Unusual late presentation of X-linked Chronic Granulomatous Disease in an adult female with a somatic mosaic for a novel mutation in CYBB

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Abstract

Most patients with chronic granulomatous disease (CGD) have mutations in the X-linked CYBB gene that encodes gp91\textsuperscript{phox}, a component of the phagocyte NADPH oxidase. The resulting X-linked form of CGD is usually manifested in boys. Rarely, X-CGD is encountered in female carriers with extreme expression of the mutated gene. We report here a woman with a novel mutation in CYBB [CCG(90-92)\textarrow{\rightarrow}GGT], predicting Tyr30Arg31\textarrow{\rightarrow}stop, Val in gp91\textsuperscript{phox}, who presented with clinical symptoms at the age of 66. The mutation was present in heterozygous form in genomic DNA from her leukocytes but was fully expressed in mRNA from these cells, indicating that in her leukocytes the X chromosome carrying the non-mutated CYBB allele had
been inactivated. Indeed, only 0.4-2% of her neutrophils showed NADPH oxidase activity. This extreme skewing of her X-chromosome inactivation was not found in her cheek mucosal cells, and is thus not due to a general defect in gene methylation on one X chromosome. Moreover, the CYBB mutation was not present in the DNA from her cheek cells, and was barely detectable in the DNA from her memory T lymphocytes. Thus, this patient shows a somatic mosaic for the CYBB mutation, which probably originated during her lifetime in her bone marrow.

**Introduction**

Chronic Granulomatous Disease (CGD) is a rare, heterogeneous, inherited disorder that affects about 1 in 250,000 individuals. The main defect in CGD is a failure of neutrophils, monocytes, macrophages and eosinophils to mount a respiratory burst and, therefore, to generate superoxide anions and other reactive oxygen species derived from superoxide, such as hydrogen peroxide. This renders the patients susceptible to severe, recurrent bacterial and fungal infections. The enzyme that generates superoxide is a phagocyte-specific NADPH oxidase, consisting of two membrane-bound subunits and four cytosolic proteins involved in activity regulation of the enzyme. The central, catalytic subunit is gp91phox, a flavocytochrome that forms a heterodimer with p22phox in the plasma membrane of phagocytic leukocytes. Defects in gp91phox are inherited in an X-linked fashion because its gene, CYBB, is located on the X chromosome. About 70-75% of the CGD patients are X-CGD patients. Autosomal forms of CGD are due to mutations in CYBA, the gene that encodes p22phox in ~3% of the CGD patients or to mutations in the genes encoding the cytosolic proteins p47phox (~25%) or p67phox (~3%).

For many years the onset of CGD was regarded to occur early in infancy, with fatal outcome in adolescence, due to the recurrent severe infections and the secondary
granulomatous and fibrotic tissue formation that develops in many organs. Changes frequently occur in the skin, lymph nodes and lungs, but also in bones, joints, liver and kidneys, leading eventually to pulmonary, gastrointestinal, musculoskeletal and renal insufficiency.1,2,5,8,9

In the last decades, with the rapid development of sophisticated diagnostic tools and techniques, a better understanding has been gained of the pathophysiological mechanisms of the disease, and the genetic basis of CGD has been elucidated.1,2,6 Moreover, a more accurate determination of the infectious etiological agents has led to a more appropriate, specific therapy.8-11 Together with improved techniques of bone marrow transplantation this has brought about the prolongation of life and/or the correction of the basic disorder in CGD.12-16

CGD is a very heterogeneous disease, with different genetic causes and with a variable and heterogeneous clinical spectrum, from a mild disorder to a very severe form. The patient here reported is unique because the disease manifested very late in life and the genetic cause of her disease is unusual. She appeared to be a carrier of the X-linked form of CGD, who usually have no or only mild clinical complaints because these women have a mixture of normal and abnormal phagocytes in their circulation. However, this woman had very few normal phagocytes, due to a skewed X-chromosome inactivation pattern. Moreover, the mutation in CYBB was found in her short-living blood cells, but not in her memory T lymphocytes or in her cheek mucosal cells. This indicates that the skewed X-chromosome inactivation pattern in her leukocytes may be due to a clonal expansion of a mutated bone marrow stem cell.

