THE MECHANISM OF THE ENTRY OF DYE INTO NEUTROPHILS IN THE NITROBLUE TETRAZOLIUM (NBT) TEST

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(Received 24 July 1973)

SUMMARY

1. Washed buffy-coat preparations of human blood suspended in different media were exposed to the Nitroblue Tetrazolium (NBT) and the mechanism of entry of the dye into neutrophil polymorphonuclear leucocytes was investigated.

2. Stimulation of neutrophils with endotoxin and the presence of heparin and/or fibrinogen in the suspending medium were necessary for dye reduction.

3. NBT complexed with and precipitated heparin and/or fibrinogen from solution. The percentage of cells reducing the dye and the degree of precipitation of fibrinogen and heparin depended on the concentration of dye; the critical concentration of NBT necessary for each effect was the same.

4. Electron microscopy of stimulated neutrophils revealed the presence of amorphous material, which was probably complexed NBT, outside the cell, in the process of endocytosis and within membrane-bound vacuoles within the cytoplasm.

5. Neither complement nor immunoglobulins were obligatory for dye reduction in this system.

6. It is suggested that NBT only enters neutrophils in quantities visible by light microscopy, after stimulation which produces phagocytosis of a macromolecular complex of the dye and heparin and/or fibrinogen.

Key words: infection, granulocytes, phagocytosis, fibrinogen, tetrazolium salts.

The Nitroblue Tetrazolium (NBT) test is a rapid, simple non-specific method of distinguishing bacterial, fungal and protozoal infections from other disease processes such as viral infections, autoimmune diseases, neoplasms and rejection of allografts (Park, Fikrig & Smithwick, 1968; Matula & Paterson, 1971; Anderson, 1971; Wollman, David, Brennan, Lewy, Stenzel, Rubin & Miller, 1972). The test is performed by incubating a small volume of heparinized blood with a dilute solution of Nitroblue Tetrazolium. This dye is water-soluble and light yellow in colour.

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but forms a dark-blue water-insoluble formazan compound on reduction. When the test is performed on the blood from a normal person, a small proportion of the neutrophil polymorphonuclear leucocytes contain formazan deposits in their cytoplasm. When more than 10% of neutrophils are found to contain formazan deposits, the test is classified as positive and this strongly suggests the presence of a bacterial or other treatable infection.

The mechanisms of the entry of the NBT into the cell and of its provocation by infection are unknown. It has been postulated that the finding of formazan deposits in neutrophils is related to their phagocytic activity and that the increased metabolic activity associated with phagocytosis is responsible for the reduction of NBT to formazan (Park et al., 1968). Park (1971) holds the view that a change of membrane permeability is necessary to allow the entry of NBT into the cytoplasm. The activation of complement has been implicated as a result of the decrease of sensitivity of the test when ethylenediaminetetra-acetate (EDTA) is used as anticoagulant instead of heparin (Park & Good, 1970). It has been suggested further, that immunoglobulins have a functional role because of the finding of false negative results in infected hypogammaglobulinaemic patients (Freeman & King, 1972).

**METHODS**

**Preparation of cells**

Blood samples were collected from healthy volunteers between the ages of 18 and 30 years. Blood samples from different individuals were used for each experiment.

A portion (25 ml) of heparinized blood (50 units/ml) was centrifuged at 450 g for 10 min at 15°C. The buffy coat (approximately 5 ml) was aspirated with a Pasteur pipette and washed three times in 50 ml of Hanks balanced salt solution at 4°C. After each wash, the suspension was centrifuged at 200 g for 10 min at 15°C. After the final wash, most of the supernatant was discarded and the washed cells were mixed by gentle shaking. A portion (0.4 ml) of the washed cell preparation was mixed with 0.6 ml of the suspending media.

**Suspending media**

The washed cells from four individuals were suspended in each of the following solutions: (i) autologous serum; (ii) autologous serum containing disodium EDTA (4.0 mg/ml); (iii) autologous serum containing mucous heparin (Weddel Pharmaceuticals) (70 units/ml); (iv) Hanks solution (Oxoid); (v) Hanks solution containing mucous heparin (10 units/ml); (vi) Hanks solution containing disodium EDTA (4.0 mg/ml). Fibrinogen (Kabi) in sodium chloride solution (0.15 mol/l) was added to aliquots of each of suspensions (ii)–(vi) to give a final concentration of 5 mg/ml. Additional studies were done with solutions (i) and (ii).

