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Phorbol myristate acetate stimulated NBT test: a simple method suitable for antenatal diagnosis of chronic granulomatous disease

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SUMMARY

When endotoxin was compared with phorbol myristate acetate (PMA) for stimulation of phagocytes in the nitroblue tetrazolium (NBT) test, both methods discriminated between affected patients with X-linked chronic granulomatous disease (CGD) and controls, but only the PMA NBT test distinguished female carriers of CGD. Endotoxin provided no stimulation of normal fetal blood whereas PMA was an effective stimulator. Our results indicate the superiority of the PMA NBT test for diagnosis of patients and carriers of CGD and should allow accurate antenatal diagnosis of the disease.

Keywords PHA stimulated NBT chronic granulomatous disease antenatal diagnosis

INTRODUCTION

Chronic granulomatous disease (CGD) is characterized by repeated severe bacterial infections (especially to catalase producing organisms) and may be fatal in childhood. The inheritance is most commonly X-linked although an autosomal recessive variety is also described. The polymorphonuclear leucocytes (neutrophils) of affected patients fail to produce the respiratory burst required for bacterial killing and the underlying defect has been attributed to lack of cytochrome b (Segal *et al.*, 1983). The easiest and most reliable tests for diagnosis of X-linked CGD depend upon the inability of stimulated neutrophils to reduce yellow nitroblue tetrazolium (NBT) to blue black formazan deposits. Neutrophil reduction of NBT may be stimulated by adherence to glass (Gifford & Malawista, 1970), phagocytosis of latex (Baehner & Nathan, 1968), exposure to endotoxin (Park & Good, 1970), or immune complexes (Nydegger *et al.*, 1973). Many of these tests are satisfactory for diagnosis of the affected male patients, but diagnosis of the female heterozygous carriers is more difficult as overlap occurs between them and healthy subjects. Phorbol myristate acetate (PMA), the active principle of croton oil, is an exceptionally powerful stimulant and when used in conjunction with glass adherence causes nearly 100% of all normal neutrophils to become transformed and reduce NBT to formazan deposits (Repine *et al.*, 1974). This test has been used for antenatal diagnosis of CGD (Newberger *et al.*, 1979). However, fetal blood of less than 18 weeks gestation contains very few neutrophils (Linch *et al.*, 1982), and there is the theoretical possibility that only a selected population of these adhere to glass. In addition we have found the reading of these slides difficult, so we have developed a PMA stimulated NBT test for whole blood cells in suspension which combines the advantages of PMA stimulation with better staining of all the cells available. We have compared this test with the *E. coli* endotoxin stimulated NBT test on blood samples from adults, children, patients and carriers of X-linked CGD, cord blood, and mid-trimester fetuses.

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MATERIALS AND METHODS

Six boys with the characteristic syndrome of CGD, and negative NBT test, defective bacterial killing, and absent neutrophil cytochrome b were studied; most were reported by Segal *et al.* (1983). We also studied eight heterozygotes for CGD; six were obligate (five mothers and one maternal grandmother) and two were sisters, previously shown to be affected. Eleven blood samples obtained from fetuses of gestational age ranging from 19 to 24 weeks, taken by fetoscopy (Rodeck, 1980) for exclusion of other inheritable diseases, six cord blood samples from healthy babies, 18 healthy adult laboratory workers and 80 children investigated either because of suspected bacterial infection, or suspected immunodeficiency, none of whom had CGD, were also tested.

PMA stimulated NBT test. One hundred microlitres of EDTA blood was mixed with 100 μl of a solution of 125 mg NBT, 1 μg of PMA and 17·5 mg of human serum albumin, in 1 ml normal saline. The tubes were mixed and incubated at 37°C for 20 min and then at room temperature for 10 min. After mixing, smears were prepared, the slides were air dried and stained with Leishmans. The percentage of neutrophils (100 cells counted) containing the formazan black deposit in the cytoplasm were counted.

Endotoxin stimulated NBT test (modified from Park & Good, 1970). One hundred microlitres EDTA blood was mixed with 1 mg of NBT in 50 μl of saline and 10 μg of endotoxin in 50 μl of saline. The tubes were incubated and analysed as above.

