Carrier screening and prenatal detection of chronic granulomatous disease in Iran

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ABSTRACT

Objectives: To develop an application that is simple and reliable using the nitroblue tetrazolium (NBT) method that clearly differentiates chronic granulomatous disease (CGD) patients with heterozygous carriers in groups suspected with CGD.

Methods: This study was carried out in Shiraz University of Medical Sciences from October 2002 and March 2004. The study included 260 samples consisting of 123 children (2-24 months) and 106 neonates (<2 months), either suspected with bacterial infection or are immunodeficient, and 31 cord blood samples. Fifty healthy adult individuals were also diagnosed as normal control. Neutrophil reduction of NBT can be stimulated in vitro by protein kinase agonists such as phorbol myristate acetate (PMA), resulting to superoxide anion release.

Results: The PMA is an exceptionally powerful stimulant and when we used it in conjunction with adherence of glass slides, it causes transformation of nearly 100% of all normal neutrophils, and reduces NBT to formazan deposits. Of 260 blood samples, 12 unrelated CGD patients and 16 carriers of X-linked or autosomal recessive CGD patients were diagnosed. The carriers had a range of 15-75% stimulated neutrophils.

Conclusion: We have established a PMA-stimulated NBT test for the detection of CGD patients, which clearly differentiate the CGD patients from heterozygote carriers. The results in the cord fetal blood samples indicate that this test may be used for antenatal diagnosis of affected boys, carrier females and autosomal recessive variants of CGD. The technique is simple, fast, inexpensive, and requires only a few microliters of blood.

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Chronic granulomatous disease (CGD) is a primary immunodeficiency that affects the oxidative mechanism of microbial killing of phagocytic cells. The defect is characterized by a lack or severely reduced superoxide anion (O_2^-) production by phagocytes.[1] The main manifestations of the disease consist of infections of the lung, gastrointestinal tract, and skin. In most 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 H_2O_2 generated by the bacteria to H_2O, hence, it can not be used by the phagocytes for the formation of oxidase.[2]

Early studies of the function of leukocytes in vitro from patients with CGD revealed that there is no respiratory burst upon stimulation, suggesting that the defect is associated with the respiratory burst oxidase system.[3] It was shown that in this system, both the membranes and the cytosol are needed for the oxidase activity.[4] Some patients have a defect

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associated with the membranes, whereas others have a defect associated with the cytosol. Furthermore, it was established that patients with X-linked CGD were characterized by a membrane defect and absence of cytochrome b558. Autosomal inheritance, on the other hand, was associated with normal levels of cytochrome b558 and a defect in the cytosol, which later was shown to be a molecular defect found in any of the gp91phox, p22 phox, P47 phox or P67 phox subunits.[5]

The standard method of laboratory testing for phagocytosis is the reduction of a colorless substance known as nitroblue tetrazolium (NBT) to a blue-black formazan insoluble deposit within the neutrophils.[6] Neutrophil reduction of NBT may be stimulated by adherence to glass, phagocytosis of latex, exposure to endotoxin or immune complexes.[7] Many of these tests are satisfactory for diagnosis of the affected X-linked male patients, but diagnosis of the female heterozygous carriers is more difficult as overlap occurs between them and healthy subjects. To develop an application that is simple and reliable method that clearly differentiates the CGD patients and heterozygote carriers, we have developed an 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 in slide.

Methods. Blood samples from 123 children (2-24 months) and 106 neonates (<2 months), either suspected with bacterial infection or are immunodeficient were obtained from the Hematology Research Center of Shiraz University of Medical Sciences between October 2002 and March 2004. This study was also carried out in 31 cord blood samples from babies. Fifty normal adult individuals with no signs or symptoms of CGD were selected from the laboratory personnel. Patients and normal controls were randomly selected from both males and females.

