The search for a genetic defect in Polish patients with chronic granulomatous disease

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Summary

Introduction: Chronic granulomatous disease (CGD) is a rare inherited disorder in which phagocytic cells are unable to generate superoxide anions. Patients with CGD are predisposed to recurrent bacterial and fungal infections because the superoxide-generating NADPH oxidase activity is needed for efficient killing of microbes. Among the at least 5 subunits creating a functional NADPH oxidase, a molecular defect located in any of the gp91phox, p22phox, p47phox, or p67phox subunits may cause CGD.

Materials and Methods: In this study, 8 patients were diagnosed with CGD on the basis of clinical findings and absence of nitroblue tetrazolium reduction in phagocytes. Southern blot analysis, GeneScan, and direct sequencing were performed to define particular DNA mutations.

Results: Among 6 X-linked CGD (X-CGD) patients, 4 different mutations were identified in the X-linked CYBB gene (encoding gp91phox) by direct sequencing. A novel missense mutation, located in the NADPH-binding region of gp91 phox, was found in 2 brothers. One frameshift 1578delA, one splicing 252G->A mutation, and one partial gene deletion were also identified. The molecular defect in the NCF1 gene (encoding p47phox) was established in 2 patients. One was a ∆GT/∆GT homozygote, the other carried, besides this GT deletion on one allele, a unique Phe118stop mutation on the other.

Conclusions: In general, the X-CGD patients within the group followed a more severe clinical course than the patients with an NCF1 defect. However, the lack of a straightforward genotype-phenotype correlation indicates that the clinical severity of CGD depends also on other antimicrobial host-defense systems.

Key words: CGD • NADPH oxidase • CYBB • NCF1 • molecular diagnostics
INTRODUCTION

Chronic granulomatous disease (CGD) is a rare inherited disorder of the innate immune system. In Poland, 28 patients had been diagnosed and supervised up to the year 2002 at the Department of Immunology, Children’s Memorial Health Institute in Warsaw. That accounts for ~4% of all primary immunodeficiency patients diagnosed between the years 1980–2002 at the Department.

The disease is caused by a profound defect in a respiratory burst that generates superoxide and normally accompanies phagocytosis by neutrophils. Superoxide plays a critical role in the killing of pathogenic bacteria and fungi. Thus, CGD patients suffer from severe recurrent infections, also caused by organisms not ordinary considered pathogens, and from diffuse granulomas, presumably caused by microbes. The disease is usually recognized in young children below the age of 2, but sometimes it is recognized later.

Modern therapy of CGD includes aggressive and prolonged application of antibiotics and prednisone as well as surgical drainage of abscesses and resection (when possible) of granulomas. Early and accurate diagnosis enables prophylactic use of antibacterial and antifungal drugs as well as interferon γ. Gene therapy for CGD, due to the disease’s monogenic character, may perhaps be possible in the future.

The molecular defect resulting in the lack of a respiratory burst is located in anyone of at least 4 genes whose products create a functional NADPH oxidase, the enzyme that catalyses superoxide production. The crucial subunits of the NADPH oxidase are the membrane proteins gp91phox and p22 phox (creating a phagocyte-specific heterodimeric cytochrome b558) and the cytosolic proteins p47 phox and p67 phox that translocate to the membrane upon oxidase activation. Mutations in the Xp21.1-linked CYBB gene, encoding gp91phox, result in the most frequent (about 65% of cases) form of the disease, for obvious reasons affecting mainly males. Other components participate as follows: p47phox −25% of cases (NCF1 gene, located at 7q11.23), p67phox −5% (NCF2, 1q25), and p22phox −5% (CYBA, 16q24) and cause CGD in equal male-to-female proportion (recent review in reference). About two-thirds of all identified mutations are unique to a specific kindred.

The aim of this study was to establish the molecular lesion causing CGD in a group of 8 newly diagnosed patients. We also attempted to correlate the nature of the lesion with the clinical severity of the disease.

