Mechanism of the Heparin Effect on the Nitroblue-Tetrazolium Slide Test

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The nitroblue-tetrazolium (NBT) slide test has been applied widely to assist in the diagnosis of bacterial infection. The test involves mixing a small amount of heparinized blood with a solution of NBT and noting the percentage of neutrophils that contain reduced NBT after a fixed period of incubation. Although it has been recognized that anticoagulants can influence the scores obtained, the mechanism of this effect has not been widely appreciated. We have observed that particles are formed when NBT and heparin are mixed, and that these particles are ingested by human neutrophils. Major changes in human neutrophil metabolism result from minor changes in the relative concentrations of heparin and NBT. These observations may explain many discrepancies that have been reported on the NBT slide test by various investigators.

Since its introduction by Park et al. (8) as a means of distinguishing bacterial infection from other causes of febrile illness, the nitroblue-tetrazolium (NBT) slide test has received both enthusiastic support and severe criticism. Two recent extensive studies have indicated that the NBT test may be less accurate than other clinical and hematologic indicators for the diagnosis of bacterial infection (10, 11). Many modifications in the test and much controversy have centered on the type of anticoagulant used (3–5). Although most investigators have used heparinized blood for testing, too little attention has been directed to the heparin concentration, and, as a result, this has often varied in different studies. In the methods of Park et al. (8) and of Matula and Paterson (7), the need for rigid standardization of heparin concentration has not been stated, but 75 to 100 U of heparin per ml of blood is recommended.

In examining the mechanism of NBT uptake by neutrophils, we have observed heparin and NBT to form an insoluble particulate complex that was ingested by human neutrophils (PMN). We examined the effect of heparin concentrations on NBT particle formation and measured the effect of the ingestion of these complexes on the metabolism of neutrophils. We found the particles to be ingested and to produce a metabolic response resembling that induced by other particles. Our observations can explain many of the discrepancies in the NBT slide test and suggest that so-called "spontaneous" NBT reduction is measured under conditions wherein PMN are partially stimulated by NBT-containing particles.

MATERIALS AND METHODS

Formation of heparin-NBT particles. NBT (Sigma, St. Louis, Mo.) was dissolved in phosphate-buffered saline (PBS Ca2+ and Mg2+ free) at a concentration of 0.1% and was filtered through a membrane filter (0.22 μm pore size; Naglene, Rochester, N.Y.) prior to use. Powdered heparin (Sigma) was dissolved in either PBS or fresh human plasma anticoagulated with 1 U of heparin per ml of blood. Heparin solutions contained 10, 100, and 500 U/ml. Particles were formed by adding equal volumes of NBT and the respective heparin stock solutions. Our final concentrations of heparin employed were comparable to those used in the clinical study of Hellum and Solberg (4), and include the concentrations used in the method of Park et al. (8).

Quantitative NBT reduction. The effect of heparin-NBT particles on the quantity of NBT reduced by PMN was evaluated by the method of Baehner and Nathan (1) with several modifications: Hanks balanced salt solution, pH 7.4, was substituted for Krebs-Henseleit buffer; erythrocytes were sedimented with 3% dextran in normal saline rather than by fibrinogen; contaminating erythrocytes were removed by hypotonic lysis instead of by treatment with 0.87% ammonium chloride, and a single extraction with 3 ml of hot pyridine (100 C, 15 min) was performed to solubilize the reduced formazan. Cell volumes, incubation conditions, and cell concentrations were those of Baehner and Nathan (1). Particles were generated by adding 0.1-ml portions of appropriate heparin stock solutions prior to the addition of PMN. Optical density of reduced formazan was measured at 515 nm. Comparative values were obtained by adding 50
μlites of a 10% suspension of washed 0.79-μm polyvinyl latex spherules (Dow Chemical Co., Midlands, Mich.). Cell-free plasma caused appreciable NBT reduction and partially solubilized the formazan produced, preventing accurate measurement of quantitative NBT reduction by PMN in the presence of plasma.

Oxygen consumption. The effect of heparin NBT particles on oxygen consumption by neutrophils was measured with a Gilson model KM oxygraph (GME, Middleton, Wis.) fitted with a Clark-YSI electrode. Each sample contained 10⁷ PMN and 0.05% NBT in PBS or PBS-50% plasma. Neutrophils were stimulated by the addition of heparin or a 10% suspension of latex spherules. Basal and stimulated rates of oxygen consumption were measured for 7 to 10 min at 37°C and calculated from the slopes.

