Severe Clinical Forms of Cytochrome $b$–Negative Chronic Granulomatous Disease (X91$^{-}$) in 3 Brothers with a Point Mutation in the Promoter Region of CYBB

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Chronic granulomatous disease (CGD) is a rare congenital syndrome that results in severe, recurrent bacterial and fungal infections. The most common form is caused by defects in the CYBB gene, leading to the absence of gp91phox associated with totally abolished NADPH oxidase activity (X91$^{-}$ CGD). We report 3 brothers with atypical cases of X-linked CGD, characterized by low levels of expression of gp91phox (X91$^{-}$ CGD). A point mutation (T-55C) identified in the CYBB gene’s promoter region appears to prevent the full expression of this gene in neutrophils. This results in low levels of expression of gp91phox protein that are correlated with residual oxidase activity in the whole population of neutrophils. The total $O_2^-$ production in these cells was $\sim$5% of normal. Despite this oxidase activity, the patients experienced severe and life-threatening infections. It was concluded that the $O_2^-$ production in the neutrophils of these patients was not sufficient to protect them against infections, and this X91$^{-}$ CGD phenotype must be considered to be a severe clinical form of CGD.
deep organs (lung, liver, bone, and brain) are frequent and are difficult to fight [13]. The majority of affected individuals receive a diagnosis early in life, but, in some rare cases, CGD is discovered during adulthood, when clinical signs are not severe and appear infrequently [14, 15].

CGD is a very heterogeneous genetic disease caused by a large variety of mutations—such as deletions and splice-site, missense, or nonsense mutations— localized in the 4 genes encoding NADPH oxidase components, with no “hot-spot” localization, except for the NCF1 gene encoding p47phox protein [16]. There are 2 types of CGD transmission that make up autosomal forms (mutations in genes encoding p47phox, p67phox, or p22phox proteins) and the most common (60% of CGD cases) X-linked CGD type (defects in CYBB encoding gp91phox). The CYBB gene encompasses 13 exons spanning ~30 kb of human X-chromosome genomic DNA. In the majority of cases, cytochrome b558 is absent and no oxidase activity can be detected (X91+) [16–21]. A few rare cases of mutations in CYBB (<10%), resulting in low levels of expression of cytochrome b558 have been identified (X91−) [22–30]. In some cases, X91− CGD results in weak oxidase activity detected in the entire population of neutrophils, weak activity that is related to low levels of expression of cytochrome b558 [25, 28, 29]. Only 4 different mutations in the promoter region of CYBB that are related to CGD disease have been described [23, 26, 27, 30]. Mutations C-52T and C-53T have been reported to prevent the expression of gp91phox in neutrophils of patients with CGD and leave it intact in their eosinophils, which retained oxidase capacity [30]. This probably explains the relatively mild course of these CGD types. However, X91− CGD due to T-55C or A-57C mutations, which are characterized by a small subset (5%–10%) of neutrophils expressing a near normal level of oxidase activity, have been described as severe clinical forms [23, 24].

In the 3 brothers described here, the neutrophil population had a diminished but measurable level of NADPH oxidase activity (3%–9% of normal). Neutrophils from these patients showed a homogeneous population, with slight O2− production related to low levels of expression of cytochrome b558 (X91− CGD). We have examined the molecular defect in the 3 brothers, who have “variant” X-linked CGD. No mutations were found in the reverse-transcriptase (RT) polymerase chain reaction (PCR)–amplified fragments from gp91phox mRNA after sequence analysis. By amplification and sequencing of the corresponding genomic DNA, a point mutation was identified in the promoter region of CYBB, changing thymidine to cytosine at position −55 (T-55C). This mutation is located between the “CCAAT” and the “TATA” boxes in a consensus binding site for the ets family of transcription factors of the gp91phox promoter responsible for CYBB transcription [23]. Despite the low levels of expression of gp91phox protein and the residual NADPH oxidase activity in phagocytes, the clinical appearance of CGD in these 3 patients was severe, with multiple life-threatening infections occurring during their lives.

