Origin of Mutations in Two Families With X-Linked Chronic Granulomatous Disease

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The most common X-linked recessive form of chronic granulomatous disease (X-CGD) is characterized by the absence of cytochrome b$_{558}$ in neutrophils. In a rare variant form of X-CGD, cytochrome b$_{558}$ is present but not functional. The gene (locus symbol CYBB) was localized to band Xp21 by studies of patients with small chromosome deletions. The gene was cloned based on its location and found to encode the 91-Kd subunit of the membrane cytochrome b$_{558}$ complex. Most female carriers for X-CGD can be identified by their X-inactivation mosaicism; on average 50% of their neutrophils express the mutant phenotype and fail to reduce nitroblue tetrazolium (NBT). In 2 of 4 families studied, the maternal grandmothers had normal NBT tests, suggesting either nonrandom X-inactivation or new mutations. Restriction fragment length polymorphism analysis using closely linked flanking markers or the NsiI polymorphism detected by the CYBB probe itself, allowed us to identify the X chromosome carrying the mutation as derived from a healthy NBT-positive maternal grandfather. The mothers of the affected boys have received a paternal X chromosome carrying a new mutation, consistent with the maternal grandmothers’ normal NBT tests. In all of eight potential carriers studied, the results of the NBT and DNA marker testing were in complete agreement. Prenatal diagnosis by DNA testing can be performed in early gestation obviating the need for fetal blood sampling.

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MATERIALS AND METHODS

Family 1 contains two affected brothers, born in 1954 and 1956. Both have a history of recurrent pulmonary infections, frequent fevers, lymphadenopathy, and liver abscesses." In 1969, the younger patient presented with symptoms resembling Crohn’s disease." The diagnosis of CGD was confirmed by defective bacterial and metabolic activity of neutrophils, inability to reduce NBT, abnormal superoxide anion formation, and the presence of lipid-laden histiocytes in rectal and small bowel biopsies. Neutrophils of both patients had normal levels of cytochrome b$_{558}$. Recent DNA sequence analysis identified a single nucleotide change, a C → A transversion that results in a Pro → His substitution at residue 415.
of the 91-Kd protein. Two sisters and one brother are healthy. The mother has discoid lupus and is otherwise healthy. A maternal aunt and the maternal grandfather are alive and well. The maternal grandmother, now deceased, was part of a large sibship; none of her five brothers had unusual infections or died in infancy.

Family 2 has one affected boy, born in 1984 and diagnosed with CGD at the age of 20 months when he had persistent skin infections, lymphadenopathy, and osteomyelitis. The diagnosis was confirmed by demonstrating decreased bacteriocidal activity of leukocytes, inability to reduce NBT, and absence of cytochrome b558. His three sisters and both parents, four maternal uncles, two maternal aunts, and both maternal grandparents are healthy. None of the maternal grandmother’s sons died in early infancy or childhood.

Methods. NBT reduction was determined histochemically for individual neutrophils using an NBT slide test as previously described. Coverslips were coated with either endotoxin or phorbol myristate acetate before allowing a drop of blood to clot. After removing the clot and washing off the red blood cells, the coverslips were inverted and incubated on a glass slide with a suspension of NBT, fresh serum, and phosphate-buffered saline, pH 7.4. The cells were fixed with methyl alcohol and counterstained with safranin. The proportion of neutrophils capable of reducing NBT was determined by counting at least 500 cells.

DNA was extracted from peripheral blood leukocytes and Southern blots were prepared as described previously. Samples were tested for the following RFLPs previously mapped to band Xp21: MspI and BamH1/OTC; Psi1/754; HindIII/754-6; EcoRI/754-11; TaqI/JX1.1; BstNI and XmnI/pERT87-1; BsrXI and TaqI/pERT87-8; TaqI and XmnI/pERT87-15; BglII/pERT87-30, BamH1/J-Bir; EcoRV/C7; and BstNI/B24. Locus symbols, origin of probes, and allelic fragment sizes have been summarized. In addition, we have used a human cDNA clone of the X-CGD gene that encodes the large subunit of cytochrome b558, kindly made available by Dr S.H. Orkin (Boston Children’s Hospital), to search for deletions or structural rearrangements and for the recently described NsiI RFLP.

RESULTS

The results of NBT reduction tests and DNA marker analyses are illustrated in Figs 1 and 2. The numbers above the symbols designating individuals indicate the percent of neutrophils that were positive in the NBT slide test. Numbers greater than 95% represent the normal control range. Affected males are unable to reduce NBT (0% positive). Females with 50% (Fig 1), or 32%, 53%, and 35% (Fig 2) positive cells were diagnosed as probable heterozygotes. It is noteworthy that in both families the maternal grandmothers had NBT test results in the normal range. While this may indicate that they are not carriers of the X-linked CGD mutation, other possibilities cannot be ruled out, such as nonrandom X-inactivation or loss of heterozygosity due to clonal selection of bone marrow cells.

Therefore, molecular genetic studies were performed with the goals to evaluate the NBT slide test for carrier detection, determine the grandparental origin of the mutations, and look for deletions or structural rearrangements of the CYBB gene in affected males. No deletions or abnormal size fragments were detected on Southern blots in DNA from affected males and carrier females of both families. Both mothers of affected males were informative for four RFLPs, including the CYBB polymorphism in family 2. Maternal grandfathers and fathers were available to unequivocally establish haplotypes in females. The maternal grandmother in family 1 died before the DNA studies were initiated and her alleles are inferred.

