

The cytocentrifuge NBT test

P. A. GORDON, J. STUART, T. R. LEE, G. R. BREEZE, AND R. N. H. PUGH

From the Department of Haematology, The Children's Hospital, Birmingham and the Department of Communicable and Tropical Diseases, East Birmingham Hospital, Birmingham

SYNOPSIS The NBT test is a non-specific test of neutrophil membrane stimulation which has application to the study of neutrophil function, particularly in the septicæmic patient. An improved cytochemical test which eliminates potential sources of laboratory error has been developed. Venous or capillary blood samples may be studied and the technique can be applied to the neutropenic patient since available neutrophils are concentrated by cytocentrifugation. Clinical evaluation in 443 patients is described.

The nitroblue tetrazolium (NBT) test was introduced by Park *et al* (1968) as a rapid diagnostic test for systemic bacterial infection. Its potential value was soon anticipated (*Lancet*, 1971) and early reports supported its value (Feigin *et al*, 1971; Freeman *et al*, 1973; Wollman *et al*, 1972; Gordon *et al*, 1973).

Subsequent reports have, however, shown overlap between control and infected patients and have illustrated numerous examples of false positive and false negative results (Segal, 1974). Several authors have concluded that the test is not of significant diagnostic value as a routine screening test for systemic infection (Segal *et al*, 1973; Steigbigel *et al*, 1974). Reports from different laboratories using the same basic method have conflicted so markedly, however, as to suggest that technical factors inherent in the original method might be responsible for some of the discrepant results. The present study was therefore undertaken to identify and eliminate as far as possible those variables in the test procedure which are potential sources of laboratory error.

Materials and Methods

Venous blood was taken into disposable plastic syringes and transferred to plastic tubes containing heparin (mucous, preservative-free, Weddel Pharmaceuticals) at a final concentration of 20 units/ml, unless otherwise specified. The tests were performed immediately or within 2 hours' storage at 4°C.

Heparinized blood (100 µl) was then pipetted by means of a plastic-tipped micro-pipette (Finnpipette, Jencons) into a 10 ml capacity plastic tube and allowed to warm for 5 minutes in a waterbath at

37°C. Nitro-blue tetrazolium (Sigma, St Louis) was dissolved by gentle shaking at room temperature in equal volumes of phosphate buffer (0.15 moles per litre, pH 7.2) and 0.85% sodium chloride to give a concentration of 0.075% w/v. The NBT solution was pre-warmed to 37°C, and 100 µl were then pipetted into the tube containing 100 µl heparinized blood to start the incubation reaction.

This reaction was terminated after exactly 10 minutes at 37°C in a waterbath by adding 2 drops (40 µl) of formol-saline fixative (0.5 ml 40% w/v formaldehyde added to 4.5 ml physiological saline). Fixation was allowed to continue at room temperature for 3-4 minutes, and 3 ml of distilled water was then added and mixed by gentle inversion for 20 seconds. Isotonicity was restored by the immediate addition of 1.0 ml of 3.4% sodium chloride and gentle inversion (Nelken *et al*, 1960). After standing for approximately 5-10 minutes at room temperature until haemolysis was complete, aliquots of the haemolysate were then spun on to glass slides in a cyto-centrifuge (Shandon Southern Ltd) at 750 rev/min for 5 minutes. The slides were air-dried and counter-stained with 0.1% w/v aqueous neutral red for 45 seconds. Neutrophils containing any formazan deposit, regardless of size and distribution, were regarded as positive, and the score for 100 neutrophils was determined using a ×50 oil-immersion objective.

In vitro stimulation of polymorphs with *Escherichia coli* endotoxin (026:B6 Difco) was achieved by adding 15 µg of endotoxin (in 50 µl phosphate-buffered saline) to 0.5 ml blood and incubating at 37°C for 10 minutes.

Three types of collecting pipette for capillary blood were studied—a heparinized glass micro-

haematocrit capillary tube (Red-Tip, Sherwood Medical Industries, St Louis) which gave a final heparin concentration of approximately 24 units per ml of blood; a Miale siliconized glass prothrombin pipette (Dade) into which was taken 10 μ l (2 units) of heparin solution (diluted to 200 units/ml phosphate-buffered saline, pH 7.2) followed by 0.1 ml of blood; and, finally, a plastic tube of 7.5 cm length and an internal diameter of 2.0 mm (from Ortho Monospot kit) and marked at 3.0 and 6.0 cm. This plastic tube was used as a pipette and initially rinsed several times with heparin solution (40 units/ml phosphate-buffered saline, pH 7.2) before filling to the 3.0 cm mark. Capillary blood was then taken until the heparin-blood mixture reached the 6.0 cm mark to give a final heparin concentration of 20 units/ml blood. Each of the three pipettes was filled by capillary action holding the pipette horizontally and using the second and subsequent drops of blood obtained after thumb prick by a disposable lancet. The contents were expressed immediately into an empty plastic pot and mixed by flicking, and 100 μ l was subsampled for the NBT test.