Case Study
We report an 80-year old woman of Iraqi origin who was born to related parents. As shown in the family pedigree (Figure 1), she is one of two pairs of twin siblings; one
of each pair died soon after birth from an undetermined etiology. Nine of her offspring were healthy and one of twins died at the age of 3 months from a severe bacterial infection. A great-grandchild died at 10 days of age from a fulminant infection.

Figure 1. Pedigree of the patient and her family.

The patient is indicated in black. Deceased family members are indicated with a diagonal in their symbol. Age at death of #3, 54 years; #6 and #8, at birth; #11, 3 months; #22, 10 days. Healthy grandchildren and great-grandchildren of the patient are not indicated.

The patient had a normal and healthy life until age 66; since then she underwent about 30 hospitalizations within 8 years, for *Serratia marcescens* sepsis, recurrent pneumonia (5 times) and sinusitis (twice), *Staphylococcal aureus* pre-tibial abscess, *Acinetobacter* skin abscess, *E. coli* and *Candida albicans* urinary tract infection, *Providentia* osteomyelitis and septic arthritis, suppurative adenitis, liver cysts and calcified lesions (detected by computerized tomography), pan-uveitis, anthralgia, vaginal ulcers, aphthous stomatitis, pyoderma gangrenosum and vasculitis-
like skin rash in face and limbs. Elevated RBC sedimentation rate, leukocytosis, neutrophilia and remarkable dimorphic anemia manifested by anisocytosis as sometimes seen in chronic disorders (microcytic-hypochromic anemia associated with macrocytic red blood cells) as well as hypergammaglobulinemia became apparent with time. Many tentative diagnoses were raised, such as Behçet’s disease, vasculitis, SLE and Wegener granulomatosis. Insufficient clinical and laboratory manifestations supported these tentative diagnoses. She was treated with antibiotics, surgical drainage, NSAIDs, steroids and colchicine.

Following comprehensive studies of leukocyte functions, Western blot analysis of neutrophil extracts and molecular DNA analysis, the diagnosis of chronic granulomatous disease was established at the age of 74 years. Since then, she was successfully treated with trimethoprim-sulphamethoxazole on a prophylactic daily basis, and no more admissions or relevant infections were recorded. At present in her eightieth, she is leading a completely normal life.

**Methods**

**Neutrophils** were purified from heparinized venous blood of the patient and her relatives. Simultaneously, a healthy volunteer served as control. Informed consent was obtained from all participants. Neutrophils were isolated from ten milliliters of heparinized blood by dextran sedimentation, followed by erythrocyte lysis, as described by Böyum. The neutrophils were washed and resuspended in phosphate buffered saline with 1% (v/v) albumin and kept on ice until tested. The purity of these cells was 99% and the viability >95%.

**Purification of other blood cells.** Monocytes and lymphocytes were purified as described by Roos and de Boer. CD45RO+ and CD45RO− T lymphocytes were
purified from mononuclear leukocytes by incubation with CD45RO magnetic microbeads (Miltenyi, Gladbach, Germany) and isolation over a VarioMacs column. The fraction with CD45RO+ cells was further purified by incubation with CD45RO-PE and CD3-FITC (Becton Dickinson, San Jose, CA, USA) and FACS sorting of the double-positive cells in a MoFlow high-speed cell sorter (Dako Cytomation).

**Superoxide production** by neutrophils was measured as superoxide dismutase-inhibitable reduction of ferricytochrome c by the method of Weisbart et al.\textsuperscript{19}

Neutrophils (10\textsuperscript{6}) were suspended in Hanks Balanced Salt Solution with 60 μM ferricytochrome c, with (control) or without 214 U of superoxide dismutase. The rate of superoxide anion release was measured after addition of 100 nM N-formyl-methionyl-leucyl-phenylalanine (fMLP), or 10 ng/ml phorbol myristate acetate (PMA), for 10 min at 37°C in a UV-260 Shimadzu spectrophotometer, at 550 nm. The superoxide anion release was calculated with the Massey extinction coefficient for ferrocytochrome c of 2.1 x 10\textsuperscript{4} M\textsuperscript{-1} cm\textsuperscript{-1}.