RESULTS

Fig. 1 shows the results of the *E. coli* endotoxin stimulated NBT test. The range of activity in 18 normal healthy adults was 22–96% (median 33%) and in 80 children suffering from minor infections, age range a few months to 14 years, the range was 18–92% with a median of 59%. Six patients with chronic granulomatous disease gave no stimulation and the range in the eight CGD carriers was 4–38% with a median of 17%. Four of these carriers had values which overlapped with the normal adult control range. Six cord blood samples had a range of 10–50% with a median of 24% and surprisingly 11 fetal blood samples from babies screened for metabolic diseases other than

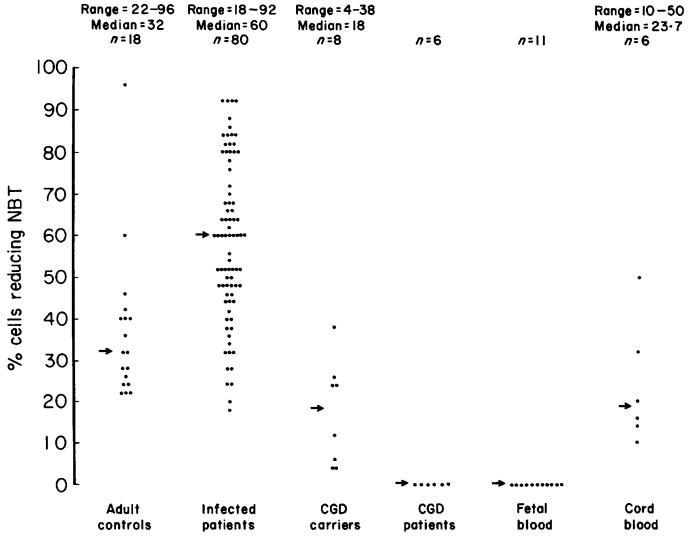

Fig. 1. NBT reduction of polymorphs following *E. coli* endotoxin stimulation. Results expressed as percentage of polymorphs showing black formazan deposits.

CGD gave no NBT reduction after endotoxin stimulation. When PMA was used as a stimulant the 18 normal adults all gave 100% NBT reduction, the 80 children had a range between 96 and 100%, as did the normal fetal bloods, while the CGD patients gave no reduction and the carriers had a range of 14–82% with a median of 42%. With PMA stimulation there was no overlap between the carrier state and the normal range (Fig. 2). The slides were easy to read since the stained formazan deposits stood out and the positive cells were transformed, enlarged and had lost the nuclear lobulation. This transformation did not occur with endotoxin stimulation. The CGD cells which failed to reduce NBT did not transform either. In the heterozygotes, two populations of cells were clearly seen; those that reduced NBT and transformed and those that did not reduce NBT and remained lobulated (Fig. 3).

DISCUSSION

This PMA stimulated NBT test, on blood cells in suspension, has all the advantages of the PMA test on glass adherent cells for diagnosis of patients and carriers with CGD (Repine *et al.*, 1975; Segal *et al.*, 1983), but the staining of the unselected cells is better, and as their morphology is less distorted, counting becomes easier. It clearly differentiates the X-linked CGD heterozygotes from controls, and the positive results in fetal blood indicate that it may be used for antenatal diagnosis of affected boys, and perhaps of carrier females, though we have not yet had an opportunity to do this.

The degree of transformation of the neutrophils is less than that seen in the adherent cell tests, as contact with glass alone stimulates some of the cells (Gifford & Malawista, 1972). This step is clearly unnecessary for the consistent stimulus of NBT reduction achieved by PMA and has the disadvantage of selecting a small population of cells. It is interesting that transformation is defective in CGD cells (best seen in the two cell populations found in female carriers), suggesting that transformation is dependent, in part, on the respiratory burst.

In contrast to other reports where fetal blood neutrophils have shown NBT reduction following endotoxin coated glass slide stimulation (Newburger *et al.*, 1979) we have consistently failed to achieve this in liquid suspension. This suggests that the stimulation for NBT reduction was caused by adherence mechanisms and that the cell surface receptors which respond to endotoxin are still immature at this gestational age.