Patients

A total of 443 patients was studied. They consisted of 177 uninfected hospital patients (5-68 years) undergoing diagnostic venepuncture, 162 children (3 months-14 years) requiring diagnostic capillary blood collection, and 39 healthy antenatal patients. Venous blood samples from an additional group of 65 patients (3 months-72 years) admitted to an infectious diseases unit were coded, and the NBT score was determined without knowledge of the clinical state of the patient. All tests were performed immediately or within 12 hours' storage at 4°C.

Results

EFFECT OF ANTICOAGULANT

A final heparin concentration of 20 units/ml blood was selected after studying the effect of different heparin concentrations on resting and endotoxin-stimulated polymorphs (fig 1). This concentration was adopted since it provided adequate anti-coagulation, clear separation of NBT scores between resting and endotoxin-stimulated polymorphs, and also gave a score of less than 5% for unstimulated cells.

EFFECT OF VARYING NBT CONCENTRATION

The effect of varying the NBT concentration is seen in figure 2. Both resting and stimulated polymorphs showed an increase in NBT positivity with increasing concentration of NBT. A concentration of 0.075%

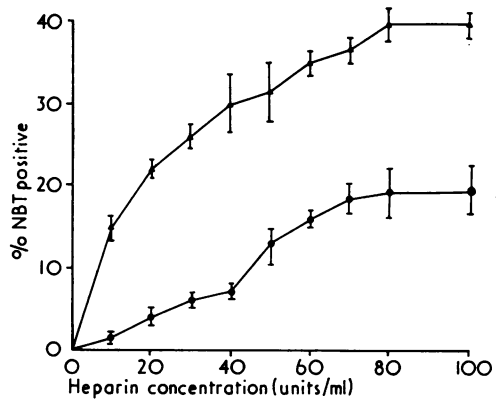


Fig 1 Effect of heparin concentration on NBT-positive score in unstimulated (●—●) and endotoxin-stimulated (▲—▲) neutrophils. Mean of four studies \pm SEM.

v/v NBT (75 mg/100 ml) was therefore adopted since this gave clear separation between resting and stimulated polymorphs, was readily dissolved in the buffered saline at room temperature, and gave a score of less than 5% for unstimulated cells.

EFFECT OF INCUBATION TIME

The effect on the NBT score of increasing the incubation time is seen in figure 3. When both the NBT solution and the test blood sample were pre-warmed to 37°C, then clear separation of the NBT score between resting and stimulated polymorphs was demonstrable after only 10 minutes' incubation. Incubation for 20 minutes or more resulted in NBT-positive scores greater than 10% in resting cells. The pattern of formazan precipitation in resting polymorphs was predominantly, but not exclusively,

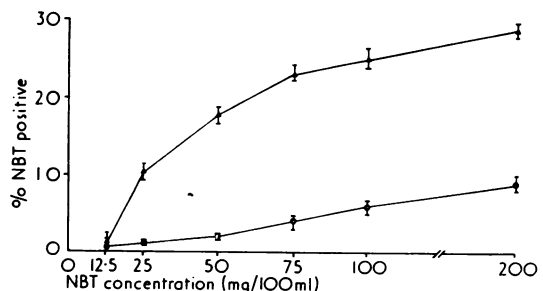


Fig 2 Effect of NBT concentration on NBT-positive score in unstimulated (●—●) and endotoxin-stimulated (▲—▲) neutrophils. Mean of two studies \pm SEM.

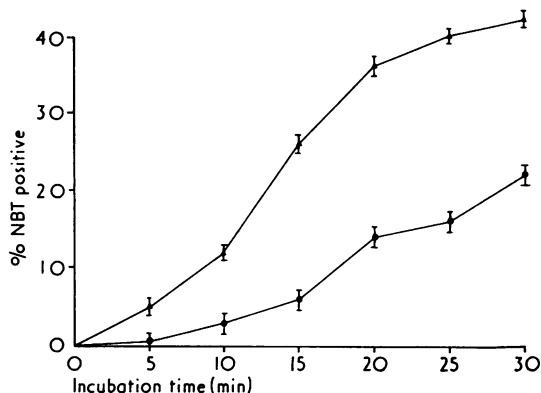


Fig 3 Effect of incubation time at 37°C on NBT-positive score in unstimulated (●—●) and endotoxin-stimulated (▲—▲) neutrophils. Mean of three studies \pm SEM.

a localized deposit. With an increase in NBT-positivity both localized formazan deposits and diffuse granular formazan precipitation developed. Formazan precipitation of either pattern was scored as NBT-positive.

EFFECT OF STORAGE

The effect of storage on resting polymorphs is seen in table I. Heparinized samples were stored at 4°C and at room temperature (22°C). The mean NBT scores for resting polymorphs increased after 2 hours at room temperature but remained virtually constant until 12 hours when stored at 4°C. After 12 hours, the mean NBT scores fell slightly in both groups and the morphological quality also deteriorated.