Superoxide production by neutrophils was also measured as nitroblue tetrazolium (NBT) reduction by the NBT slide test reported by Baehner et al.\textsuperscript{20} with slight modifications.

**Hydrogen peroxide production** by neutrophils was measured with the dihydrorhodamine-1,2, 3 (DHR) assay as described by Vowells et al.\textsuperscript{21} In short, neutrophils were incubated with DHR and catalase, and were activated with 100ng/ml PMA. The reaction was stopped at various times and the amount of DHR oxidation product rhodamine was measured by FACS analysis.

**The bactericidal activity** was assessed as previously reported\textsuperscript{22} and expressed as the decrease in the number of viable bacteria after incubation of bacteria with neutrophils in the presence of autologous and homologous serum.
**The chemotactic response** was assessed by 48-well chemotaxic microchamber (Neuro Probe, Inc., Bethesda, MD) to determine random migration and chemotaxis as previously reported.\(^{23}\)

**Western blot** was performed for protein analysis of the NADPH-oxidase system.\(^{24}\) Neutrophil lysate (20 µg) was electrophoresed on 10% SDS-polyacrylamide gel and transferred to a nylon membrane. The membrane was blocked with 5% skimmed milk, incubated with a monoclonal antibody against gp91\(^{\text{phox}}\) (mAb 48) or p22\(^{\text{phox}}\) (mAb 449), washed, incubated with AP-conjugated rabbit-anti-mouse-Ig , and developed with Alkaline Phosphatase Stain.

**DNA and RNA analysis.** DNA was purified from leukocytes, cultured fibroblasts or cheek mucosal cells by standard methods.\(^{25}\) PCR amplification and sequence analysis of all \(CYBA\) and \(CYBB\) exons with their intron/exon boundaries and of ~400 basepairs of the \(CYBB\) promoter region was performed as described previously.\(^{7,26}\) RNA was isolated from mononuclear leukocytes and converted to cDNA by standard methods.\(^{7,27}\) A 627-basepair fragment containing the nucleotide sequence of the first five-and-a-half exons of \(CYBB\) was amplified and sequenced as described previously.\(^{26}\)

**Detection of X-chromosome inactivation at the HUMARA locus.** The HUMARA locus (human androgen-receptor gene) on the X chromosome contains two methylation-sensitive \(Hpa\)II restriction sites and is 90% polymorphic in Caucasian females for varying allele sizes.\(^{28}\) Genomic DNA was incubated with \(Hpa\)II overnight to digest all unmethylated, active DNA at this locus. The mixture of digested and undigested DNA was PCR amplified with FAM-labeled fluorescent primers flanking a region comprising both the polymorphic and the restriction sites, according to Allen et al.\(^{28}\) Capillary PCR conditions were as follows: five sec at 95 °C, 30 sec at 60 °C,
and 15 sec at 72 °C, for 50 cycles. The PCR products were diluted 1:5 with loading buffer. The samples were then heat-inactivated at 98 °C for 3 min and cooled on ice. Two µl of each sample was run on an ABI Prism 377 sequencer (Applied Biosystems) for separation of the PCR products. The intensities of the bands corresponding to the two alleles before and after HpaII digestion were compared to determine the X-chromosome inactivation ratio between the two alleles in each female by means of the program Genescan 3.1 (Applied Biosystems).

Polymorphic marker analysis. Isolated DNA was amplified with the commercially available fluorescent STR multiplex systems SGM+ (Applied Biosystems) and PowerPlex 16 (Promega, Madison, WI, USA). The set-up of the SGM+ kit was modified by reducing the amplification mixture per sample to 10 µl and a template volume of 1 µl. The PowerPlex-16 set-up was modified by reducing the mixture per sample to 10 µl, with 1:2 diluted 10 primer-pair mix. PCR products were separated on the ABI 310 analyzer (Applied Biosystems).