In family 1 (Fig 1), the mother of the two affected males was heterozygous for four RFLPs flanking the CYBB locus. At DXS28 in band Xp21.3, probe C7 distinguishes the EcoRV alleles A1/8.0 kilobases (kb) and A2/7.5 kb. At DXS164 within the dystrophin (DMD) gene, probe pERT87-1 sees XmnI alleles A1/8.7 kb and A2/7.5 kb. At DXS84, located at Xp21.1 close to CYBB, probe 754-11 defines EcoRI alleles D1/2.4 kb and D2/4.2 kb. The ornithine transcarbamylase locus (OTC) is definitely centromeric to CYBB, as patient B.B. who suffered from DMD and CGD, was missing DXS84 but not OTC. The informative MspI alleles at OTC were A1/6.6 kb and A2/6.2 kb.

Haplotypes have been constructed for the Xp21 region as indicated by different bar symbols on Fig 1. The haplotype...
that contains the X-CGD mutation, identified as present in both affected males, is shown in black. It is evident that the mutation must have originated in the sperm of the clinically unaffected grandfather who has the black haplotype. Because the mother's sister had a normal NBT slide test, her paternal haplotype does not carry the mutation. In the third generation, the unaffected son has inherited the other maternal haplotype. However, both sisters have received a recombinant chromosome resulting from a crossover between DXS84 and OTC. Their normal NBT tests indicate that they have not received the mutant CYBB allele. DNA studies alone would be noninformative regarding the carrier status of these two females at risk. The older of the two affected males has two daughters, and both showed mosaicism on the NBT slide test. These results further confirm X-linked inheritance of CGD in this family.

The informative RFLPs in family 2 (Fig 2) included two within the DMD gene: DXS270, probe J-Bir, with BamHI alleles A1/21 kb and A2/5 kb; and DXS164, probe pERT87-30, with BglII alleles N1/8.0 kb and N2/30 kb. The NsiI RFLP at the CYBB locus with alleles A1/1.7 kb and A2/1.3 kb is illustrated in Fig 3. Based on estimates of the size of the B.B. deletion, these four markers should span no more than 5 megabases. Within this region, no recombination event was

\[ Fig 2. \] Results of NBT tests (given in percentage of positive neutrophils) and Xp21 RFLP haplotypes in family 2. CYBB indicates the NsiI RFLP detected with the X-CGD gene probe. The mutation is associated with the A1 CYBB allele that is part of the haplotype shown as a black bar in the affected male and the three female carriers (circles with dots). The same haplotype is present in the grandfather and two of his daughters who are not carriers. These findings place the origin of the X-CGD mutation in the grandpaternal sperm.
identified in the 11 meioses represented in this pedigree. The haplotype associated with the mutant CYBB allele and identified by the A1 NsiI allele (shown in black) was concordant with the NBT test results in the carrier mother, her affected son, and her two carrier daughters. Her noncarrier daughter has received the other haplotype.

As in family 1, the haplotype carrying the mutation was traced to the unaffected grandfather. The two noncarrier maternal aunts share the same haplotype without the CYBB mutation as suggested by their normal NBT slide tests.

**DISCUSSION**

In this study, the phagocyte functional assays and gene marker studies complement and confirm each other in a remarkable fashion. Because Southern analysis with the CYBB probe did not disclose a specific change in the DNA from the affected males, the X-linked pattern of inheritance of CGD could not have been established in these pedigrees without the NBT slide test that identified heterozygous females. While in family 2 the absence of cytochrome b heavy chain is consistent with X-CGD, in family 1 the cytochrome b levels were normal. X-linked inheritance was proven by demonstration of X inactivation mosaicism in the mother and in both daughters of the older affected male. However, it remained uncertain whether the mutation involves the CYBB gene or another locus on the X chromosome. The results of the haplotype analysis are consistent with the mutation residing in the Xp21 region. While this report was in preparation, Dinauer et al. reported normal size and abundance of the 91-Kd subunit messenger RNA in the affected males and identification of a nonconservative amino acid substitution in the 91-Kd gene. The Pro → His change at residue 415 does not appear to affect a known functional site and no formal proof exists that it represents the mutational basis of the disorder in this family, other than its absence in normal controls and in cytochrome b negative males. Our data locating the mutation in this family to the Xp21 region lend further support to the notion that there is only one X-linked CGD gene (CYBB).

The origin of both mutations has been traced to a grandpaternal sperm. Previous studies of dystrophin gene mutations where the origin could be unequivocally determined have shown grandpaternal origin in 5 of 13 cases. Thus, it appears possible that, similar to the dystrophin gene, the CYBB gene undergoes frequent mutations in male gametes. Haplotype analyses in autosomal disorders have also shown predominantly paternal origin of new mutations for retinoblastoma and neurofibromatosis. Deletions involving the entire CYBB locus together with the adjacent Xq locus (McLeod syndrome) have been reported. In contrast to DMD, partial deletions or structural abnormalities resulting in a truncated gene product seem to be rare in X-CGD.

Our determinations of carrier status by the NBT slide test and by DNA haplotype analysis are in complete agreement. Prenatal diagnosis of X-CGD, which previously required fetal blood sampling and NBT test in mid-gestation, can now be done early in pregnancy by chorionic villus biopsy and DNA haplotype analysis, in particular, using the NsiI RFLP at the X-CGD locus.

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