**Neutrophil stimulation**

To cause stimulation, 10 μg of *Escherichia coli* endotoxin (type O127 : B8, Difco) in 0.05 ml of phosphate (0.075 mol/l)–saline (0.15 mol/l) mixture (pH 7.2) was added to a 1.0 ml sample of each of the above suspensions in a disposable plastic test tube (Luckham L/P 3S) and the tubes were incubated for 10 min at 37°C. This amount of endotoxin was used because in a dose-response study it was the minimum necessary to give a maximal response. Similar amounts of suspension were incubated without endotoxin.
Entry of dye into neutrophils in NBT test

Incubation with NBT

To 0.5 ml of the stimulated and unstimulated cell suspensions was added 0.5 ml of a 0.1% solution of NBT (Sigma) in phosphate (0.075 mol/l)-saline (0.15 mol/l) mixture (pH 7.2), which was centrifuged at 1000 g for 10 min immediately prior to use. The mixture was gently shaken and incubated at 37°C for 30 min.

Staining and counting of neutrophils

Thin smears were made on glass slides, air-dried and stained with Leishman's stain. Then 100 neutrophils were counted on each slide and those containing a densely staining formazan deposit at least the size of one lobe of the nucleus of the cell were classified as positive. All counts were performed by the same person (A.W.S.). Twenty slides were re-examined and the standard deviation of a single count (percentage of positive cells) was estimated to be 2.2.

Precipitation of fibrinogen by NBT

To 0.6 ml samples of fibrinogen (5 mg/ml) in Hanks solution with and without EDTA (4.0 mg/ml) and to plasma from blood anticoagulated with EDTA (2.4 mg/ml) was added 1.0 ml of 0.1% solution of NBT in phosphate-buffered saline. These mixtures were gently shaken and incubated in a water bath at 37°C for 30 min. After centrifugation at 500 g for 10 min, the deposit was washed three times with distilled water. The washed deposit was dissolved in Hanks solution by incubation in a water bath at 37°C for 30 min. The solution was then centrifuged at 1000 g for 10 min. Thrombin (bovine, Parke-Davis, 50 units/ml) was added to the supernatant and the mixture observed for the presence of a clot. Precipitates produced by the addition of NBT to plasma anticoagulated with EDTA were redissolved in a similar manner. Electrophoresis of these redissolved precipitates and a standard solution of fibrinogen (Kabi) in saline (5.0 mg/ml), against whole human antiserum (Burroughs Wellcome) was performed by a modified Laurell technique (Minchin Clarke & Freeman, 1968).

Precipitation of heparin by NBT

To 0.6 ml of a solution of heparin (70 units/ml) in Hanks solution and 0.6 ml of plasma from blood anticoagulated with heparin (50 units/ml) was added 1.0 ml of a 0.1% solution of NBT in phosphate-buffered saline. The solutions were examined microscopically, using ordinary and polarized light, for precipitated particles, as were control solutions of heparin in Hanks solution and plasma, and the 0.1% solution of NBT. Reduction of the solutions was performed by the addition of phenazine methosulphate (BDH) and NADH (Boehringer). To investigate whether or not the precipitates obtained above contained NBT, they were dialysed against water for 72 h using a cellulose membrane and then reduced by the addition of phenazine methosulphate and NADH and examined for blue coloration.

Measurement of the relationship between the concentration of NBT, the precipitation of fibrinogen and heparin, and the percentage of positive neutrophils

A portion (1.0 ml) of solutions of NBT in phosphate-buffered saline in concentrations of 0.1-0.0032 g/100 ml was added to 1.0 ml of whole blood anticoagulated with EDTA (2.4 mg/ml) or heparin (50 units/ml). Endotoxin stimulation, incubation, staining and counting were carried out as described above. Portions (1.0 ml) of solutions of NBT in phosphate-buffered
saline in concentrations of 0.2–0.0032 g/100 ml were added to 0.6 ml of plasma to which was added 0.03 μCi of \(^{125}\text{I}\) labelled fibrinogen (The Radiochemical Centre, Amersham) and to 0.6 ml of plasma from heparinized blood (50 u/ml) containing 0.03 μCi of \(^{125}\text{I}\) labelled fibrinogen plus 0.28 μCi of heparin \(^{35}\text{S}\) sulphate (corresponding to 60 μg, The Radiochemical Centre, Amersham). After incubation for 30 min at 37°C the mixtures were centrifuged at 4000 g for 30 min. The gamma activity in the supernatant was counted using a well-type sodium iodide detector. The samples containing no NBT gave about 14 000 c.p.m. and those with the highest concentration of NBT gave about 1200 c.p.m., the background being 20 c.p.m. The \(^{35}\text{S}\) activity was counted in a liquid-scintillation system, the corresponding count rates being about 25 000 c.p.m. to about 1000 c.p.m. with a background count of 25 c.p.m. Each sample was counted for 100 s with a cut-off at 10 000 counts. The observed beta counts were corrected for the contribution due to \(^{125}\text{I}\), which amounted to 13·6% of the observed gamma counts.