Results

The patient’s neutrophils displayed a significantly reduced bactericidal activity both in the presence of autologous and homologous serum (Table 1). The NBT slide test with PMA stimulation showed only 2% positive cells, and the DHR test with PMA only 0.4% positive cells. The fMLP- or PMA-stimulated superoxide generation by the patient’s neutrophils was undetectable (Table 1). As shown in Table 1, all tests
Table 1. Results of family studies

<table>
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<tr>
<th>Subject</th>
<th>DHR (% pos.)</th>
<th>$\text{O}_2^-$ generation$^a$</th>
<th>bactericidal activity$^b$</th>
<th>mutation present</th>
<th>skewing$^d$ (%)</th>
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anti-gp91[^p91] or mAb 449 anti-p22[^p22] showed negative results with the patient’s neutrophils. This test was normal in her daughters (the sons were not investigated) (Figure 2).

**Figure 2. Western blot analysis of neutrophil membranes from the patient and four of her daughters.**

Neutrophil membranes were solubilized and subjected to electrophoresis, blotted and probed with mAbs against gp91phox (A) or against p22phox (B) as indicated under Methods. Lane 1, control neutrophil from a patient with X-CGD; lane 2, control neutrophil from a healthy individual; lane 3, neutrophil from the patient (#9); lane 4, neutrophil from daughter #20; lane 5, neutrophil from daughter #16; lane 6, neutrophil from daughter #17; lane 7, neutrophil from daughter #15.

Thus, the patient suffers from CGD caused by a defect either in gp91[^p91] or p22[^p22], because these two proteins stabilize each other expression in the neutrophil membrane: absence of one of these leads to simultaneous absence of the other.24

Sequencing of the patient’s CYBA (encoding p22[^p22]) did not reveal any mutations. However, in the patient’s CYBB (encoding gp91[^p91]) we found an unusual substitution of three nucleotides (Figure 3), i.e. the sequence TACCGG at position 88-93 (A of the start codon ATG is number 1), which encodes Tyr30 and Arg31, was changed into TAGGTG, coding for Stop and Val. This predicts a premature termination of protein synthesis at amino-acid position 30. The patient was found to be heterozygous for this mutation. None of her relatives, including five grandsons and three granddaughters, had this mutation in their DNA (not shown). In contrast to the findings with the genomic DNA of the patient, the cDNA from the patient showed an
apparently homozygous expression of the mutated sequence (Figure 3), which explains the total lack of NADPH oxidase activity in her neutrophils.

**Figure 3. Nucleotide sequence of CYBB around position 88-93 from the patient and a healthy control.**

A. Genomic DNA from the leukocytes of a healthy control. B. Genomic DNA from the leukocytes of the patient (#9). The normal CYBB sequence TACCGG is changed in the patient into a mixture with TAGGTG, indicating that she is a heterozygote for this 3-nt mutation. C. Leukocyte cDNA from a healthy control. D. Leukocyte cDNA from the patient (#9) shows an apparent homozygous mutant sequence TAGGTG. The underlying signal is from another exon, apparently due to some incorrect mRNA splicing.

We then investigated whether this strongly skewed expression of one of her two CYBB loci was due to a generalized defect in X-chromosome inactivation in all somatic cells, or confined to her blood cells. For this purpose, we analyzed a highly polymorphic microsatellite region near a cytosine methylation-sensitive restriction site for HpaII at the HUMARA locus on the X chromosome (Figure 4). Predigestion of DNA with HpaII cuts those alleles that are not inactivated by X-chromosome inactivation-related methylation and are therefore not amplified. In DNA isolated from the patient’s leukocytes, the ratio between her paternal and maternal microsatellite signal was strongly affected by the HpaII treatment: one of these signals was almost completely suppressed whereas the other was unaffected. This confirms our cDNA findings that in her leukocytes only one of both CYBB alleles on
her X chromosomes is active and transcribed into mRNA. In contrast, in DNA isolated from a buccal smear, *HpaII* treatment did not affect the ratio between the two microsatellite signals, indicating that in her cheek mucosal cells no skewing in X-chromosome inactivation has taken place. Thus, the skewed X-chromosome inactivation in the patient is not a phenomenon found in all of her somatic cells. In the leukocyte DNA from the four daughters (Table 1) and three granddaughters (not shown) that we investigated, the ratio between maternal and paternal region changed much less dramatically by the *HpaII* treatment, indicating that they had not inherited this trait from their mother or grandmother. In contrast, in DNA isolated from the leukocytes and from cultured fibroblasts of a patient with a proven generalized defect in X-chromosome inactivation,31 we found that the *HpaII* treatment eliminated the formation of one of the parental microsatellite PCR products in both DNA preparations.

