RFLP and Deletion Analysis for X-Linked Chronic Granulomatous Disease Using the cDNA Probe: Potential for Improved Prenatal Diagnosis and Carrier Determination

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The molecular basis of X-linked chronic granulomatous disease (X-CGD) has recently been elucidated and the defective gene identified and isolated. Two restriction fragment-length polymorphisms have been identified using the X-CGD cDNA probe. We have analyzed eight families with X-CGD and seven normal, unrelated females and have demonstrated that these polymorphisms are not in linkage disequilibrium. This should increase to approximately 50% the proportion of families to whom first-trimester prenatal diagnosis can be offered. Unambiguous determination of carrier status in related females in informative families will also be possible. In addition, we have identified an apparently unique small deletion in the X-CGD gene in a family affected by this disease, members of which are not informative for either polymorphism. This will allow prenatal diagnosis and carrier determination in this family.

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CHRONIC granulomatous disease (CGD) is a rare, inherited disorder of granulocyte function that results in recurrent and chronic bacterial and fungal infections in affected individuals. In the majority of families (65% to 70%), CGD is inherited as an X-linked trait, the remainder of families showing autosomal recessive inheritance patterns. Carrier females in families with the X-linked variety and affected males are identifiable using the nitroblue tetrazolium (NBT) reduction test. This test assesses activity of the phagocyte NADPH oxidase system, which is defective in all forms of CGD. Prenatal diagnosis has until recently only been possible by fetal blood sampling and NBT testing at 18 weeks gestation, since there has been no method available for the detection of the disorder at an earlier stage. The gene for X-linked CGD was localized to the short arm of the X chromosome in 1985. This followed identification of a patient with an interstitial deletion of Xp21 who exhibited symptoms of CGD, Duchenne muscular dystrophy (DMD), retinitis pigmentosa, and the McLeod red-cell phenotype. The gene has recently been isolated by the use of so-called "reverse-genetic" techniques and codes for the β chain of cytochrome b_558, a component of the NADPH oxidase system. Several additional patients have now been described who suffer from CGD and have cytogenetically detectable deletions of the X chromosome, and one patient has been described who has CGD and the McLeod phenotype with a deletion only detectable by absence of hybridization to the X-CGD cDNA.

Using the X-CGD cDNA as a probe, a restriction fragment-length polymorphism (RFLP) has recently been described using the restriction enzyme NsiI, which should aid in antenatal diagnosis in informative families. We have described a further polymorphism, also detected using the enzyme NsiI, that does not appear to be in linkage disequilibrium with the polymorphism described previously. This should increase the proportion of families to whom antenatal diagnosis may be offered in the first trimester of pregnancy. In addition, the establishment of carrier status in related females should be unequivocal in informative families, whereas use of the phorbol myristate acetate (PMA)-stimulated NBT reduction test is not always necessarily conclusive, as we show here for two families affected by this disease. We also describe a family with X-linked CGD whose members are not informative for either of the above polymorphisms but in whom we have detected a deletion within the X-CGD gene. An altered band pattern was revealed on hybridization with the X-CGD cDNA probe in affected and carrier-family members. No other clinical or cytogenetic abnormalities were observed. Prenatal diagnosis and carrier determination should be possible for this family on the basis of the altered band pattern.

MATERIALS AND METHODS

Southern blot analysis. High molecular-weight genomic DNA was extracted from whole peripheral blood and digested with restriction enzymes according to manufacturer's recommendations. DNA fragments were size separated by electrophoresis in 0.8% agarose gels. The gels were denatured, neutralized, blotted onto Hybond N nylon filters (Amersham International, UK), and hybridized according to manufacturer's instructions. DNA probes were labeled to high specific activity with 32P-dCTP by random priming. Following hybridization, the filters were washed as follows: 3 x SSC (0.45 mol/L sodium chloride, 0.045 mol/L sodium citrate, pH 7.4) and 0.1% sodium dodecyl sulphate (SDS) at room temperature for 20 minutes three times, followed by one wash with 3 x SSC and 0.1% SDS at 65°C for 15 minutes. Filters were autoradiographed using Kodak X-OMAT film and Cronex (Dupont) intensifying screens for between 16 hours and 7 days. Molecular weight standards were lambda DNA restricted with HindIII.

