INTRODUCTION

Chronic granulomatous disease (CGD) is a congenital disorder characterized by severe and recurrent infections, including pneumonia and deep tissue abscesses, leading to early death from sepsis. (Curnutte and Babior 1987). Two forms of inheritance have been noted. Most cases (80-90%) show X-linked transmission, with the remaining cases being autosomal recessive. Both forms have in common a deficiency in neutrophil and macrophage NADPH-oxidase activity. The X-linked form has a defective b-type cytochrome, while autosomal recessives probably carry a defective flavoprotein which cannot reduce the cytochrome. Together these two enzymes normally constitute a plasma-membrane-bound short electron-transport chain, unique to phagocytes, which transports electrons from NADPH to O₂. The net reaction is as follows:

\[ O_2 + \text{NADPH} \rightarrow 2O_2^- + \text{NADP}^+ + H^+ \]

H₂O₂ is generated from the O₂⁻ by superoxide dismutase, and is used as a substrate by myeloperoxidase to generate microbicidal compounds.

NADPH-oxidase is normally induced when phagocytes are stimulated by a variety of natural and experimental agents and accounts for the so-called respiratory burst of phagocytes. In CGD, NADPH-oxidase activity is absent or very low in unstimulated phagocytes, but more importantly, its activity is not induced when phagocytes are stimulated in vitro and in vivo. Phagocytes from CGD patients can kill micro-organisms that generate their own H₂O₂ and that lack catalase, but cannot kill catalase-positive micro-organisms. CGD patients respond to these latter micro-organisms with excessive but ineffective granulomatous inflammation.

Diagnosis is called for in three circumstances. Early diagnosis is critical in patients suspected of having CGD because prophylactic antibiotics can often prevent death during childhood from acute infections. Nevertheless, these patients still suffer excessive morbidity, and eventually succumb to the cumulative effects of partially-suppressed chronic infections such as bronchiectasis, pulmonary fibrosis, and GI and urinary strictures. The only definitive therapy is bone marrow transplantation, a procedure that is far from routine as yet. Because of this grim prognosis for CGD patients, it is highly desirable to identify female carriers within an affected family. Furthermore, the ability to make the diagnosis in utero would greatly expand the options open to these women.

DIAGNOSIS of CGD

A variety of techniques to diagnose CGD have been devised that measure the in vitro production of extra-cellular free radicals by stimulated phagocytes. The most widely used is the Nitroblue Tetrazolium (NBT) test. Nitroblue tetrazolium is a yellow water-soluble dye that is reduced to insoluble blue formazan by the O₂⁻ produced by NADPH oxidase activity. Two versions of the NBT test have been used, a histochemical slide test and a quantitative spectrophotometric method. In the latter, NBT dye is added to stimulated leukocytes purified from a patient's whole blood. After incubation at 37° the leukocytes are lysed and the reduced NBT is extracted and quantitated spectrophotometrically. In the histochemical procedure, a drop of blood is washed from a coverslip and remaining adherent leukocytes are exposed to latex
particles and NBT. After incubation at 37° the cells are counterstained, and the percentage of neutrophils containing blue-black formazan deposits are recorded. A modification using phorbol myristate acetate (PMA) to stimulate neutrophils in suspension in the presence of NBT before making the smears is reportedly easier to score (Pham Huu et al. 1987).

A significant disadvantage of the spectrophotometric procedure is that it requires a large quantity of blood, and the patient in question usually is a small child. This is not a problem with the slide procedure, but the slide procedure does have a problem with subjectivity in the interpretation of a positive versus a negative neutrophil, with positivity ranging potentially from cells having only formazan stippling to cells having solid clumped formazan pigment.

Despite these problems, numerous reports in the literature (reviewed up to 1975 in Lace et al. 1975) show a clearcut differentiation between CGD patients and controls by a variety of investigators using either procedure. It should be pointed out here that most of these studies, as well as the others mentioned in this review, involve very small numbers of patients due to the rarity of the disease. It should also be mentioned that the actual numerical results differed among the different studies; hence a laboratory that embarks on this test for diagnostic purposes must define its own normal and abnormal values.

The recent application of single cell analysis by flow cytometry to the diagnosis of CGD appears to successfully circumvent both the subjectivity of the histochemical NBT test and the requirement for sizeable quantities of blood in the spectrophotometric NBT test. The procedure quantitates generation of intracellular H$_2$O$_2$ by using the compound dichlorofluorescin diacetate (DCFH-DA), which diffuses into neutrophils and is rendered fluorescent upon oxidation by H$_2$O$_2$. One hundred microliters of whole blood can be used without the necessity for leukocyte purification. In a study by Hassan et al. 1988, three CGD patients showed 27-34% less cellular fluorescence than 2 normal controls after stimulation of PMN's by PMA. Fluorescence histograms of stimulated cells from the CGD patients did not overlap with those of the controls, making it possible to distinguish patients in the two groups. The one disadvantage of this technique is the requirement for a flow cytometer.