When we investigated the cheek mucosal DNA from the patient, we found that this DNA did not contain the TAGGTG sequence in *CYBB*, although polymorphic marker analysis proved this mucosal DNA to be otherwise identical to her leukocytic DNA (not shown). This indicates that the patient has a somatic mosaicism for the *CYBB* mutation and prompted us to search for this mutation in DNA isolated from various purified blood cells of the patient. In DNA from purified neutrophils and monocytes, we found the signals of the three aberrant nucleotides (GGT) to be about equal in intensity to the signals of the wild-type CCG, indicating that all neutrophils and monocytes contained a mutated X chromosome and an X chromosome with a normal *CYBB* gene. In DNA from CD45RO-positive memory T lymphocytes (still containing 10% monocytes), the mutation signals were only about 20% of the wild-type signals. In DNA from CD45RO-negative, naïve lymphocytes, the mutation
signals were about half of the wild-type signals. These results indicate that the CYBB mutation is present in heterozygous form in the short-living myeloid cells, but not in the long-living memory T lymphocytes and only to a limited extent in the shorter-living naïve lymphocytes. Similarly, the skewing of the X-chromosome inactivation was found to be extreme in the myeloid cells and in the naïve T lymphocytes but not in the memory T cells. Examination of bone marrow taken in 1992 revealed normocellular tissue with diffuse fibrosis replacing normal trabecular bone and architecture, as well as an increased (left-shifted) myeloid/erythroid ratio without increased blast counts.

Figure 4. X-chromosome inactivation pattern of the patient and a control with an X-chromosome inactivation defect.

DNA was isolated from the leukocytes and from a buccal smear of the patient (#9), as well as from the leukocytes and from cultured fibroblasts of a control with an X-chromosome inactivation defect. The DNA samples were incubated with or without the restriction enzyme HpaII and subjected to PCR amplification of the HUMARA locus (see Methods). The labeled products were separated by size chromatography and scanned for comparison. Panels A and B, DNA from patient (#9) buccal smear; panels C and D, DNA from patient (#9) leukocytes; panels E and F, DNA from control fibroblasts; panels G and H, DNA from control leukocytes. The patient shows equal inactivation of X chromosomes in the buccal smear but strongly skewed X-chromosome inactivation in the leukocytes. The control shows strongly skewed X-chromosome inactivation in both fibroblasts and leukocytes. Peaks indicated in grey are the main PCR product peaks, those in white are shorter products due to incorrect polynucleotide synthesis. The calculation of skewing percentage (Table 1) is based on the peak heights of the main PCR products.
Discussion

The impaired respiratory burst of CGD phagocytes is caused by a defect in one of the four components of the phagocyte NADPH oxidase, either in one of the two subunits that reside in the cell membrane (gp91\textsuperscript{phox}, p22\textsuperscript{phox}), or in one of two cytosolic subunits (p47\textsuperscript{phox}, p67\textsuperscript{phox}). Our patient has a gp91\textsuperscript{phox} deficiency, type X91\textsuperscript{0} (no gp91\textsuperscript{phox} protein expression). She occasionally suffered from banal infections until the age of 66 years. Since then, she was hospitalized about 30 times for severe, recurrent bacterial or fungal infections. No clinical or laboratory evidence of an autoimmune disorder or other disease was established. The family pedigree here reported showed two twin siblings who died at birth for reasons not well established, and two additional members, one son and one grand-grandson who died at 3 and 10 months of age, respectively, from a severe generalized infection of non-determined etiology. Although the primary disorder causing the fatal infections could not be established, knowing the family history, we could not discard the possibility of a primary immune deficiency. Therefore, we performed a full screen for phagocytic disorders and indeed, the functional, biochemical and molecular diagnosis of CGD was established in the indicator patient. She was put on trimethoprim-sulphamethoxazole prophylaxis and her clinical condition remarkably improved.