MATERIALS AND METHODS

Patients

Eight patients (7 boys and 1 girl) were included into the study. The diagnosis of CGD was made in all of them on the basis of clinical findings and negative nitroblue tetrazolium (NBT) test results. This microscopic test scores the ability of neutrophils to reduce the pale-yellow NBT with superoxide to black formazan deposits on a cell-to-cell basis. X-CGD heterozygotes display a mosaic of NBT-positive and -negative cells. Following the NBT test (Table 1) and chemiluminescence test with different stimulators (Table 2), 7 boys were classified as X-linked CGD (X-CGD) and 1 child (female) as presumably autosomal recessive CGD patients. Molecular diagnostics challenged these assumptions.

Clinical course

The mean age of diagnosis in the group was 5.4 years. Three boys with X-CGD presented the first symptoms in their first year of life, while the 4 others did so in
their second year. The only girl with autosomal recessive inheritance demonstrated the first symptoms at the age of 7 years. Pneumonia was the most common type of infection encountered in this group of patients, occurring at least once in 6 patients, with 4 boys with X-CGD having suffered from recurrent pneumonias. Cutaneous abscesses represented the next most common type of infections – in 4 children. Recurrent organ abscesses were often recognized within the group, mainly in the liver (in 3 boys with X-CGD, and in 1 with autosomal recessive CGD), but also in the brain (1 X-linked, 1 autosomal) and lung (2 brothers with X-CGD). One boy developed kidney abscesses. Three of the 8 children presented with sepsis, the same number with lymphadenitis, 2 had perianal abscesses. Granulomas were diagnosed in 2 patients. According to these data, the children with X-CGD demonstrate a more severe clinical course and earlier onset of the disease than those with the autosomal form of CGD.

**Mutation analysis**

Molecular tests were performed on the basis of biochemical findings and the general frequency of defected genes observed in CGD patients worldwide. The research project was approved by the Bioethics Committee of the Children’s Memorial Health Institute in Warsaw.

Genomic DNA was isolated from blood leukocytes by phenol-chloroform extraction. The CYBB exons with flanking intronic regions were amplified with the appropriate primer combination and standard polymerase chain reaction (PCR) conditions. The PCR products were subsequently purified with the QIAquick PCR Purification Kit (Qiagen, Germany) and cycle-sequenced by means of the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, USA). The products were purified with Centrisep columns (Princeton Separations, USA) and run on either an ABI 377XL or an ABI 310 Automated DNA Sequencer (PE Applied Biosystems, USA). Sequence analysis was performed by means of the Sequence Analysis and Sequence Navigator software (PE Applied Biosystems, USA). The most frequent NCF1 dinucleotide deletion and a novel point mutation were identified as described elsewhere by GeneScan quantitative analysis with labeled primers and direct sequencing. Southern blot analysis was performed by digestion of the DNA with the restriction enzyme BamH1, separation of the fragments, blotting and probing with a 3.5 kb KpnI/PstI fragment of gp91phox cDNA labeled by random priming.

**RESULTS**

Within our group of X-CGD patients (6 cases, 2 sibling pairs included) 4 different mutations were identified (Table 3). The mutation identified in the family of patients 13109 and 13059 turned out to be a G->T transversion in exon 12 of the CYBB gene (the 1498 position of the coding sequence) and was not previously described. The mutation changes codon 500 of the CYBB gene to encode for tyrosine instead of asparagine. Also affecting exon 12 was a novel 1578delA deletion found in patient 13278 (Fig. 1). Frameshift led to the creation of a stop codon 6 codons later and truncation of the original protein. The 252G->A transition detected here in 1 patient has been observed previously. It is a well-known point mutation (13 unrelated cases reported) in the consen-

| Table 2. Chemifluorescence test results for the family of patient 14009 |
|---------------------------------|--------------------|--------------------|
| Family member                  | Unstimulated (0.05–0.5) | fMLP (0.1–0.7) | OZ (0.6–2.5) | PMA (0.2–1.5) |
| Patient 14009                  | 0.00                | 0.00              | 0.00          | 0.00          |
| Mother                         | 0.05                | 0.08              | 0.26          | 0.33          |
| Father                         | 0.08                | 0.17              | 0.53          | 0.94          |