DNA probes. The probes used were as follows: X-CGD cDNA, a 3.5-kb PstI-KpnI fragment of the X-CGD cDNA that includes the major part of the coding region and the 3' untranslated region of the subunit of cytochrome b_558; X-CGD5', a 0.5-kb PstI-NcoI fragment from the 5' end of the X-CGD cDNA; X-CGD3', a 1.0-kb NheI-KpnI fragment from the 3' end of the X-CGD cDNA; 754, a 2.3-kb HindIII fragment mapping to Xp21 (DXS84) and closely linked to X-CGD.
ARP AND DELETION ANALYSES IN X-CGD

*NBT reduction test.* The PMA-stimulated NBT reduction test was performed on peripheral blood neutrophils as described. Results are expressed as percentage of cells reducing NBT. Normal individuals demonstrate close to 100% reduction, while carriers show values ranging from 14% to 82%, and affected individuals give no reduction.

RESULTS

_Nsil RFLP._ Digestion of genomic DNA with _Nsil_ and probing with the X-CGD cDNA probe revealed invariant hybridizing fragments of 7.2, 6.2, 3.7 and 0.9 kb. In addition, two pairs of polymorphic fragments were identified, of 2.9 and 2.5 kb and 1.7 and 1.3 kb (Fig 1). The smaller polymorphic bands (1.7 and 1.3 kb, B1 and B2, respectively) were much more difficult to visualize reproducibly than the larger pair (2.9 and 2.5 kb, A1 and A2, respectively).

The frequencies with which the various alleles occurred was as shown in Table 1. No significant deviation between the observed haplotype distribution and that expected on the basis of the above allele frequencies was observed, implying that there is not strong linkage disequilibrium between the two polymorphisms, although the size of the sample is too small to be statistically significant. The predicted frequencies for heterozygotes in the white population based on the allele frequencies shown are 31.2% for polymorphism A and 18.2% for polymorphism B, so approximately 50% of families may be expected to be informative for one or other of these polymorphisms. Of eight families known to carry the X-linked CGD defect, four families were found to be informative for polymorphism A and three for polymorphism B (one family was informative for both polymorphisms). Figure 2A and B shows representative Mendelian X-linked inheritance for both polymorphisms, which was demonstrable in three generations in four of eight families available for study (two families for each polymorphism). In one family the grandmother of an affected child had previously been labeled as a probable carrier of the disease on the basis of a PMA-stimulated NBT reduction result of 83% (which falls on the borderline between normal and carrier levels); however, RFLP analysis clearly demonstrated that she does not possess the allele associated with the disease in this family (Fig 2C). This suggests that the mutation most probably arose in the subsequent generation. In a second family the sister of an affected male shows 93% NBT reduction but is clearly a carrier based on RFLP analysis (Fig 2A). Paternity was confirmed by hybridizing _HinI_ -digested genomic DNA from this family with the locus-specific minisatellite probe p[AG3] (F. Katz, unpublished observations). Diagnosis of

![Fig 1. Nsil-digested genomic DNA from normal, unrelated individuals hybridized with the probe X-CGD, demonstrating two separate pairs of polymorphic fragments (A1, A2 and B1, B2). Lanes 1, 2, and 4: males; lanes 3, 5, and 6: females. All band sizes are in kilobases (kb).](image)

![Fig 2. (A and B) Family pedigrees demonstrating Mendelian inheritance of (A) polymorphism A and (B) polymorphism B. (A and C) Pedigrees demonstrating the discrepancy between carrier prediction based on NBT testing and RFLP analysis: The female marked with an arrow in panel A is a definite carrier of the affected X chromosome in spite of an NBT result of 93%. The female marked with an arrow in C does not possess the allele associated with the affected X chromosome in spite of an NBT result of 83%. Allele sizes are as in Fig 1. NBT results are expressed as percentage of cells reducing NBT (normal value, 100%). Females marked with an asterisk in panels A and C indicate new mutations.](image)

| Table 1. Allele Frequencies for Two _Nsil_ X-CGD Polymorphisms |
|------------------------|------------------------|------------------------|------------------------|
| Allele | Size (kb) | No. of Chromosomes | % |
| A1 | 2.9 | 6/32 | 19 |
| A2 | 2.5 | 26/32 | 81 |
| B1 | 1.7 | 26/29 | 90 |
| B2 | 1.3 | 3/29 | 10 |

13 females, 6 males
12 females, 5 males
carrier status should, therefore, be improved by the use of these RFLPs in informative families.

