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

DAVID C. HOHN AND ROBERT I. LEHRER
Departments of Surgery and Medicine, and the Cancer Research Institute, University of California,
San Francisco, San Francisco, California 94131

Received for publication 16 May 1974

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-tetra-
zolium (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 diag-
nosis 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 atten-
tion has been directed to the heparin concen-
tration 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 phos-
phate-buffered saline (PBS Ca⁺⁺ and Mg⁺⁺ free) at
a concentration of 0.1% and was filtered through a
membrane filter (0.22 μm pore size; Naglene, Roch-
ester, 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 compa-
rable to those used in the clinical study of Hellem and
Solberg (4), and include the concentrations used in
the method of Park et al. (8).

Quantitative NBT reduction. The effect of hepa-
in-NBT particles on the quantity of NBT reduced by
PMN was evaluated by the method of Baehner and
Nathan (1) with several modifications: Hanks bal-
anced 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, incu-
bation 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
μliters 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 O₂ 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.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants CA 11067, AI 10547, and GMO 1474-08 SUR from the National Cancer Institute, the National Institute for Allergy and Infectious Diseases, and the National Institute for General Medical Sciences, respectively. Additional support was from the American Cancer Society (CF-3258) and a grant from the Research Corporation (Brown-Hazen Fund).

LITERATURE CITED