Effect of centrifugation of the NBT solution on the reduction of NBT by neutrophils

A solution of 0.1% NBT in phosphate-buffered saline was centrifuged at 30 000 g for 16 h. A portion (1.0 ml) of this solution was added to 1.0 ml of blood anticoagulated with either EDTA (2.4 mg/ml) or heparin (50 u/ml) with and without prior stimulation with endotoxin as described above. Incubation, staining and counting were performed as above. After centrifugation the solution of NBT was examined microscopically, using ordinary and polarized light, for the presence of particles.

Electron-microscopic studies of leucocytes after exposure to NBT

A portion (1.0 ml) of 0.1% NBT was added to 1.0 ml of whole blood, using EDTA (2.4 mg/ml) and heparin (50 u/ml) as anticoagulant after prior incubation of the blood with endotoxin (10 μg/ml) at 37°C for 10 min. The mixture was incubated in a water bath at 37°C for 15 min, centrifuged at 400 g for 60 min and the buffy coat aspirated with a Pasteur pipette, fixed by the method of Hirsch & Fedorko (1968), embedded in Epon (G. T. Gurr and Searle Scientific Supplies, Bucks.). Thin sections were cut and examined unstained and after staining with uranyl acetate and lead citrate (Renolds, 1963) in an A.E.I. EM6B electron microscope. Control samples were prepared by excluding the NBT or endotoxin from the above mixtures.

RESULTS

Reduction of NBT by stimulated neutrophils suspended in various media

A small percentage of neutrophils were found to be NBT-positive when suspended in serum or Hanks solution with or without the addition of disodium EDTA (Table 1). Prior stimulation with endotoxin did not affect these results. The addition of fibrinogen resulted in a small increase in the percentage of positive cells in the absence of stimulation but in a marked increase with stimulated cells. In the presence of heparin, a large percentage of the neutrophils suspended in serum or Hanks solution became positive regardless of whether or not fibrinogen was added. The discrepancy between the percentage of positive cells found when stimulated cells were suspended in serum plus heparin (70 units/ml) or Hanks solution plus heparin (10 units/ml) can be attributed to the different concentrations of heparin added to the two solutions, a lower concentration being added to Hanks solution to prevent the clumping of cells caused by higher concentrations.
Precipitation of fibrinogen by NBT

Solutions of fibrinogen in Hanks solution with or without added EDTA, or plasma anticoagulated with EDTA, all yielded a precipitate after incubation with NBT. The precipitates were dissolved and subsequently formed clots upon the addition of thrombin, indicating the presence of fibrinogen. Immunoelectrophoresis of the redissolved precipitate from plasma showed that fibrinogen, identified by its electrophoretic mobility, formed the major protein component. A number of minor protein peaks were also seen. These probably result from entrapment of protein within the fibrinogen precipitate but could be due to precipitation of other proteins by NBT on a smaller scale than in the case of fibrinogen.

<table>
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<th>Additions</th>
<th>Serum</th>
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<th>Hanks</th>
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<tbody>
<tr>
<td></td>
<td>+ EDTA</td>
<td>+ heparin</td>
<td>solution</td>
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<tr>
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<td>0 (0)</td>
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<td>1 (0-2)</td>
<td>0 (0-1)</td>
<td>12 (0-32)</td>
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<td>16 (10-25)</td>
<td>28 (18-45)</td>
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No. of experiments
10 6 4 4 4 4

Precipitation of heparin by NBT

Numerous small precipitated particles could be seen on microscopy with both ordinary and polarized light after the addition of NBT to the solution of heparin. These particles developed a dark-blue colour upon reduction.

Demonstration of NBT in the precipitates of fibrinogen and heparin

Unreduced NBT was found to be freely dialysable across a cellulose membrane. After dialysis against water for 72 h the precipitates of fibrinogen and heparin were incubated with a reducing solution. The development of a deep-blue coloration of these precipitates indicated the presence of formazan. Thus NBT was not dialysed off the precipitates.