Deletion within the X-CGD gene. One family investigated in this study, which was informative for neither of the RFLPs, was shown to have a deletion involving the X-CGD gene, as revealed by an altered band pattern following hybridization of genomic DNA with the X-CGD cDNA probe. Genomic DNA prepared from the blood of the affected boy (PT), digested with the enzyme NsiI and hybridized with the X-CGD probe, showed that he had an additional hybridizing fragment of approximately 11 kb and that he lacked the 7.2, 6.2, 3.7, 2.9/2.5, and 1.7/1.3 kb species (Fig 3A). His sister and mother, both of whom are carriers for the disease as defined by a lack of PMA-stimulated NBT reduction (27% and 38%, respectively), both possess all the hybridizing species by this analysis and, in addition, the 11-kb species.

In normal individuals hybridization of NsiI-digested DNA with the X-CGD 5' probe revealed only the constant 7.2 and 0.9 kb bands, while the X-CGD 3' probe detected only the invariant 6.2-kb fragment (Fig 3). Both of the NsiI polymorphisms must therefore lie within the central portion of the gene. In carriers of the deletion, the X-CGD 5' probe detected the 11-kb band in addition to the 7.2- and 0.9-kb bands found in normal individuals, implying that the deletion involves the region extending downstream from the 5' end of the gene (Fig 3 A and B). No hybridizing species were detected in the affected boy using the X-CGD 3' probe.

Using a variety of other restriction enzymes, including EcoRI, HindIII, PstI, SstI, and KpnI (results not shown), we have confirmed the findings of altered band patterns using both the X-CGD and X-CGD 5' probes. DNA from the affected boy consistently contained identically sized restriction fragments on hybridization with these two probes (results not shown). No hybridizing species were detected in DNA from the affected boy using the X-CGD 3' probe in conjunction with any of these enzymes (results not shown). From these studies we conclude that the deletion must extend

![Fig 3](https://www.bloodjournal.org/)

**Fig 3.** (a) NsiI-digested genomic DNA from a family affected by X-CGD and one normal individual, demonstrating an altered band pattern in the affected male (PT) and two related carrier females on hybridization with the X-CGD, X-CGD 5', and X-CGD 3' probes. A, Affected male (PT); B and C, related carrier females; D, Normal unrelated female (heterozygous for polymorphism A). All band sizes are in kb. (b) Schematic map of NsiI fragments in normal and deleted (PT) DNA. All bands shown hybridize with X-CGD; an asterisk indicates hybridization with X-CGD 5' and a + indicates hybridization with X-CGD 3'. Bands for which the order has not been determined are enclosed in brackets.
from within the first 500 bp of the 5' end of the cDNA probe (ie, the region hybridizing to the X-CGD 5' probe) to beyond the 3' end of the coding region and into the 3' untranslated region of the cDNA. However, we have been unable so far to define the exact extent of the deletion beyond the 3' end of the gene. This interpretation of our results is supported by RNA hybridization studies that show that polyA+ messenger RNA (mRNA) isolated from an Epstein-Barr virus (EBV)-transformed B-cell line established from the peripheral blood of the affected boy does not contain any CGD hybridizing transcripts (Levinsky et al, unpublished observation, February 1990). The spectrum of cytochrome b_245 is also absent in neutrophils from the affected boy (A. Segal, personal communication). No other clinical abnormalities exist in this family. The nearest flanking genetic disease markers (the McLeod red-cell phenotype and ornithine transcarbamylase [OTC] deficiency; Fig 4) are therefore not affected by this deletion. Using the closely linked probe, 754, which lies telomeric to X-CGD, no differences were observed between the patterns obtained in the affected family and normal individuals (results not shown). No probes were available that map between the X-CGD gene and the locus for OTC deficiency, so we were unable to define the extent of the deletion in the direction of the centromere.