**PATIENTS, MATERIALS, AND METHODS**

**Patients.** The pedigree of family S is depicted in figure 1. Patient A was born in May 1976. Diagnosis of CGD was made when his brother, patient B, received a diagnosis. Patient A exhibited no complications of his immune defect until he was 11 years old. He first experienced an episode of bacterial pneumonia and then multiple hepatic abscesses that were successfully treated by antibiotics (1 g of oxacillin 4 times daily and 300 mg of rifampin twice daily). *Staphylococcus aureus* was involved in these 2 infections. Surgical treatment was required for new hepatic abscesses when he was 18 years old, and *Klebsiella pneumonia* was isolated from the pus; 2 g of ceftriaxone was administered intravenously (iv) 4 times daily with 500 mg of ciprofloxacin orally twice daily, for 2 months. Today, patient A is well and is undergoing prophylactic antibiotic and antifungal therapies (160 mg of cotrimoxazole and trimethoprim twice daily and 400 mg of itraconazole daily).

Patient B was born in April 1978. Diagnosis of CGD was made when he was 8 months old. He had staphylococcal wrist arthritis. He previously had had recurrent carbuncles. Then he had minor infections, including repeated adenitis, bronchopneumonia, dental abscesses, and recurrent mouth ulcerations, but hospitalization was not required. The first major infection...
occurred when he was 18 years old. It was a primitive femoral osteitis, which was treated with a single antibiotic for 8 months (200 mg of ofloxacin orally twice daily and 600 mg of rifampicin twice daily). Two years later, he had hepatic abscesses that required drainage, needle puncture, and antibiotic therapy (4 g of piperellin-tazobactam three times daily and 200 mg of ciprofloxacin iv twice daily). Interferon (IFN)–γ–1b was added to the antibiotic therapy (80 μg subcutaneously 3 times/week) over the next year. Recently (at 21 years old) he developed a disseminated candidiasis, with cutaneous localizations and positive blood cultures with liver and spleen involvement. Although this severe episode was controlled by fluconazole (800 mg iv, the first few days, followed by 400 mg orally), a bacteremia due to Salmonella typhimurium occurred a few weeks later. Antibiotic therapy (200 mg of ofloxacin twice daily) was added to the previous antifungal therapy, and IFN-γ was used again at the same dosage. Today, patient B is well and is undergoing prophylactic antibiotic and antifungal therapies associated with IFN-γ.

Patient C was born in April 1979 and has a more severe medical history than his brothers did. Because he was the youngest child, diagnosis of CGD was made during the first week of life by a nitroblue tetrazolium (NBT) test. He showed early and frequent mouth ulcerations and adenitis. At 7 years old, he developed a viral meningomyelitis due to complications of Epstein-Barr virus (EBV) infection. The outcome was positive and free of sequelae. Five years later, he had severe pneumonia due to Aspergillus fumigatus. He was treated with amphotericin B (50 mg iv daily) for 6 months. This severe fungal pneumonia was followed by multiple hepatic abscesses (S. aureus) requiring surgical excision. New hepatic abscesses occurred 1 year later (Serratia marcescens), which were controlled by treatment with a single antibiotic (2 g of ceftriaxone iv four times daily with 500 mg ciprofloxacin orally twice daily, during a 3-month period). Today, he shows a delayed consolidation of a leg fracture and is receiving 160 mg of cotrimoxazole and trimethoprim twice daily and 400 mg of itraconazole daily, as a prophylactic regimen.

As seen in figure 1, their grandmother (I2) and mother (I2) are carriers of the disease, according to their intermediate values on the NBT test. The maternal aunt of the 3 patients (I3) is under investigation; she is 45 years old and has no children. Their 2 maternal uncles (II4 and II5) have always been in good health. Patient A has a daughter (IV1) who is a carrier of CGD, and patient C has a healthy, oxidase-normal son (IV2) (see family S). The pedigree of family S is shown in figure 1. Nos. in parentheses are percentage of control values, unless otherwise noted. CGD, chronic granulomatous disease; ND, not done.