Effect of decreasing the concentration of NBT on the precipitation of fibrinogen and heparin and on the reduction of NBT by stimulated neutrophils

In EDTA anticoagulated blood and plasma, concentrations of NBT greater than 0.05% caused almost total precipitation of fibrinogen and resulted in a significant percentage of stimulated neutrophils becoming NBT-positive. Concentrations of NBT of 0.025% or less resulted in little fibrinogen precipitation or NBT reduction by neutrophils. The critical con-
centration of NBT necessary for reduction by a significant percentage of neutrophils and for marked fibrinogen precipitation was similar (Fig. 1). In heparinized plasma, NBT caused the precipitation of both fibrinogen and heparin. The precipitation of fibrinogen and heparin and the percentage of positive cells were decreased in parallel by dilution of the dye (Fig. 2).

![Graph](image)

**Fig. 1.** Relationship between the percentage of NBT-positive neutrophils in blood anticoagulated with EDTA and stimulated with endotoxin (○, mean of five studies ± 1 SEM) and the percentage of ¹²⁵I-labelled fibrinogen precipitated from EDTA-treated plasma upon the addition of varying concentrations of dye (▲, mean of duplicate values shown).

**Effects of centrifugation of the NBT solution**

No particulate matter could be detected on microscopic examination of the solution of NBT after centrifugation at 30,000 g for 16 h. Use of this NBT solution did not decrease the percentage of NBT-positive neutrophils in stimulated blood when compared with solutions of NBT prepared in the standard manner.

**Electron-microscopic studies of leucocytes after exposure to NBT**

Membrane-bounded vacuoles, containing densely staining amorphous material, were found in the cytoplasm of a large proportion of the monocytes and neutrophils from blood exposed to both NBT and endotoxin. Similar amorphous less densely staining material was also seen both extracellularly and undergoing phagocytosis (Fig. 3).

The neutrophils appeared swollen and their outer membranes were partially ruptured. The
Entry of dye into neutrophils in NBT test

**Fig. 3.** Stained electron micrograph of a neutrophil after exposure to NBT and endotoxin, showing membrane-lined vacuoles containing amorphous material (V), amorphous material outside the cell (A) and undergoing phagocytosis (P), nucleus (N) and outer membrane (M). Magnification $\times 12\,000$.

**Fig. 4.** Unstained electron micrograph of a neutrophil after exposure to NBT and endotoxin showing amorphous material outside the cell (A), amorphous material containing electron-dense particles within the cell (P), nucleus (N) and outer membrane (M). Magnification $\times 12\,000$.

(Facing p. 822)
amorphous material was seen purely extracellularly in blood exposed to NBT alone but was not seen after exposure to endotoxin in the absence of NBT. Neither cellular swelling nor abnormalities in the outer membrane of neutrophils were found in cells that were not exposed to a combination of both endotoxin and NBT. Unstained sections showed the amorphous material to contain electron-dense, elongated, somewhat needle-shaped particles approximately 150–500 nm in length. These particles were most numerous when the amorphous material was within phagocytic vesicles and could represent formazan deposits (Fig. 4).

**DISCUSSION**

The availability of a rapid indicator of bacterial infection has important clinical application. It gives direction to further lines of investigation and helps safeguard against indiscriminate therapeutic manoeuvres. An understanding of the mechanism of such a test delineates exactly what it is that is being measured and makes the interpretation of the results obtained more
logical. In order to investigate this a model system was used. This system is basically similar to
the standard NBT test, but differs in that endotoxin was used as an artificial stimulus of neutro-
phil activity.

The present study shows that NBT has a dual action. It causes the precipitation of a macro-
molecular complex, the nature of which depends upon the anticoagulant used. In the case of
heparin, both heparin and fibrinogen are precipitated, whereas in the presence of EDTA,
fibrinogen alone is precipitated. The cationic precipitation of heparin (Scott, 1968) and fibrino-
gen (Surgenor, 1952) have been described and although precipitation of these substances by
NBT has not been investigated, this would seem a likely mechanism. The NBT could not
be dialysed from the macromolecular precipitate and it is therefore either bound to or enmeshed
within this precipitate. Phagocytosis of the precipitated material results in concomitant
phagocytosis of dye which turns dark blue after intracellular reduction. The blue colour is
easily seen under the light microscope and the cell containing it is labelled NBT-positive.

