

Original Article

A Fast and Easy Nitroblue Tetrazolium Method for Carrier Screening and Prenatal Detection of Chronic Granulomatous Disease

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Background: Analysis of the functional activity of neutrophils is of great importance in the differential diagnosis of patients with recurrent bacterial infections. It has long been established that stimulated polymorphonuclear leukocytes reduce nitroblue tetrazolium. Application of a simple and reliable nitroblue tetrazolium method that clearly differentiates the chronic granulomatous disease patients and heterozygote carriers in some groups suspected to have chronic granulomatous disease was investigated.

Methods: This study consisted of 197 samples taken from 100 children (2 – 24-month-old) and 81 neonates (aged <2 months) referred to our center either due to a suspected bacterial infection or suspected immunodeficiency. The sample also included 16 cord blood samples. Fifty healthy adult individuals were enrolled in this study and were diagnosed as normal control. Neutrophil reduction of nitroblue tetrazolium can be stimulated *in vitro* by protein kinase agonists such as phorbol myristate acetate, resulting in release of superoxide anion.

Results: Phorbol myristate acetate is an exceptionally powerful stimulant and when used in conjunction with glass adherence, caused nearly all normal neutrophils to become transformed and reduced nitroblue tetrazolium to formazan deposits. Of 197 blood samples, 9 were diagnosed as having unrelated chronic granulomatous disease and 7 were carriers of X-linked or autosomal recessive chronic granulomatous disease. The carriers had a range of 15 – 75% stimulated neutrophils.

Conclusion: We have established a phorbol myristate acetate-stimulated nitroblue tetrazolium test for detection of chronic granulomatous disease patients, which clearly differentiates the chronic granulomatous disease patients from heterozygote carriers. The results in cord fetal blood indicate that this test may be used for antenatal diagnosis of affected boys, carrier females, and autosomal recessive variants of chronic granulomatous disease. The technique is simple, fast, inexpensive, and requires only a few microliters of blood.

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Introduction

Chronic granulomatous disease (CGD) is a rare inherited disorder caused by defective respiratory burst oxidase (RBO) and affects about 1 in 500,000 individuals.¹ The main manifestations of the disease consist of

infections of the lung, gastrointestinal tract, and skin. In the majority of the cases, the manifestations of the disease appear during the first year of life. Infections are caused by a variety of microorganisms such as bacteria and fungi. Some of the main pathogens contain catalase, the enzyme that converts the H₂O₂ generated by the bacteria to H₂O and as a result, it can not be used by the phagocytes for the formation of oxidase.^{2,3}

Early studies *in vitro* on the function of leukocytes taken from patients with CGD revealed that there is no respiratory burst upon stimulation, suggesting that the defect is associated with the RBO system.⁴ It was shown that in this system,

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both the membranes and the cytosol are needed for the oxidase activity.⁵ Some patients have a defect associated with the membranes, whereas others have a defect associated with the cytosol. Furthermore, it was established that patients with X-linked CGD are characterized by a membrane defect and absence of cytochrome b_{558} . Autosomal inheritance, on the other hand, is associated with normal levels of cytochrome b_{558} and a defect in the cytosol, which later was shown to be due to the absence of P47_{phox} or P67_{phox}.⁶

The standard method of laboratory testing for phagocytosis is to determine the ability to reduce a colorless substance, nitroblue tetrazolium (NBT), into a blue-black formazan insoluble deposit within the neutrophils. This process may be stimulated by adherence to glass, phagocytosis of latex, and exposure to endotoxin or immune complexes.⁷ Many of these tests are satisfactory for diagnosis of the affected X-linked male patients, but the diagnosis of female heterozygous carriers is more difficult as overlap occurs between them and healthy subjects. We have developed a NBT test by using phorbol myristate acetate (PMA), the active principle of croton oil as the stimulator of neutrophils in whole blood cells. This PMA-stimulated NBT reduction test combines the advantages of PMA stimulation with better staining of all polymorphonuclear cells on slide.

Materials and Methods

Blood samples taken from 100 children aged 2 – 24 months and 81 neonates <2 months of age, referred to our center either for a suspected bacterial infection or suspected immunodeficiency were obtained from the Hematology Research Center of Shiraz University of Medical Sciences between October 2002 and December 2003. This study was also carried out on 16 cord blood samples taken from newborns. Fifteen normal adult individuals with no signs and symptoms of CGD were also selected from the laboratory personnel. Patients and normal controls were randomly selected from both males and females.