### Materials

Phorbol 12-myristate 13-acetate (PMA), formyl methionyl leucyl phenylalanine, and cytochrome c (horse’s heart, type VI) were purchased from Sigma Chemical. Diisopropyl fluoroephosphate was purchased from Sigma-Aldrich. NADPH, avian myeloblastosis virus RT, and Taq DNA polymerase were obtained from Roche Molecular Biochemical. Reagents and molecular weight markers for SDS-PAGE were obtained from Amersham. Nitrocellulose sheets for Western blotting were purchased from Bio-Rad Laboratories. Trizol reagent was obtained from Life Technology. Monoclonal antibodies (MAbs) 449 and 48 were provided by D. Roos and A. J. Verhoeven. Phycoerythrin (PE)–conjugated MAb against CRTH2 receptor was a gift from G. Bouvier and C. Canino (Beckman Coulter).

### Cell preparations

Human neutrophils and mononuclear cells (lymphocytes plus monocytes) were isolated from 25 mL
of citrated or heparinized blood obtained from the patients, their parents, and healthy donors, as described elsewhere [31], after their informed consent was obtained. Lymphocytes from 5 mL of heparinized sterile venous blood were collected by Ficoll-Hypaque density gradient centrifugation and were infected with the B95–8 strain of EBV, as described elsewhere [32]. The EBV–B lymphocyte cell line was grown in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 50 μg/mL streptomycin, at 37°C in a 5% CO2 atmosphere.

**NBT reduction and phagocytosis slide test.** The phagocytic capacity and the O2•− production of the neutrophils from the patients, their relatives, and healthy donors were estimated by NBT reduction and opsonized latex bead phagocytosis slide tests [33]. Also, cells were activated with PMA.

**Superoxide assay.** NADPH oxidase activity of intact neutrophils was assessed by measuring the rate of superoxide-sensitive cytochrome c reduction at 550 nm (ε 550 = 21.1 mmol/L−1 × cm−1), in the presence of superoxide dismutase (SOD) [32].

**Flow cytometric detection of peroxide in purified granulocytes.** To evaluate cellular hydrogen peroxide levels, dihydro-}

**RESULTS**

**Diagnosis of CGD for patients A, B, and C.** Diagnosis of CGD for the 3 brothers (patients A, B, and C) was established by use of an opsonized latex bead and NBT reduction test. Patient C's neutrophils phagocytosed normally opsonized latex beads (figure 2C) but had very weak staining in presence of...
Figure 2. Opsonized latex bead phagocytosis and nitroblue tetrazolium (NBT)-reduction slide test in patients with chronic granulomatous disease (CGD). Purified neutrophils ($1 \times 10^6$ cells) from a patient with X910 CGD (A), from a healthy donor (B), and from patient C (D) were stimulated with opsonized latex beads, in the presence of NBT dye, as described in Patients, Materials, and Methods. The percentage of neutrophils that phagocytose the latex beads and reduce the NBT were counted by microscopy. Neutrophils from patient C were stimulated with opsonized latex beads without NBT (C). The same test was performed with neutrophils from patients A and B (data not shown). Arrows indicate violet formazan precipitates.

NBT (figure 2D). Each cell had only a few grains of violet formazan precipitate, rather than the heavy deposits seen in those from the healthy donor (figure 2B and 2D). Patient C’s neutrophils had a uniformly weak staining, unlike the absence of NBT reduction in the more common form of X-linked CGD (X910) (figure 2A and 2D). The same result was observed when cells were stimulated with PMA. Similar results were obtained from patients A and B (data not shown). The result of the NBT slide test on their mother’s neutrophils (60% of her neutrophils intensively reduced the NBT with normal phagocytosis) confirmed that she is a carrier of the disease. This mode of inheritance supported an X-linked mutation, because the daughter of patient A (IV1) is a carrier, and the son of patient C (IV2) is normal. Purified neutrophils from their father have normal phagocytosis of opsonized latex beads and NBT reduction, compared with the healthy donor’s neutrophils (table 1).