RESULTS

Heparin-NBT particle formation. When sufficient heparin was added to NBT in buffer or plasma, a particulate suspension developed (Fig. 1). At low heparin concentrations the particles formed rapidly, were large and widely dispersed in the suspending medium, and sedimented promptly. With increasing heparin concentration many more particles were formed, but they were smaller and formed more stable turbid suspensions. In plasma, visible particle formation did not occur at 5 U of heparin per ml but was evident at the higher concentrations.

Oxygen consumption and NBT reduction. The effect of heparin-NBT particles on O₂ consumption and NBT reduction is illustrated in Fig. 2. In control experiments, heparin or NBT alone did not stimulate O₂ consumption by PMN. Addition of increasing quantities of heparin to PMN in a standard concentration of NBT caused a progressive increase in neutrophil O₂ consumption, whether measured in buffer or plasma. When 50% plasma was present, the increase was of lower magnitude, with no measurable increase at 5 U of heparin per ml. At heparin concentrations of 50 U/ml, O₂ consumption was more than twice the resting value either in plasma or buffer. Addition of latex
spherules to cells in the presence of NBT caused a 3.8-fold increase in $O_2$ consumption.

Heparin-NBT particles produced a larger increment in measured NBT reduction by PMN than did latex spheres. At a heparin concentration of 50 U/ml, the increment was double that produced by latex spheres.

**Ingestion.** The particles formed by NBT and heparin were amorphous, nearly colorless, and poorly refractile, making direct light microscopic observation of ingestion impossible. We noted that particles were also formed if heparin was mixed with 0.1% toluidine blue. These particles were dark blue and their ingestion by neutrophils was readily observed microscopically.

**DISCUSSION**

We have demonstrated that particles containing NBT are formed when NBT and heparin are mixed under conditions used in the NBT slide test, as introduced by Park et al. (8). Undoubtedly, the presence of erythrocytes in the blood-NBT mixtures has obscured the detection of these particles by users of the test, for they would be readily apparent if the test were performed with leukocytes suspended in plasma. Segal and Levi have demonstrated the ingestion of heparin-NBT particles by PMN by electron microscopy (9). Our studies demonstrate that these particles, once ingested, suffice to initiate the metabolic response produced by the phagocytic uptake of other particles. This includes augmented oxygen consumption and the reduction of NBT to an insoluble colored formazan. Formation of NBT-containing particles appears to be essential for the process of NBT reduction. Segal and Levi found that endotoxin did not cause augmented NBT reduction by leukocytes when soluble NBT was present in the mixture, but did under conditions wherein particulate NBT was formed (9).

Nitroblue tetrazolium is a basic dye that bears a positive charge in the oxidized state (2). Heparin, a sulfated mucopolysaccharide with strongly acidic characteristics in solution, has the capacity to form ionic complexes with cationic proteins (2) and fibrinogen (9). Jaques, in 1943, first demonstrated that heparin also forms ionic complexes with various positively charged dyes and organic bases, such as benzidine and toluidine blue. He showed these salts to vary widely with respect to solubility and dissociation (6). The formation of a particulate complex with heparin and NBT provides another example of this phenomenon.

In their studies on the effect of heparin concentration on NBT scores, Hellum and Solberg found good correlation of NBT scores and infection, and little overlap with control values only when heparin concentration was controlled at 10 U per ml of blood (4). Recent critical clinical studies of the value of the NBT test by Steigbigel et al. (11) and by Segal et al. (10) have employed the methods of Matula and Paterson (7) and Park (8), respectively. In neither of these papers is there mention of the heparin concentrations actually employed.
In theory, the NBT test should discriminate situations wherein phagocytic activity and oxidative metabolism by PMN are increased over control values. The sensitivity of the test has been greatly impaired by its performance under conditions that minimize or abolish these differences between control PMN and those being tested. Based on our studies of the effect of heparin concentration on NBT reduction, we believe that it is mandatory to rigorously control this concentration. We would suggest that blood to be tested be anticoagulated with heparin at a concentration of 10 U/ml. This level caused no stimulation of oxidative metabolism by normal PMN suspended with NBT in 50% plasma and proved satisfactory in the clinical experiments of Hellum and Solberg (4). A final judgment of the clinical value of the NBT test should be reserved until an adequate trial utilizing controlled, low concentrations of heparin has been performed.

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LITERATURE CITED