One hundred microliters of EDTA blood was mixed with 100 μ L of a PMA/NBT solution. Polymorphonuclear leukocytes (PMNL) are stimulated in the presence of NBT dye. Phagocytosis is accompanied by superoxide anion release due to stimulation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase present in the membrane of PMN. Superoxide production

assay was performed by two methods: PMA-stimulated NBT test of Levinsky et al method with minor modifications,^{8, 9} and phagocytosis of the latex-stimulated NBT test.⁷ Neutrophils are allowed to adhere to glass and stimulated with PMA in the presence of NBT dye. NBT is an electron acceptor used to indirectly detect the production of superoxide by stimulated PMNL. The soluble, yellow dye is converted to the blue-black formazan—an insoluble substance which precipitates intracellularly. Slides with NBT-treated cells were prepared, stained, and examined under light microscope for the percentage of cells containing black formazan deposits. The NBT slide test provides an easy method to screen PMNL for the capacity to undergo oxidative metabolism.

Results of NBT screening test were reported as (i) normal, if cells took up dye or (ii) abnormal, if cells failed to take up dye or if the percentage of the cells reducing NBT differed markedly from the control.

Results

In the absence of PMA, the cell size remained normal; the cell had a discrete nucleus and was light red. After stimulation, the cells became swollen, the nucleus lost its characteristic multilobulated appearance, and cells became diffusely blue with spots of deeper intensity. Normal individuals reduced NBT in greater than 90% of their PMN, whereas granulocytes from patients with CGD or other defects in phagocytosis/oxidative metabolism did not produce blue formazan cells.

In normal subjects, all cells became swollen and blue. In CGD, all cells remained red and retained their normal architecture. The CGD heterozygote patients showed a mixture of positive and negative cells.

The superoxide anion production in NBT reduction test of our samples was estimated by counting all the PMNL-containing formazan deposit. All normal adults gave 100% NBT reduction. The slides were easy to read since the stained formazan deposits stood out, and the positive cells were transformed, enlarged, and lost their nuclear lobulation (Figure 1). The healthy children had a range between 96% and 100% of stimulated PMN cells, as did the normal fetal blood. Of 197 blood samples, nine unrelated CGD patients and seven carriers of X-linked or autosomal recessive CGD patients were diagnosed

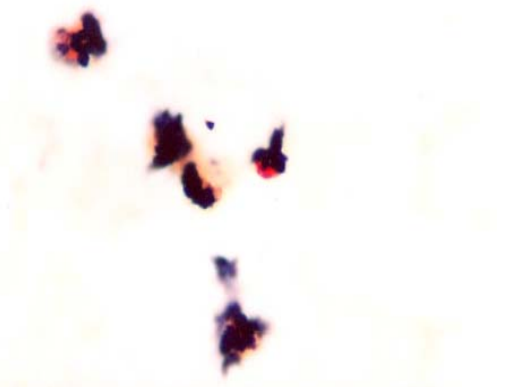


Figure 1. PMA-stimulated NBT reduction of neutrophils from a normal adult individual. Nearly 100% of all normal neutrophils gave NBT reduction. The positive cells were transformed, enlarged, and had lost their nuclear lobulation and reduced NBT to formazan deposits. The patient was followed up about a month after the course of treatment.

(Table 1). The patients with CGD gave no reduction and their cells, which failed to reduce NBT, were not transformed (Figure 2). In the heterozygote state, however, two cell populations were clearly seen: those that can reduce NBT and transformed, and those that cannot reduce NBT and remained lobulated (Figure 3). The carriers had a range of 15 – 75% with a mean of 45%. With PMA stimulation, there was no overlap between the carrier state and the normal range.

Discussion

Professional phagocytes use reactive oxygen species derived from superoxide anion (O_2^-) for killing bacteria and fungi. The O_2^- -generating enzyme NADPH-oxidase, is a multicomponent system composed of membrane-bound cytochrome b_{558} —a heterodimer of gp_{91} and p_{22} —and cytosolic phox (phagocyte oxidase) factors, p_{67} , p_{47} , p_{40} , and a small G-protein Rac, which is a regulator of activation process.^{10, 11} Much of the present

Table 1. Estimation of NBT reduction test by counting all the polymorphonuclear leukocytes containing formazan deposit.

Studied individuals	No. tested	No. stimulated PMN
CGD patients	9	<10
Carriers of CGD	7	45
Cord blood	16	98
Normal controls	50	100

PMN = polymorphonuclear; CGD = chronic granulomatous disease.