To confirm the defect in $O_2^-$ production, NADPH oxidase activity was measured by the SOD-sensitive cytochrome c reduction test, in purified neutrophils from patients A, B, and C (figure 3). Neutrophils were stimulated with a soluble stimulus PMA (20 ng/mL), and the specificity of the reaction was evaluated by addition of SOD (10 μg/mL) at the end of the kinetic reaction. As seen in figure 3, the respiratory burst measured in the neutrophils of patients A, B, and C was very low (0.48, 0.33, and 1.1 nmol/min/10$^6$ cells, respectively), compared with the $O_2^-$ production in purified neutrophils from the healthy donor (11.8 nmol/min/10$^6$ cells). In contrast, no NADPH oxidase activity was detected in neutrophils from the patient with X910 CGD. This result is representative of 3–6 different experiments. The mean ± SD NADPH oxidase activity is shown in table 1. These values for the patients’ neutrophils represent 3%–9% of the total NADPH oxidase activity of control neutrophils.

Flow cytometric detection of hydrogen peroxide production in granulocytes was measured by the DHR oxidation assay (figure 4A). Patient C’s granulocytes (figure 4A, bottom) showed a unique activity peak after stimulation with PMA (20 ng/mL), with a mean fluorescence intensity of 33 arbitrary units (AU), compared with that for those from the healthy donor (1935 AU) (figure 4A, top). In our conditions of measurement, 70% of the healthy donor’s granulocytes showed maximal activation.
Identification of granulocyte population and membrane expression of cytochrome b$_{558}$ were checked by double-staining flow cytometric analysis (figure 4B). The specificity of the MAbs against the CRTH2 receptor was evaluated by a granulocyte preparation containing 20% eosinophils, determined by morphological criteria, obtained from a hypereosinophilic patient. No positive granulocyte population was detected in the patient with X91° CGD (4 AU) after PMA stimulation (figure 4A, middle).

For the patient with X91° CGD (figure 4B, bottom left), 2% of granulocytes expressing CRTH2 and gp91phox were characterized as eosinophils. Likewise, the level of eosinophils in patient C's granulocytes was very low (2%), making the detection, by flow cytometric analysis, of oxidase activity in these cells difficult (figure 4A, bottom). For the patient with X91° CGD (figure 4B, top right), this population, also representing 2% of granulocytes, was CRTH2° and 7D5°. No 7D5 binding was measured in neutrophils from the patient with X91° CGD related to the absence of cytochrome b$_{558}$ expression and O$_2^-$ production (figure 4B, top right). In the case of patient C (figure 4B, bottom left), despite the low but measurable levels of NADPH oxidase activity in his granulocytes, as shown above, gp91phox was not detected in the CRTH2° population (97%). The level of gp91phox protein expressed in each neutrophil was probably too low, making its detection impossible by flow cytometry. The same results were obtained from patients A and B (data not shown).