Polymorphonuclear leucocytes (PMNL) are stimulated in the presence of NBT dye. Phagocytosis is accompanied by superoxide anion release due to stimulation of NADPH oxidase present in the membrane of PMNL. We carried out superoxide production assay using 2 methods: 1. PMA stimulated-NBT test with some modification of the Levinsky et al[8] method,[9] and 2. phagocytosis of the latex stimulated-NBT test.[7] One hundred microliters of EDTA blood was mixed with 100 μl of NBT/PMA solution. The tubes were mixed and incubated at 37°C for 20 minutes, and then at room temperature for 10 minutes. After mixing, smears were prepared. Neutrophils are allowed to adhere in glass and stimulated with PMA in the presence of NBT dye. The NBT is an electron acceptor used to detect indirectly the production of superoxide using stimulated PMNL. The soluble, yellow dye is converted to blue-black formazan, an insoluble material that precipitates intracellularly. Slides with NBT treated cells are prepared, stained with safranin dye, which stains only nucleated cells, so better estimation of neutrophil count was achieved. Slides were 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.

In the absence of PMA, cells remain in normal size with a discrete nucleus and are light red in color. After stimulation the cells become swollen, the nucleus loses its characteristic multilobed appearance and the cell becomes diffusely blue with spots of deeper intensity. Normal individuals reduce NBT greater than 90% of their PMNL, whereas granulocytes from patients with CGD or other defects in phagocytosis-oxidative metabolism do not produce blue formazan cells. Results of NBT screening test are reported as (i) normal if cells take up dye or (ii) abnormal if cells fail to take up dye or if the percentage of cells, which reduced NBT differs markedly from control. In normal subjects, all the cells become swollen and blue colored. In CGD, all the cells remain red and retain their normal architecture. The CGD heterozygotes show a mixture of positive and negative cells.

Results. The slides with NBT treated cells were examined under light microscope. The superoxide anion production in NBT reduction test of our samples was estimated by counting all the PMNL containing formazan deposit (**Table 1**). Normal adults all 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 had lost

Table 1 - Estimation of nitroblue tetrazolium (NBT) reduction test by counting all the polymorphonuclear leukocytes containing formazan deposit.

Studied individuals	NBT-stimulated PMNLs (%)
CGD patients	<(10)
Carriers of CGD	(45)
Normal children	(95)
Normal adult	(100)

PMNL - Polymorphonuclear leukocytes, CGD - Chronic granulomatous disease

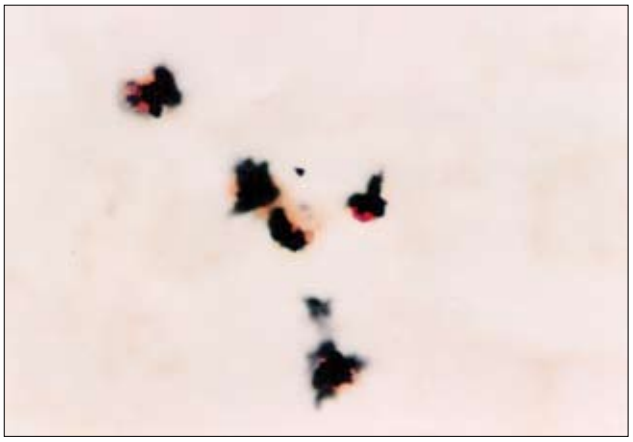

Figure 1 - Phorbol myristate acetate stimulated nitroblue tetrazolium (NBT) reduction of neutrophils from a normal adult individual. Nearly 100% of all normal neutrophils gave NBT reduction. The positive cells were transformed, enlarged and had lost the nuclear lobulation and reduced the NBT to formazan deposits.