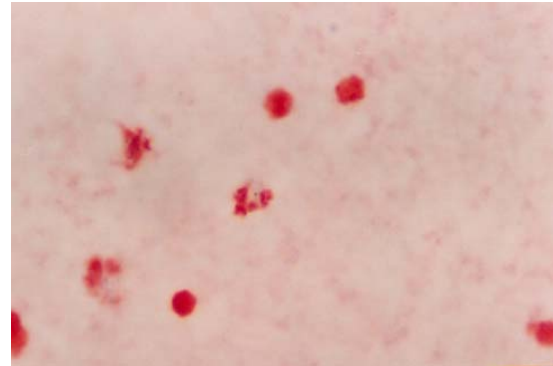


Figure 2. PMA stimulated NBT reduction of neutrophils from a CGD patient. Neutrophils show no NBT reduction. These cells were not transformed and failed to reduce NBT.

understanding of this system is derived from evidences found in patients with CGD, whose phagocytes have a defective NADPH-oxidase due to mutations in genes of either gp_{91} , p_{22} , p_{67} , or p_{47} .¹²

Phagocytic function can be assessed by some procedures. The standard method of laboratory testing for phagocytosis is the reduction of a colorless substance known as NBT.¹ Chemiluminescence (CL) and flow cytometry can also be used to measure oxidative mechanisms.¹³ Some of the reactive oxygen intermediates generated during phagocytosis exist for just a short period at a higher energy state, but release this energy as CL, which can be quantified.¹³ Contamination by erythrocytes reduces CL, because the extinction coefficient of hemoglobin in the blue region of the visible light is absorbed. In flow cytometric method, we need dichlorofluorescein diacetate

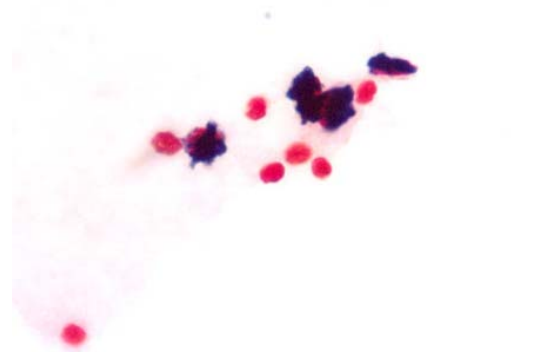


Figure 3. PMA-stimulated NBT reduction of neutrophils from a heterozygous carrier of CGD. This shows the two cell populations of a) unaffected polymorphs showing transformation with black formazan deposition and b) affected polymorphs showing no NBT reduction.

(DCFH-DA) probe, which is used to measure intracellular H_2O_2 .¹⁵

The multisubunit NADPH-oxidase complex can be activated *in vitro* by phagocytosis of latex, exposure to endotoxin or immune complexes, and a protein kinase agonist such as PMA, resulting in superoxide anion release.⁷ We have compared the PMA-stimulated NBT test with the phagocytosis of the latex-stimulated NBT test on blood samples from adults, children, patients, and carriers of X-linked CGD. The degree of transformation of normal neutrophils is less than that of PMA, because phagocytosis of latex and/or contact with glass alone can only stimulate some of the cells. Endotoxins provide no complete stimulation of normal blood. When endotoxin was compared with PMA for stimulation of phagocytes in the NBT test, both methods discriminated between the patients with X-linked CGD and controls. However, only the PMA-stimulated NBT test could distinguish female carriers with CGD.

We have developed a PMA-stimulated NBT test on blood cells that has all the advantages of the PMA-stimulation test on glass adherent cells for diagnosis of patients and carriers of CGD. It clearly differentiates the X-linked and autosomal recessive heterozygotes with CGD from controls. This technique is simple, inexpensive, requires 100 μ L of blood and takes only a few hours for completion.

It has been suggested that blood of fetus aged less than 18 weeks of gestation, contains very few neutrophils.¹⁶ Theoretically, there is a possibility that only a selected population of these cells adhere to glass. The PMA is an exceptionally powerful stimulant and when used in conjunction with glass adherence, can cause nearly 100% of all normal neutrophils to become transformed and reduce NBT to formazan deposits. A positive result in cord fetal blood indicates that this test may be used for antenatal diagnosis of affected boys and carrier females. Hence, we suggest that this test is a suitable NBT method and could allow antenatal diagnosis of the disease.

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