To demonstrate the presence of cytochrome b$_{558}$ in the patients' neutrophils, Western blot analysis and reduced minus-oxidized difference spectra were performed in 1% Triton X-100 soluble extract. As illustrated in figure 5A and table 1, difference spectra revealed a low level of cytochrome b$_{558}$ (2–5 pmol/mg proteins) in the neutrophils of patients A, B, and C, compared with the soluble extract from a healthy donor (110 ± 30 pmol/mg proteins). In contrast, the spectrum of X91° CGD neutrophils did not have the $\gamma$, $\beta$, and $\alpha$ peaks characteristic of cytochrome b$_{558}$. The cytochrome b$_{558}$ content in patients A, B, and C was 2%–5% of the normal neutrophils. The presence of a low level of cytochrome b$_{558}$ in the neutrophils of these patients was confirmed by the results of Western blot analysis that used an identical amount of 1% soluble extract in all samples (50 μg), as seen in figure 5B. We illustrated the low level of the heavy chain of cytochrome b$_{558}$ (gp91phox) and the light chain subunit $\alpha$ (p22phox), in the content of patients A, B, and C, compared with that in the healthy donor’s content. The specificity of MAbs directed against the 2 subunits was demonstrated by the absence of gp91phox and p22phox in the X° CGD–soluble extract, compared with that in the control neutrophils. A similar experiment was performed with antibodies directed against the cytosolic factors p67phox and p47phox, with an identical result occurring in all samples (data not shown). The above results suggest that the phenotype was compatible with a mutation located in CYBB encoding gp91phox, leading to a low level of expression of cytochrome b$_{558}$ as found in the X91° CGD type.

Genetic analysis of patients A, B, and C. RT-PCR and sequence analyses were performed to localize the mutation in the mRNA encoding gp91phox. The mRNAs of healthy donors and patients were reverse-transcribed into 3 overlapping cDNA fragments, with 3 pairs of oligonucleotide primers derived from the gp91phox cDNA, and were sequenced as described else-
Figure 4. Flow cytometric analysis of granulocytes from patients with chronic granulomatous disease (CGD). A, Hydrogen peroxide production. Dihydro-rhodamine (DHR)–loaded granulocytes (1 × 10⁶ cells) from a healthy donor (top), from a patient with X91⁰ CGD (middle), and from patient C (bottom) were incubated for 10 min at 37°C with PBS (white histogram) or with 20 ng/mL phorbol myristate acetate (shaded histogram), as described in Patients, Materials, and Methods. The fluorescence of the H₂O₂ oxidized DHR was measured by flow cytometry. B, Double staining of granulocytes with anti-gp91phox (7D5) and anti-CRTH2 receptor. The same granulocyte preparations as described in A were used for this double-staining experiment. Granulocytes (5 × 10⁶ cells) from a healthy donor (top left), a patient with X91⁰ CGD (top right), patient C (bottom left), and a hypereosinophilic patient with 20% of eosinophils (bottom right) were stained with a monoclonal antibody directed against gp91phox (7D5), combined with a fluorescein isothiocyanate-conjugated goat-F(ab′)² specific to IgG1 isotype and a phycoerythrin-conjugated anti-CRTH2 receptor. The granulocytes from patients A and B were analyzed by use of the same methodology (data not shown). This result represents 1 experiment of 3 experiments performed. AU, arbitrary units.

where [39]. No mutation in the cDNA of gp91phox protein was found. A part of the gp91phox promoter region in genomic DNA (CYBB) was amplified by PCR using the 2 primers described in Patients, Materials, and Methods. By use of sequence analysis, a point mutation was found to be localized at position −55, changing a thymidine to a cytosine in patients A, B, and C, as seen in figure 6A. This mutation is localized in a region between the “CCAAT” and the “TATA” boxes in a consensus binding site for the ets family of transcription factors of the gp91phox promoter responsible for CYBB transcription (figure 6B). Fifty control genomic DNA were checked, and no mutations were found for the father of patients A, B, and C.

Clinical appearance of CGD in patients A, B, and C. Details of the clinical history of the 3 brothers are described in case reports. Some characteristics of family S must be emphasized. The 3 brothers have always been treated in the same medical center. The medical histories of patients A, B, and C are comparable, including severe and recurrent pneumonia or hepatic abscesses. All 3 patients exhibited ≥1 life-threatening infection. Their CGD must be considered to be a clinically severe form of the disease.