The failure of NBT uptake and reduction by stimulated cells when the dye is presented to
them in solution emphasizes the necessity for the phagocytosis of NBT in association with
particulate matter. The phagocytosis of these complexes with their associated dye explains
how the NBT enters the cell, and why the formazan 'granules' assume such bizarre formations
within the cytoplasm. The neutrophils cluster around particulate matter, probably in attempted
phagocytosis, and this clumping leads to difficulty in making accurate counts of positive cells
in a blood smear. This is the main technical problem in the test of Park et al. (1968). NBT tends
to crystallize out of solution in the concentrations in which it is used in the Park et al. (1968) test.
Phagocytosis of NBT crystals is not a significant mode of entry of dye into neutrophils because
significant NBT reduction does not occur in the control tests in the absence of heparin or
fibrinogen and removal of these residual crystals by high-speed centrifugation does not
decrease the number of neutrophils becoming NBT-positive.

When the NBT test is performed on the blood of patients with bacterial infection a smaller
percentage of the neutrophils become NBT-positive if EDTA is used as anticoagulant instead
of heparin. This has been interpreted as resulting from complement inactivation by EDTA
(Park & Good, 1970). The demonstration that the addition of ficoll to the NBT solution can
reverse this effect (Gordon, Rowan, Brown & Carson, 1973), that a normal NBT response can
be obtained in the blood of C6 (complement fraction)-deficient rabbits and C4-deficient
guinea-pigs (A.W. Segal, unpublished work), and in cells suspended in a balanced salt solution,
suggests that EDTA does not mediate its effect through complement inactivation. It is more
likely that the nature of the precipitates presented to the neutrophils for phagocytosis deter-
mines the degree of dye reduction.

Similarly, as washed cells can reduce NBT when suspended in Hanks solution in the presence
of heparin, it is unlikely that soluble immunoglobulins are essential for NBT reduction.
Although these experiments do not completely exclude the possibility that adsorbed immuno-
globulins play a role, in another study, using similar methods, it was found that in only four
of twenty hypogammaglobulinaemic patients was a negative NBT test obtained after endotoxin
stimulation in vitro. The reduction of NBT was unrelated to the pattern of immunoglobulin
deficiency and occurred despite immeasurably low (less than 2 mg/100 ml) serum levels of
IgG, IgA or IgM (immunoglobulins G, A and M respectively). When neutrophils from those
patients in whom impaired NBT reduction was observed were suspended in normal plasma
the deficient reduction was not corrected. Nor was NBT reduction decreased in normal cells
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suspended in their hypogammaglobulinaemic plasma (Segal & Webster, unpublished work). These facts imply that the primary defect of NBT reduction by the neutrophils of hypogammaglobulinaemic patients is cellular rather than humoral.

A positive NBT test depends upon enhanced phagocytic activity of neutrophils. This may result from the presence of bacteria, or products thereof, in the peripheral circulation (Cohn & Morse, 1960). The minimum concentration of E. coli endotoxin necessary to produce a positive test in vitro in this model system is 1 ng/ml (Segal, unpublished work), which is in the concentration range found in endotoxaemia in vivo (Levin, Poore, Zauber & Oser, 1970; McGill, Porter & Kass, 1970). Circulating bacterial products could be the natural stimulus of phagocytosis that is demonstrated by a positive NBT test in patients with bacterial infection. The percentage of positive cells never reaches 100% under any of the experimental circumstances investigated even when a large excess of endotoxin was used in vitro. This raises the possibility that there may be more than one functional population of neutrophils.

The conversion of a neutrophil into an NBT-positive cell encompasses a wide spectrum of neutrophil function, from the ability to be stimulated to phagocytic activity, through phagocytosis, to reduction of the dye by functional intracellular reductase enzyme systems. Thus an in vitro stimulation test is a good screening test of normal neutrophil function, and quantitation of such a test has enabled the demonstration of partial defects of neutrophil function in a series of patients (Segal, unpublished work).

ACKNOWLEDGMENTS

We would like to thank Dr E. Wills for electron-microscopic studies, Dr H. Minchin Clarke for immunological studies, the staff of the Radioisotopes Division for the dispensing and supply of the radioactive materials and the radioactive sample counting, and Dr G. L. Asherson, Dr P. Lachman, Dr A. S. McFarlane and Dr. N. Veal for valuable discussion and advice. A.W.S. held a Vera Levi Memorial Fellowship.

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