing. As expected, the PCR products from the patients and their carriers had the predicted sizes on the gel. Incubation of exon 12 of patient T.M. with MspI resulted in fragmentation into two pieces: distinct 84 bp and weak 40 bp bands (Fig 6, lane 2) in contrast to the two controls, which gave a single band of 124 bp (lanes 1 and 4 in Fig 6). Exon 12 of the mother of T.M. (Fig 6, lane 3) showed partial fragmentation into weak 84 and 40 bp bands, providing evidence for the carrier state. The restriction enzyme TaqI did not digest the PCR product of exon 9 of patient T.H. (Fig 7, lane 2), but completely digested those of exon 9 of his father and a healthy control to yield a 228 bp product (with slight 26 bp) (Fig 7, lanes 1 and 4). The PCR product of his mother showed partly digested products as evidence of the
carrier state (Fig 7, lane 3). The PCR products of exon 10 of patient J.O. and his mother were directly sequenced because no available restriction enzyme was found for the mutated site. The T1271 to C substitution of patient J.O. was identified in the read sequence of exon 10, and the mother was heterozygous for the mutation (data not shown).

We identified missense mutations in the gene encoding gp91- phox in the three patients with X-linked CGD, as summarized in Table 1. In addition to the three families, we recently reported another variant CGD (patient T.K.) in which neutrophils have similar amounts of both p22-phox and gp91-phox, but show no O₂ forming activity or the heme spectrum of cytochrome b⁵₅₈ in contrast to the present variant CGD (T.H.). The mutated site in patient T.K. was found at residue 101 causing replacement of His by Tyr, probably one of the heme binding residues for bis-histidine coordination of cytochrome b⁵₅₈. In the present study, the predicted mutated site, H101Y, was confirmed at a position in exon 4 with the PCR product of genomic DNA, as shown in Table 1.

### Table 1. Gene Mutations and Heme Spectra of the Patients With CGD

<table>
<thead>
<tr>
<th>Patient</th>
<th>O₂ Generating Activity</th>
<th>Exon</th>
<th>Nucleotide Change</th>
<th>Predicted Amino Acid Change</th>
<th>Immunoblot-cyt b</th>
<th>Heme Spectrum 558 nm</th>
<th>CGD Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>T.M.</td>
<td>-</td>
<td>12</td>
<td>T1558 = C</td>
<td>Trp516 = Arg</td>
<td>-</td>
<td>-</td>
<td>X91°</td>
</tr>
<tr>
<td>T.H.</td>
<td>±</td>
<td>9</td>
<td>G937 = A</td>
<td>Glu309 = Lys</td>
<td>±</td>
<td>±</td>
<td>X91°</td>
</tr>
<tr>
<td>J.O.</td>
<td>-</td>
<td>10</td>
<td>T1271 = C</td>
<td>Leu420 = Pro</td>
<td>-</td>
<td>-</td>
<td>X91°</td>
</tr>
<tr>
<td>T.K.</td>
<td>-</td>
<td>4</td>
<td>C313 = T</td>
<td>His101 = Tyr</td>
<td>±</td>
<td>±</td>
<td>X91°</td>
</tr>
<tr>
<td>T.K.*</td>
<td></td>
<td>4</td>
<td>C313 = T</td>
<td>His101 = Tyr</td>
<td>±</td>
<td>±</td>
<td>X91°</td>
</tr>
</tbody>
</table>

Abbreviations: detectable, (+); undetectable, (−); slightly detectable, (±); α (p22-phox) and β (gp91-phox) subunits of cytochrome b⁵₅₈ (cyt b).

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Fig 3. Reduced minus oxidized difference spectra of neutrophils from patient J.O. (a), patient T.H. (b) and a healthy control (c). Neutrophil sonicates (6 × 10⁶ cells/250 μL) were treated with CO and then the reduced minus oxidized difference spectra were measured. The inset shows the α-band region enlarged 5.4-fold.

Fig 4. Reduced minus oxidized difference spectra of neutrophils from variant CGD patient T.H. (P) and his mother (M) and father (F). The inset shows the α-band region enlarged fivefold. The difference spectra were measured as in Fig 3.
DISCUSSION

All four missense mutations are illustrated in Fig 8 with the predicted domains. Sequence analyses of the two typical cases of X-linked CGD (T.M. and J.O.) showed novel missense mutations; W516R and L420P, respectively. The absence of the heme spectrum (X910) in the two patients suggests that a replacement at residue 420 or 516 causes a very unstable gp91-phox protein, leading to the complete absence of cytochrome b558 in the membranes. Residue 420, where Leu is replaced by Pro in patient J.O. is involved in the predicted NADPH-binding domain.19-21 The substitution of a positively charged Arg for the nonpolar hydrophobic residue Trp at residue 516 might alter the folding properties of the peptide backbone.