**DISCUSSION**

Prenatal diagnosis and establishment of carrier status in families with X-linked CGD has until now been achieved with the use of the NBT reduction test performed on fetal blood samples at 16 to 18 weeks gestation. This has meant second-trimester abortions for carriers of affected fetuses. In addition, as shown here, the results of NBT reduction testing for carrier status may not always be unequivocal. Prenatal diagnosis has been described using RFLP analysis with closely linked probes in one case, but until recently no polymorphism had been described within the X-CGD gene. The finding of RFLPs detected by the cDNA corresponding to the X-CGD gene is therefore of major importance for potential early prenatal diagnosis and hence first-trimester abortions. The RFLP described by Battat and Francke occurs at a frequency of 72% to 28% in their population and 90% to 10% in our population, suggesting a frequency of approximately 20% to 40% heterozygotes in the white population overall. In our experience the polymorphism that they described requires relatively long autoradiographic exposure times (of the order of 5 days) to be detected. The polymorphism we have described in this report and elsewhere seems to occur at a frequency of 81% to 19% in the population we investigated, predicting a heterozygote frequency of 31%, and seems to require relatively short autoradiographic exposure times (1 to 2 days). None of the individuals studied by Battat and Francke appear to have been informative for this polymorphism, which presumably reflects a slight difference in the genetic makeup of the two populations. Although the 2.9- to 2.5-kb polymorphism described by us seems to be more easily detectable, in light of the above discrepancy in the relative frequency of these polymorphisms, it is not possible to say how useful it will be globally. Nonetheless, the existence of two separate RFLPs that are apparently not in linkage disequilibrium with each other will certainly increase the number of families to whom prenatal diagnosis can be offered in the first trimester. In addition, we have identified one female who had been labeled as a carrier on the basis of an NBT reduction result of 83% but who does not carry the affected X chromosome according to the results of our RFLP analysis (Fig 2C). Conversely, a female sibling of an affected male (Fig 2A) is clearly a carrier based on RFLP analysis, although she demonstrates 93% NBT reduction. Since the probe detecting the polymorphism is contained within the X-CGD gene, diagnosis of carrier status in informative families should in the future prove to be unequivocal.

Several patients have previously been described with deletions associated with X-CGD. In most cases these patients exhibit complex syndromes (Fig 4). Material from two of these patients was used in cloning the gene for X-CGD. One patient (BB) has retinitis pigmentosa, DMD, CGD, and the McLeod red-cell phenotype; the other patient (NF) has DMD, CGD, and McLeod syndrome. Both of these patients have cytogenetically detectable deletions in Xp21. A female with mild mental retardation and heterozygosity for ornithine transcarbamylase deficiency and CGD also has a cytogenetically detectable deletion within Xp21. A further patient (OM) has been described with CGD and the McLeod syndrome and who has no cytogenetically detectable abnormality but has a deletion of the entire X-CGD gene demonstrated by absence of any hybridization with the X-CGD cDNA probe. The patient (PT) described in this study appears to have a smaller deletion than any of those described previously, probably extending from close to the 5' end of the gene to beyond the 3' untranslated region, and is not associated with a complex syndrome or the McLeod red cell phenotype, although the precise extent of the deletion has not been defined. The size of the X-CGD gene has been estimated to be of the order of 25 to 30 kb of genomic DNA, and we estimate a minimum deletion size of 9.7 kb. Although this family is not informative for either of the above polymorphisms, as a result of this deletion they may be offered antenatal diagnosis and carrier detection.

**ACKNOWLEDGMENT**

We are grateful to clinical colleagues, particularly Dr Y.L. Lau, for providing blood samples from patients, to members of the Clinical Immunology laboratory for providing NBT reduction results, to Dr F. Katz (Department of Hematology, Institute of Child Health) for confirming paternity in one of the X-CGD families, to L.A. Alterman and G. Whyman for technical assistance, and to Professor S.H. Orkin for providing the X-CGD cDNA probe.
REFERENCES


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