We feel confident that this test will be able to provide accurate antenatal diagnosis of CGD where there is a family history of the X-linked variety. The test only requires 200 μl of fetal blood

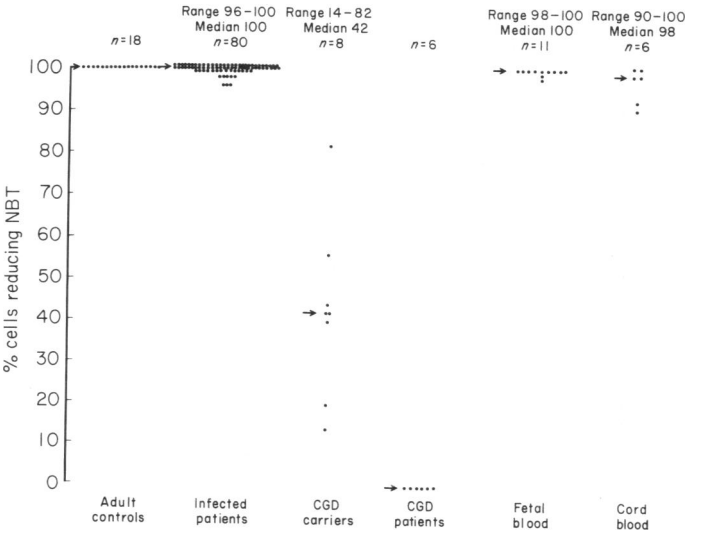

Fig. 2. NBT reduction of polymorphs following PMA stimulation. Results expressed as in Fig. 1.

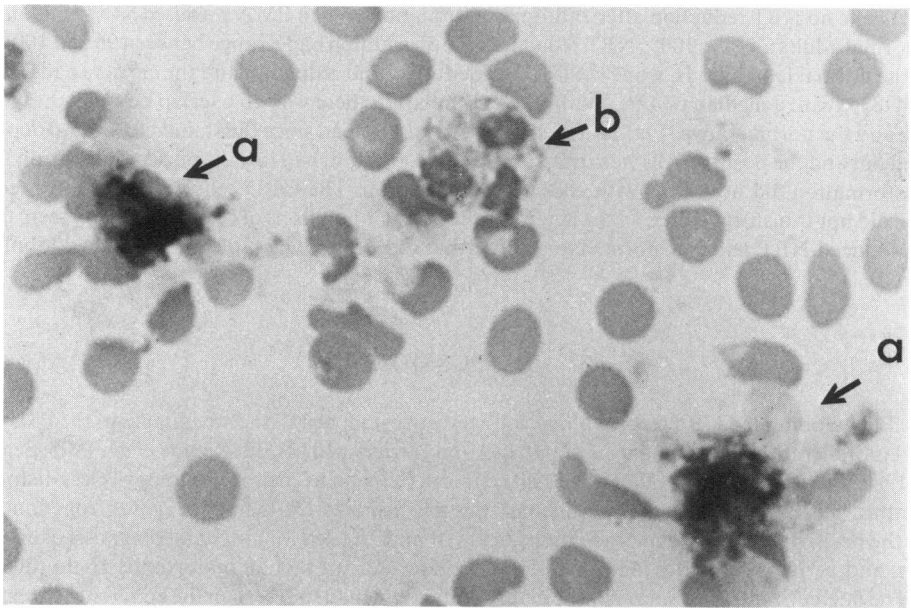

Fig. 3. PMA stimulated NBT reduction of polymorphs from a carrier of chronic granulomatous disease. This shows the two populations of cells (a) unaffected polymorphs showing transformation and black formazan deposition and (b) affected polymorph showing no NBT reduction.

and this should be obtained after 18 weeks' gestation, since prior to this fetal blood contains very few neutrophils (Linch *et al.*, 1982). The risk of causing a spontaneous miscarriage by fetal blood sampling at this stage is less than 2% and most parents with a family history of CGD would find this acceptable.

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