In the other variant CGD patient (T.H.), the missense mutation at residue 309 is interesting, having common characteristics with two other patients with variant CGD reported previously, showing the same replacement of Glu by Lys.22 This conservative substitution of a positively charged residue for a negatively charged one might alter the ionic properties of the peptide, whereas all E309K cases have a small amount of cytochrome b558 and a low level of O2 generating oxidase activity. This fact suggests that residue Glu 309 and its adjacent residues in gp91-phox are neither involved in the domain forming a stable complex with p22-phox nor in the domain holding flavin adenine dinucleotide (FAD) because the conversion of charged residues is critical for electron and proton transfer reactions. Finegold et al23 suggested from the similarity in residues between the yeast iron reductase (FRE1) and gp91-phox that the corresponding residues coordinating heme may be two pairs of histidines (101 and 115; 209 and 222) buried in the separate hydrophobic domains. However, there is no direct evidence for
the heme-binding ligands in gp91-phox except our results on the heme spectra of phagocyte cytochrome b<sub>558</sub> from the two variant CGD patients. From the differential spectra, it is possible to distinguish between the presence and absence of heme, even in the presence of a minute amount of cytochrome b<sub>558</sub> from CGD patients. Neutrophils from the variant CGD with a novel mutated site at His<sup>101</sup> showed a similar differential spectrum<sup>11</sup> to those of typical X-linked CGD patients whose phagocytes have no subunits and show no heme spectrum (Fig 5, inset). In contrast, the present variant CGD showed a distinct differential spectrum with a peak in the left of the α-band (550 to 560 nm) (Fig 5), indicating the presence of the heme of cytochrome b<sub>558</sub>, which may account for the O<sub>2</sub> forming oxidase activity. Comparative studies on these heme spectra confirmed that one of the heme binding histidines is His<sup>101</sup>. Bolscher et al<sup>24</sup> reported a typical X-linked CGD patient with a point mutation at the same His<sup>101</sup> to arginine in gp91-phox, suggesting that this part is important in binding of the heme or for formation of a stable complex with p22-phox. Although patients with many other mutations of histidine residues at the N-terminal have been reported,<sup>6,9</sup> most of these patients had typical X-linked CGD and their phagocytes had neither gp91-phox nor p22-phox, so the heme binding ligands could not be identified by spectrophotometry.

In addition to immunoblot analyses, we detected the gp91-phox protein in neutrophils from the two variant CGD patients (T.H. and T.K.) by flow cytometry of the cells using monoclonal antibody 7D5<sup>25</sup> (data not shown), which has recently been identified as an anti-gp91-phox antibody (M. Nakamura, personal communication, March 1998). Yu et al<sup>26</sup> have recently been investigating the biosynthesis of cytochrome b<sub>558</sub>. Their results suggest that heme incorporation is important in the assembly of cytochrome b<sub>558</sub> to form a stable complex of gp91-phox and p22-phox. Consistent with their report, in the present variant CGD (T.H.), the proteins of both subunits and the heme in the cytochrome b<sub>558</sub> were closely associated, as shown by immunoblots and spectra. In contrast, the other variant CGD (T.K.) showed similar amounts of the two subunits, but no heme.<sup>11</sup> The point mutation at residue 101 with replacement of His by Tyr is probably similar to FRE1 mutants that express FRE1 protein, but do not show a heme spectrum.<sup>23</sup>

Previous electron spin resonance (ESR) studies on neutrophil cytochrome b<sub>558</sub> provided indirect evidence that His<sup>101</sup>, located in the vicinity of Cys<sup>85</sup> in a cytochrome P<sub>450</sub>-like alignment (residues 78-85: FLRGSSAC<sup>85</sup>), is the fifth (proximal) heme binding ligand, and the sixth (distal) ligand may be His<sup>210</sup>, because pyridine treatment of cytochrome b<sub>558</sub> changed the ESR signal of the bis-histidine coordination to a cytochrome P<sub>450</sub>-like signal, possibly through the replacement of His<sup>101</sup> by the nearby residue Cys<sup>85</sup>.<sup>27</sup> As shown in Fig 8, Cys<sup>85</sup> may be adjacent to His<sup>101</sup> in the two transmembrane regions because a cytosolic loop of gp91-phox (residues 87-94: STRVRRQL) is present variant CGD (T.H.), the proteins of both subunits and the heme in the cytochrome b<sub>558</sub> were closely associated, as shown by immunoblots and spectra. In contrast, the other variant CGD (T.K.) showed similar amounts of the two subunits, but no heme.<sup>11</sup> The point mutation at residue 101 with replacement of His by Tyr is probably similar to FRE1 mutants that express FRE1 protein, but do not show a heme spectrum.<sup>23</sup>

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The results of comparative studies on the heme spectra of the two variant CGDs support the prediction from previous ESR studies. Because near-infrared magnetic circular dichroism (NIR-MCD) spectra demonstrated the presence of two distinct forms of the heme with bis-histidine coordination,<sup>29</sup> another pair of heme-binding histidines may be present, probably within a short distance (4 to 5 Å) from the outer surface,<sup>30</sup> close to the glycosylation sites<sup>31</sup> (Fig 8).

ACKNOWLEDGMENT

We thank Dr M. Nakamura (Institute of Tropical Medicine, Nagasaki University, Nagasaki) for supplying monoclonal antibody 7D5.

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