The most common form of CGD is due to gp91\textsuperscript{phox} absence, as we found in the patient here reported. The mutation in this patient is unusual, because in general the mutations in CYBB are either point mutations (substitutions, deletions or insertions) or larger insertions or deletions.\textsuperscript{32} However, occasionally, more complicated mutations are found. Since CYBB is on the X chromosome, CGD patients with mutations in this gene are usually male, but a few female patients are known.\textsuperscript{1,6,32} Until now, these are all heterozygotes for CYBB mutations with a low number of functionally active
neutrophils (<15%). This is caused by skewed X-chromosome inactivation, which is a random process early in the fetal development of female individuals. The patient described by us is unusual in her extreme low percentage of neutrophils with an active NADPH oxidase (0.4-2%), in agreement with a lack of wild-type gp91phox mRNA. These findings can be due either to a random unfavorable X-chromosome inactivation, or to a concurrent deficiency of X-chromosome inactivation caused by a mutation in a gene involved in X-chromosome inactivation. X-chromosome inactivation is a process in which one or more genes on the X-chromosome itself are involved. Products from these genes inactivate the genes located on the same X-chromosome in female cells. If in our patient an additional mutation is located on the X-chromosome that carries the CYBB mutation, this could lead to a lack of inactivation of this CYBB-mutated chromosome, and thus to unique expression of proteins encoded by this CYBB-mutated chromosome. Similar cases of female patients with Wiscott-Aldrich syndrome, Duchenne muscular dystrophy or glucose-6-phosphate dehydrogenase deficiency in combination with inherited skewed X-chromosome inactivation have been described. However, in our patient this is unlikely, because in that case her clinical symptoms might be expected to have become apparent much earlier in life.

To rule out an inherited X-chromosome inactivation defect, we investigated whether the skewed X-chromosome inactivation pattern was present not only in her leukocytes but also in other somatic cells. With the HUMARA assay we found that this was not the case, in contrast to the situation in the pre-mentioned female patient with G6PD deficiency and inherited X-chromosome inactivation defect. It is therefore more probable that our patient acquired the skewed X-chromosome inactivation pattern in her leukocytes in the course of her life. Indeed, it is known that
this pattern can shift in the hematopoietic system over a woman's lifetime.\textsuperscript{37-40} This may be due to stem cell depletion, true clonal hematopoiesis or a growth advantage conferred by parental-specific X chromosomes.\textsuperscript{41-43} Two recent reports describe this phenomenon as the cause of X-CGD in adult females, at age 45 and 43, respectively.\textsuperscript{44,45} Apparently, in our patient, it took even longer before the X-chromosome inactivation pattern was skewed to a degree that caused the clinical problems.

The fact that the mutation in $\text{CYBB}$ was not transmitted from the indicator patient to any of her seven children that we investigated, is in accordance with our hypothesis that the disorder was acquired late in life. Also the two living children that were unavailable for study have no medical records indicative of CGD. Our finding that the patient has a somatic mosaicism for the $\text{CYBB}$ mutation (present in her leukocytes but not in her cheek mucosal cells) provides a clue to explain this phenomenon, because we suppose that the mutation is also absent from her germ-line cells and can thus not be transmitted to her offspring.

The most plausible explanation for the origin of the clinical symptoms observed in this patient is, in our opinion, as follows. During her lifetime, the mutation in $\text{CYBB}$ arose in the stem cells in her bone marrow. This may have happened later in her lifetime or during her embryonic development, but in the latter case these stem cells did not take part in hematopoiesis until later, because we found long-living memory T lymphocytes that did not contain the mutation. Exchange of hematopoietic stem cells with her twin sister during embryonic development\textsuperscript{46} was rendered unlikely by polymorphic marker comparison between the patients cheek mucosal DNA and her leukocytic DNA. Probably, the mutated stem cells took over hematopoiesis either by chance or by a certain clonal expansion. The latter possibility
is the most likely, given the highly skewed pattern of X-chromosome inactivation in the short-living leukocytes. This clonal expansion may perhaps be due to a concomitant growth advantage over the wild-type cells. Since there is no known growth advantage of hematopoietic cells associated with X-CGD mutations, this suggests the influence of a parental gene expressed on the active X chromosome that bears the CGD mutation.\textsuperscript{41-43} The observed increase in myeloid/erythroid ratio and the diffuse fibrosis in the bone marrow at the time of diagnosis give indications of dysplastic hematopoiesis leading to clonal expansion, but may also be due to coexisting and recurrent infections.

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**References**


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