On the basis of the chemifluorescence test, X-CGD was suggested. Maximal fluorescence is presented in relative fluorescence units. Abbreviations: fMLP – formyl methionyl leucyl phenylalanine, OZ – opsonized zymosan, PMA – phorbol 12-myristate 13-acetate.

| Table 3. Mutations identified in CYBB and NCF1 genes |
|---------------------------------|--------------------|--------------------|
| Patient’s number               | Gene exon         | Nucleotide change  | Amino-acid change | Clinical severity |
| 12640                           | CYBB exon 3       | 252G->A            | splice defect of exon 3 | severe          |
| 13109 ^                         | CYBB exon 12      | 1498G->T           | Asp500Tyr         | severe          |
| 13059 ^                         | CYBB exon 12      | 1498G->T           | Asp500Tyr         | moderate        |
| 13278                           | CYBB exon 12      | 1578delA           | frameshift        | severe          |
| 13268 °                         | CYBB exon1-3     | deletion           | n.a.              | severe          |
| 13298 °                         | CYBB exon1-3     | deletion           | n.a.              | mild            |
| 13293                           | NCF1 exon 2      | 75,76delGT        | frameshift        | moderate        |
| 14009                           | NCF1 exon 2      | 75,76delGT /      | frameshift        | moderate        |
|                                 | NCF1 exon 4      | 353,354TC->AA     | Phe118stop        |                |

Abbreviations: ^, ° – brothers, n.a. – not applicable, ^ – homozygotous.
A molecular defect in the NCF1 gene was established in two patients. One was an exon 2 ΔGT/ΔGT homozygote (13293); the other carried, besides the GT deletion on one allele, a double nucleotide substitution [TC (353,354)AA] on the other allele, predicting a unique Phe118stop nonsense mutation (14009). Biochemical findings for the family of patient 14009 misled us initially to the CYBB gene being affected. But when no mutation was found in the coding region of CYBB, we switched to the NCF1 gene and finally defined the defect and autosomal background of the disease.

**DISCUSSION**

*Mutations in CYBB gene*

Defects in the CYBB gene identified in X-CGD patients have proven to be very heterogeneous and, with a few exceptions, associated with the absence of both cytochrome heterodimer (X91) and respiratory burst activity. More than 320 different mutations had been identified by February 2001 at the CYBB locus. Several types of mutation of gp91phox have been reported in X-CGD, including deletions, insertions, splice site mutations, missense mutations, nonsense mutations, and duplications. Approximately 10% of patients with X-CGD express a small amount of residual cytochrome b with low levels of superoxide production (X91). A few cases have been reported with normal levels of a non-functional or very poorly functional cytochrome b558 (X91). In general, mutations either in CYBB or in CYA cause lack of detectable surface cytochrome, because gp91phox and p22phox need each other for stable expression.

One of the novel mutations described here affects codon 500 placed in exon 12 of the CYBB gene. Considering the 3-dimensional structure of gp91phox, the Asp500 amino-acid is located in the 20-amino-acid loop over the cleft of the NADPH binding region of gp91phox. A previously reported Asp500Gly mutation leads to the rare phenomenon of production of an inactive gp91phox subunit with defective translocation and association of cytosolic NADPH oxidase components p47phox and p67phox with gp91phox. Some other missense mutations in this region produce a similar phenotype. That strongly supported the notion that a new Asp500Tyr mutation identified in the genomic sequence of patients 13109 and 13059 would be pathogenic and manifest in the same phenotype. Indeed, recent follow-up studies have confirmed this: the expression of gp91phox was normal in patient 13109 (to be published) (13059 not examined), while the NBT test was negative in 100% of cells.

Mutation 1578delA represents a novel frameshift mutation in CYBB exon 12 and results in the termination of translation 6 codons later. Nonsense and frameshift mutations in the CYBB gene affect the level of the respective mRNA to a variable degree, but there is no evidence for the production of stable truncated protein. A truncation of the C-terminal original sequence as short as 11 amino acids long due to a frameshift or nonsense mutation manifests as an almost complete lack of the whole cytochrome b558. Our patient, with the 3’ end of the CYBB gene formed with 5 amino acids changed followed by a lack of almost 40 subsequent codons, was profoundly affected with the severe clinical course of CGD.