DISCUSSION

Here, we have reported 3 cases of identical, rare X-linked CGD in which cytochrome b₅₅₈ is expressed at low levels, as measured by immunoblotting and difference absorption spectra, levels that are correlated with weak production of superoxide anion in neutrophils (X91⁻ CGD). That patient A has a daughter (IV1) with carrier status, as does his mother (II2), and that
patient C has a healthy son (IV1) confirms an X-linked transmission of the disease (figure 1 and table 1). X-linked CGD is the most common form of this disease (~60% of cases), and, generally, cytochrome b558 (including gp91phox and p22phox proteins) is absent from patients’ neutrophils, and NADPH oxidase activity is totally abolished (X910 CGD) [16]. Occasionally, variant forms of X-linked CGD, called X91/H11002 CGD, occur, in which low levels of cytochrome b558 and proportionately decreased oxidase activity appear in phagocytes. Mutations that are associated with this phenotype are usually located in exons of CYBB [25, 28, 29]. These variants are of interest because they result from structural disorganization, leading to an incomplete loss of protein, partial dysfunction, or both.

As seen in figure 2, phagocytes from patient C have a slight ability to reduce the NBT dye when they are stimulated with opsonized latex beads (figure 2D). The violet formazan coloration is very weak and is uniform, compared with normal phagocytes in which heavy deposits can be observed (figure 2B). It seems that proportionately decreased oxidase activity is expressed uniformly in all phagocytes. The same result was obtained from patients A and B when cells were stimulated with PMA instead of opsonized latex beads (data not shown). The NADPH oxidase activity in the neutrophils of patients A, B, and C is confirmed by the SOD-sensitive cytochrome c reduction assay, as seen in figure 3 and table 1. The respiratory burst of these patients’ neutrophils is 3%–9% of normal. Flow cytometric analysis of DHR oxidation in patient C’s neutrophils also confirms the presence of a homogeneous population with a low level of oxidase activity, as observed by the NBT reduction test. The mean fluorescence intensity is increased 10-fold when PMA is added and represents 2% of normal (figure 4A).

In the study by Woodman et al. [24], flow cytometric analysis of DHR oxidation was performed in granulocytes from patients with X910 CGD with −55 or −57 mutations characterized elsewhere [23]. They also obtained a homogeneous activated-neutrophil population with a mean fluorescence intensity 5-fold higher than that in resting cells; the neutrophils of a patient with X910 CGD had no oxidase activity. They focused on a 5%–10% granulocyte population with a variable fluorescence intensity (10²–10⁴ AU), which they considered to be neutrophils with normal oxidase activity. This subgroup of granulocytes had a high NBT reduction capacity when activated with PMA and perhaps were eosinophils. Unfortunately, the authors never mentioned the percentage of eosinophils in the granulocyte preparation. All the granulocytes of a patient with X910 CGD exhibited a visible NBT reduction, but, surprisingly, no reduction appeared in the granulocytes of a patient with X91/H11002 CGD, other than the subgroup of 5%–10% with normal oxidase activity.

As we know, NBT reduction is not absolutely specific to NADPH oxidase activity, and the visible background in the granulocytes of patients with X910 CGD may be due to a non-
Figure 6. Analysis of mutations in the CYBB gene. Polymerase chain reaction products were gel-purified and automatically sequenced by Genome Express, with forward and backward primers P1 and P2*, as described in Patients, Materials, and Methods. A, Point mutation detected in the gp91phox promoter region at position —55, changing a thymidine to a cytosine, for patients A, B, and C, compared with a control sequence. B, Schematic representation of part of the gp91phox promoter region, with localization of the point mutation described in A, the “CCAAT” and “TATA” boxes, and the ATG starting codon.
specific reduction. However, this raises the question of why this phenomenon does not occur in the case of patients with X91 CGD. Weening et al. [30] have demonstrated that these kinds of point mutations, which are found in the CYBB promoter region, prevented the expression of gp91phox in patients’ neutrophils and thus made these cells incapable of generating oxygen radicals but left the gp91phox expression and the function of eosinophil NADPH oxidase intact. In a later study, the percentage of eosinophils was elevated (20% and 26%) and may explain the mild clinical forms seen in their patients with CGD.