A diagnosis of CGD should not be made in the face of an abnormally low NADPH-oxidase activity without doing an erythrocyte G6PD assay to rule out severe cases of Mediterranean (B')-variant G6PD deficiency. Patients with this disease are also subject to recurrent infections, but these usually are less severe than those in CGD. These patients also show a significant hemolytic anemia, which is not a feature of CGD. These patients show low NADPH oxidase activity because of a lack of NADPH.

DETECTION OF CARRIERS OF CGD

A sister or maternal aunt of a male X-linked CGD patient has a 50% probability of being a carrier of CGD. Such a carrier will transmit the disease to 50% of her male offspring. It is therefore of great importance for these women to know if they are a carrier.

A laboratory diagnosis rather than a clinical diagnosis is required because the carrier state is asymptomatic. Carriers of X-linked CGD are expected to have two distinct populations of neutrophils, one with normal NADPH oxidase activity and one with absent activity. The percentage of abnormal neutrophils in each individual carrier will be unique, but will vary widely among carriers, in accordance with the Lyon random X-chromosome inactivation hypothesis. This was indeed seen in a study by Levinsky et al. (1983) of eight carriers using a PMA-modified slide NBT test. In this study, the percentage of neutrophils reducing NBT ranged from 14-82%; nevertheless, these carriers were easily distinguished from the 96-100% values detected in eighteen normal adults and eighty normal but infected children, and the 0% value seen in six CGD patients. Two others studies (Hirabayashi et al, 1985; and Miyazaki et al., 1976) have also successfully used the NBT slide test to identify carriers in two families. The spectrophotometric NBT test has also been shown to distinguish the carrier state from normal in a study of one family by Miyazaki et al. (1976) and of four families by Baehner and Nathan (1968). Hassan et al. (1988) utilized flow cytometry and fluorescence in the DCFH-DA oxidation test to detect two neutrophil populations in a carrier of X-linked CGD. If confirmed in additional studies, this would appear to be an alternative to the NBT slide test in diagnosing the carrier state.
In contrast to carriers of X-linked CGD, carriers of the autosomal recessive form of the disease should have a single population of neutrophils with respect to NADPH oxidase activity. Baehner and Nathan (1968), using the quantitative spectrophotometric NBT method, failed to distinguish the parents of a female CGD patient from normal controls. In contrast, Verhoeven et al. (1988), in a study of four autosomal recessive CGD patients and their families, claimed to be able to distinguish carriers from normal controls. The technique utilized in this study was the measurement of \( \text{O}_2 \) consumption by purified PMA-stimulated neutrophils, using an \( \text{O}_2 \) electrode. It should be pointed out that diagnosis of carriers of the autosomal recessive form of CGD is usually much less critical than the X-linked form because of the low probability of both members of a couple being carriers.

PRE-NATAL DIAGNOSIS OF CGD

Prenatal diagnosis of X-linked CGD was first successfully attempted by Newburger et al. (1979) using the NBT slide test. The test was adapted to using 10-50 microliters of fetal blood obtained by umbilical venous puncture under fetoscopy at 16-18 weeks EGA. Since this procedure is invasive, with risk of fetal loss, criteria for doing the procedure must include a definitive diagnosis of the carrier state in the mother, and confirmation that the fetus is male. The study by Newburger et al (1979) diagnosed one CGD fetus, with the diagnosis confirmed after termination of pregnancy. Levinsky et al. (1986) used 100 microliters of fetal blood in the NBT slide test (modified by PMA activation in suspension, as described above) to study two carriers and diagnosed one normal fetus and one CGD fetus.

In a study of four male fetuses at risk for CGD, Pham Huu et al. (1987) used three different tests to give concordant diagnosis of one normal fetus and three fetuses with CGD. Six controls gave normal results in all three tests: the NBT slide test (using 10 microliters of fetal blood), chemiluminescence (100 microliters of blood), and detection of \( \text{O}_2^- \) production by neutrophils detected by cytochrome c reduction (100 microliters of blood). This study recommended the use of three tests rather than just one to insure a greater degree of confidence before terminating the pregnancy.

The proposed use of an NBT assay on fibroblasts obtained from the less invasive procedure of amniocentesis has been shown to be unreliable (Seger and Steinman 1981, Matthey et al. 1984).
REFERENCES

Baehner, R.L. and Nathan, D.G. (1968) Quantitative Nitroblue Tetrazolium Test in Chronic Granulomatous Disease, NEJM, 278:971-976