Mutation 252G->A, detected in one of our patients, leads to an in-frame deletion and absence of exon 3. This predicts production of a shortened polypeptide...
that is not able to create a functional cytochrome \( b \). The patient affected by the splicing mutation displayed a severe immunodeficiency phenotype. Other patients with these lesion did not express the cytochrome at all, which is probably also the case here.

Single base-pair mutations are almost randomly scattered over the entire coding region of the \( CYBB \) gene. Only a few hotspots for these mutations have been observed, mainly in CpG sequences\(^\text{18} \). Because only one polymorphic site in the coding sequence has been observed so far, it appears that the gp91\(^{phox} \) subunit is extremely sensitive to amino-acid changes\(^\text{12} \).

**Mutations in NCF1 gene**

In contrast to the variety of mutations reported in \( CYBB \), only a few mutations have been identified in \( NCF1 \). The most common mutant allele bears the deletion of a GT dinucleotide in a GTGTACA sequence at the beginning of exon 2, which results in a frameshift and premature translation termination after the synthesis of a 50 amino-acid-residue protein. All patients have grossly normal p47\(^{phox} \) RNA levels but lack detectable protein, which therefore is presumed to be unstable. Also, point mutations within this gene lead to a lack of the protein with preserved normal mRNA level\(^\text{15} \). Patients with p47\(^{phox} \) affected at the very beginning of the sequence (homozygote with a “stop” after 50 codons or in a heterozygous manner with a “stop” at codon 118 on the other allele) although severely affected at the molecular level, demonstrated a quite moderate course of the CGD.

Single or dinucleotide deletions occur at sites of repetitive sequences, which may account for the high frequency of this allele in \( NCF1 \)-dependent CGD. Since the \( NCF1 \) locus is rich in repetitive elements (50.37% of the gene) the most likely explanation of the frequency of the defect described is unequal crossing-over between \( NCF1 \) and two highly homologous pseudogenes that all carry the GT deletion. The presence of virtually identical pseudogenes (greater than 98% homology) impedes sequencing of \( NCF1 \), for only primers of restricted sequence may be used\(^\text{2} \). Stability and function of the subunit p47\(^{phox} \) seem to be less sensitive to amino-acid changes, because several polymorphisms have been identified in the \( NCF1 \) sequence\(^\text{3} \).

**Genotype-phenotype correlation**

Identification of specific mutations in CGD patients may help to clarify some of the variability in clinical severity seen in this disorder, although the clinical consequences of a given mutation remain unpredictable. In general, X-CGD patients follow a more severe clinical course than patients with an \( NCF1 \) defect\(^\text{16} \). That is also the observation within our cohort of patients. X-CGD patients with missense mutations usually exhibit a milder clinical course, associated with a residual amount of gp91\(^{phox} \). However, the level of superoxide generation does not always correlate with the clinical course. Some patients suffer from severe and recurrent infections despite having neutrophils with 10–30% of normal oxidase activity\(^\text{17} \). Within our group we could not define a direct correlation between the molecular defect and the clinical course of the disease. Either truncations or missense mutations could have resulted in severe influence on phenotype. On the other hand, identical defects have led to different clinical manifestations. The only manifestation that the 2 brothers carrying the Asp500Tyr mutation shared was a similar predisposition to respiratory tract infections. Previously published data reported another 5 mutations identified in Polish patients with X-CGD. Only the Arg157stop mutation correlated with a severe course of the disease in 2 brothers. But a truncation episode 27 codons earlier (Arg130stop mutation, patient B.PA.) gave only moderate clinical severity\(^\text{20} \).

Therefore, it is likely that many factors affecting the clinical severity of CGD are dependent on other antimicrobial host defense system\(^\text{2} \). In any case, a precise identification of the molecular defect is crucial for genetic counseling and prenatal diagnosis of CGD. In future it will be the starting point for gene therapy of the disease.

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**REFERENCES**