Yet different studies [49–51] have demonstrated that transcription factors GATA-1, GATA-2, and GATA-3 are implicated in eosinophil-specific regulation of gp91phox expression, but not in neutrophilic, monocytic, or B-lymphocytic cells. The composition of the granulocyte preparation from patient C was checked by double-staining flow cytometric analysis, to simultaneously assess cytochrome b558 and CRTH2 receptor of eosinophils (figure 4B). Granulocytes of patient C contain 2% eosinophils (the same result was obtained by morphological criteria), which are also revealed by the MAb 7D5. This low level does not explain the respiratory burst measured in these cells (9% of normal) (table 1).

Nevertheless, the presence of eosinophils in the granulocyte population has not been proven to be a consequence of the mutation in the gp91phox promoter, because the same low level of these cells (2%) is present in the granulocytes from patients with X91 and from patients with X91 CGD, as well as in those from the healthy donor. In the neutrophil population from patient C (CRTH2−), cytochrome b558 could not be detected, probably because the level of gp91phox expressed at the surface of each cell is low and is under the sensitivity limit of flow cytometry. Similar results were obtained with the granulocytes of patients A and B (data not shown). Yet cytochrome b558 is present in the 1% Triton X-100 soluble extract, as assessed by difference spectra and Western blot analysis. These 2 techniques make it possible to concentrate the cytochrome b558 content and to detect it in 10⁶ equivalent cells (or 50 μg of proteins). In the neutrophils of patients A, B, and C, the 2 subunits of cytochrome b558 are both expressed in low amounts (figure 5B). This can be explained by the fact that cytochrome b558 is a heterodimer of p22phox and gp91phox proteins, which are usually missing in A22 CGD as well as in X91 CGD, probably because these 2 subunits stabilize each other [52].

The genetic defect in CYBB encoding gp91phox protein in patients A, B, and C is a point mutation in the CYBB promoter region, localized by PCR amplification and sequence analysis (figure 6). The mutation is located at −55, in a region between the “CCAAT” and the “TATA” boxes in a consensus binding for the ets family of transcription factors of the gp91phox promoter site (5’-GAGGAAAT-3’, lower strand, −57 to −50 bp). To our knowledge, only 4 point mutations have been described in patients with CGD, corresponding to the −52, −53, −55, and −57 positions [23, 26, 27, 30]. In cotransfection experiments, Elf-1 and PU.1, members of the ets family of transcription factors abundantly expressed in myeloid cells, bind to the later ets binding sequence and transactivate the promoter [26, 27]. Interestingly, −57, −55 (as in patients A, B, and C), and −53 mutations strongly inhibit the binding of both factors, suggesting that these mutations reduce gp91phox promoter activity, leading to a low level of expression of gp91phox protein [26, 27]. These results suggest that Elf-1 and PU.1 contribute to direct the lineage-restricted expression of the gp91phox gene in phagocytes. Newburger et al. have described that −55 mutation leads to a decrease in gp91phox mRNA [23]. This phenomenon can explain the low levels of expression of gp91phox protein, levels that are correlated with a reduced NADPH oxidase activity in the patients’ neutrophils. Yet it is not clear how the binding of Elf-1 and/or PU.1 to this full consensus sequence (−57 to −50) can influence the transcription initiation by RNA polymerase II. Analysis of PU.1-associated factors should lead to additional mechanisms implicated in gp91phox gene expression. These X91 CGD with −57 and −55 mutations in the gp91phox promoter resulted in a clinically severe phenotype [23, 24].