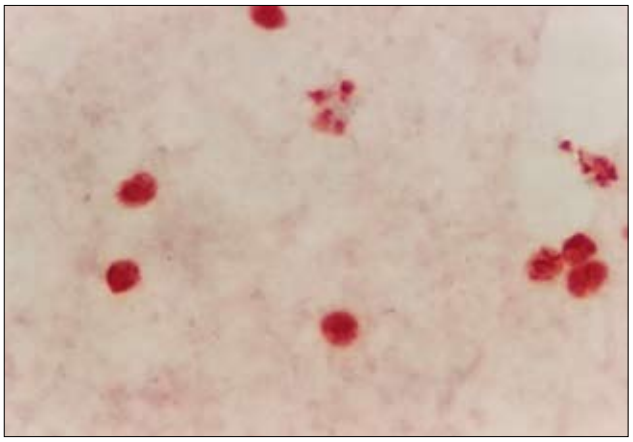

Figure 2 - Phorbol myristate acetate stimulated nitroblue tetrazolium (NBT) reduction of neutrophils from a chronic granulomatous disease patient. Neutrophils show no NBT reduction. These cells did not transform and failed to reduce NBT.

the nuclear lobulation (**Figure 1**). The blood samples of healthy children samples had a range between 96-100% of stimulated PMN cells, as did the normal fetal blood. While the CGD patients gave no reduction and these cells, which failed to reduce the NBT did not transformed (**Figure 2**). In the heterozygote state, 2 populations of cells were clearly seen: those that reduced NBT and transformed, and those that did not reduce NBT and remained lobulated (**Figure 3**). The carriers had a range of 15-75%, with a medium of 45%. With PMA stimulation, there was no overlap between the carrier state and the normal range.

Figure 3 - Phorbol myristate acetate (PMA) stimulated nitroblue tetrazolium (NBT) reduction of neutrophils from a heterozygous carrier of chronic granulomatous disease patient. This shows the 2 populations of cells: unaffected polymorphs showing transformation and black formazan deposition, and affected polymorphs showing no NBT reduction.

Discussion. Professional phagocytes use reactive oxygen species derived from superoxide anion (O_2) for killing of bacteria and fungi. The O_2^- generating enzyme NADPH oxidase, is a multi-component system composed of membrane bound cytochrome b558 (a heterodimer of gp91 and p22), and cytosolic phox (phagocyte oxidase) factors, p67, p47, p40, and a small G-protein Rac, which is a regulator of activation process.[10,11] Much of the present understanding on this system are derived from evidences found in patients with CGD, whose phagocytes have a defective NADPH oxidase due to mutations in genes of either gp91, p22, p67, or p47.[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.[13,14] Some of the reactive oxygen intermediates generated during phagocytosis exist for a short period of time in a higher energy state, but it releases this energy in the form of light known as CL, which can be quantified.[13] Contamination by erythrocytes reduces CL because extinction coefficient of hemoglobin is in the blue region of the visible regions and light is absorbed. In the flow cytometry method we need dichlorofluorescein diacetate (DCFH-DA) probe, which is used to measure intracellular H_2O_2.[15]

The multi subunit NADPH oxidase complex can be activated in vitro by phagocytosis of latex, exposure to endotoxin or immune complexes, and a protein kinase agonists such as PMA, resulting to

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 an adult and children patients and those who are carriers of X-linked CGD. The degree of transformation of the normal neutrophils is less than that with PMA, because phagocytosis of latex or contact with glass alone, stimulates only some of the cells. Endotoxin provided no completed stimulation of normal blood, and when endotoxin was compared with PMA for stimulation of phagocytes in the NBT test, both methods discriminated between affected patients with X-linked CGD and controls, but only the PMA stimulated-NBT test distinguished female carriers of 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 of CGD from controls (**Figure 3**). The technique is simple, inexpensive, requires 100 μl of blood and takes only a few hours for detection.

It has been suggested that fetal blood of less than 18 weeks gestation, contains very few neutrophils,[16] there is the theoretical possibility that only a selected population of these cells adheres to glass. The PMA is an exceptionally powerful stimulant and when we used it in conjunction to adherence of glass slides, it causes transformation of nearly 100% of all normal and reduces NBT to formazan deposits. The positive result in cord fetal blood indicates that this test may be used for antenatal diagnosis of affected boys and carrier females. We conclude that this test is a suitable NBT method and could allow antenatal diagnosis of the disease.

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