According to the total NADPH oxidase activity measured in their neutrophils (3%–9% of normal), patients A, B, and C can be compared with X-linked carriers exhibiting the same amount of O2− production because of extremely unbalanced X-chromosome inactivation [53]. In general, these carriers are free from infections or have mild infectious complications, although some may display the full-blown expression of the disease. Despite the slight NADPH oxidase activity in their neutrophils, patients A, B, and C experienced a severe clinical form of CGD and presented multiple life-threatening infections in deep organs. These different phenotypes, which are associated with the same total NADPH oxidase activity in neutrophils, can be explained by the fact that, in X-linked carriers, 5%–10% of neutrophils exhibit normal respiratory burst, which normally can fight microorganisms and can protect carriers against infections. A comparable situation can exist in the patients with X91 CGD, described elsewhere [30], with a high level of eosinophils (>5%) exhibiting normal NADPH oxidase activity. Nevertheless, the compensatory mechanism by which the eosinophils are overproduced is unknown.

In the case of patients A, B, and C, each neutrophil has a very low level of NADPH oxidase activity (as shown by NBT reduction test and by flow cytometric analysis with DHR oxidation), and the O2− production is not sufficient to kill foreign microorganisms during infections. In a recent study, Reeves et al. [54] proposed a new killing mechanism of neutrophils mediated through activation of proteases by K+ flux. In a previous report, Talkevic et al. [55] showed that, despite normal neu-
trophil development and recruitment, mice deficient in the neutrophil granule serine proteases (elastase and cathepsin G) were susceptible to fungal infections. We can speculate that the superoxide production in the neutrophils of the patients with X91^− CGD described here is not sufficient to provoke influx of K^+ as a compensatory charge process that is responsible for the release and the activation of cationic granule proteins. Consequently, the killing of microorganisms by these proteases is defective. The mechanism by which proteases are activated in the neutrophils of patients A, B, and C are currently under investigation in our laboratory. This finding has also been illustrated in an article by Bu-Ghanim et al. [25] that describes 3 cases of X91^− CGD in patients with a missense mutation or small in-frame deletions. The killing of S. aureus by cells from 1 of the patients with CGD was markedly defective and was comparable with the levels observed in cells from a patient with X91^+ CGD, even though their granulocytes were able to produce superoxide, albeit in small amounts (12% of normal). All 3 patients expressing uniform and low levels of oxidase activity in their granulocytes received early diagnoses and experienced numerous infections requiring hospitalization. The general idea is that patients with X91^− CGD with missense mutations receive a diagnosis later in life than do patients with X91^+ CGD and have a milder clinical course [13].

We believe that the number of published cases of X91^− CGD with both a well-documented clinical history and knowledge of the genetic lesion is not high enough to provide a clear and definitive opinion on the severity of the clinical course of these variants. In addition, the severity of CGD depends not only on the subtype of the disease, but also on polymorphisms in genes other than oxidase genes. The best illustration is the discrepancy between the worst clinical profile of patient C and the NADPH oxidase activity of his granulocytes, which remains higher than that of his brothers (9% vs. 3%–5% of normal) (table 1). Other pathways are implicated in the first-line defense mechanism against microbial pathogens of the mammalian host. For example, the Toll-like receptor activation [56], in particular the Toll/interleukin-1 (IL-1)–IL-1 receptor–associated kinase signaling pathway, seems to be crucial for protective immunity against specific bacteria [57].

In conclusion, in the 3 cases of X91^− CGD reported here, infections and severe clinical complications occurred, even in the presence of a low but significant O_2^−-generating oxidase activity and with constant care and follow-up at a single medical center with the same physician. In such cases, the follow-up of patients depends on early diagnosis and prophylactic antibiotic and antifungal therapies in association with IFN-γ injection [58, 59]. Systematic characterization of gene mutations in patients with CGD should help us to relate the clinical form to the type of gene affected and/or the type of mutation found in this disease. It can also be essential in genetic counseling and prenatal diagnosis. Although variant forms of CGD are rare, they contribute to our understanding of oxidase complex and of killing mechanisms in phagocytic cells.

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