Storage Time (hours)	NBT Score (%)	
	22°C	4°C
Prior to storage	4.6	4.6
2	5.4	4.4
4	8.8	5.0
8	9.8	5.6
12	7.2	5.0
24	3.0	1.7

Table I Effect of storage at 22°C and 4°C on the NBT score for unstimulated neutrophils (mean of five studies)

NORMAL RANGE FOR VENOUS BLOOD

A normal range was determined in uninfected hospital patients on two occasions, separated by six months, by different workers. Closely similar results were obtained for both mean and range, and the results were therefore amalgamated to give a control group of 177 patients (table II).

	Venous Blood			Capillary Blood		
	Study 1	Study 2	Com- bined	Plastic Pipette	Siliconized Glass Pipette	Heparinized Glass Pipette
	(103)	(74)	(177)	(77)	(71)	(14)
Mean	4.2	4.6	4.4	4.4	7.7	13.0
SD	3.3	4.3	3.7	4.3	8.5	11.0
Mean + 2SD	10.8	13.2	11.8	12.9	24.0	35.0
Range	1-15	0-18	0-18	1-20	0-48	0-43

Table II NBT results obtained for venous and capillary blood from uninfected hospital patients.

Results recorded as % neutrophils containing any formazan deposit. Number of patients studied in parentheses.

NORMAL RANGE FOR CAPILLARY BLOOD

The siliconized and heparinized glass collecting pipettes gave rise to false high NBT scores compared with venous blood. When capillary blood was collected into a plastic tube, however, the results obtained for 77 uninfected patients were closely similar to the scores for 177 venous blood specimens taken into plastic containers (table II).

RESULTS FOR PATIENT GROUPS

The results obtained for seven patient groups are given in figure 4. The 39 antenatal patients comprised 11 in the first trimester, 11 in the second, and 17 in the third. A mean NBT score of 2.7% was obtained with a range of 1.8%. NBT scores for the 65 coded

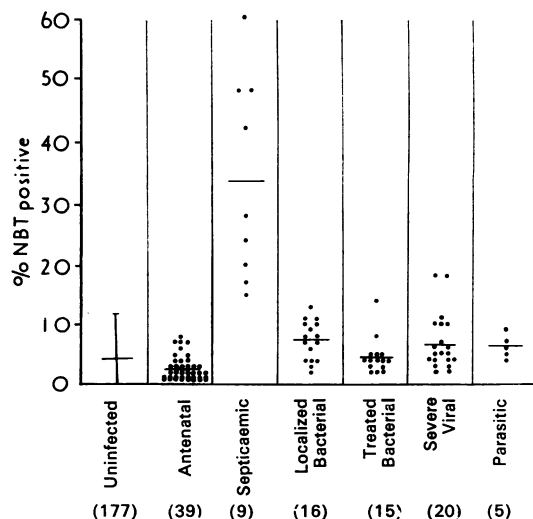


Fig 4 Individual NBT scores for seven patient groups. Number of patients studied in parentheses. Horizontal bars represent mean values. The results for 177 uninfected patients (table II) are shown as mean \pm 2 SD.

specimens for patients admitted to the infectious diseases unit were subsequently classified according to clinical diagnosis.

Blood cultures from the nine septicaemic patients were positive for meningococcus (4), *Escherichia coli* (2), *Staphylococcus aureus* (1), *Bacteroides* species (1), and *Salmonella typhi murium* (1). The mean NBT score was 33.5% with a range of 15-60%. The 16 patients with localized bacterial infection comprised pneumonia, acute follicular tonsillitis, septic arthritis, salpingitis, and infected wounds; there was no significant growth on blood culture. The mean NBT score was 7.3% with a range of 2-13%.

Fifteen patients with a treated bacterial infection gave a mean NBT score of 4.6% with a range of 2-14%. They had received antibiotics for from 36 hours to five days for either septicaemia (3 patients) or a localized bacterial infection (12 patients) before the NBT test.

Patients with severe viral infections requiring admission to hospital gave a mean NBT score of 6.8% with a range of 2-18%. Two adults with severe rubella and fatal herpes simplex encephalitis respectively gave NBT scores of 18%. The remaining patients suffered from severe measles, rubella, varicella, and infections with adenovirus, ECHO 9 and 14, and influenza C. In addition, four patients had herpes simplex, measles, or mumps encephalitis. Serological confirmation was obtained in 14 of the 20 cases.

Five patients had proven parasitic disease: four patients with an acute attack of benign tertian malaria and one patient with onchocerciasis. Their mean NBT score was 6.2% with a range of 4-9%.

Discussion

The tetrazolium salt NBT is believed to complex with heparin and fibrinogen before it enters the phagocytosing neutrophil (Segal and Levi, 1973). Subsequent reduction of NBT is then dependent on the activity of a nucleotide oxidase within the cell (Fridovich, 1974), the NBT substituting for oxygen in the oxidase reaction or being reduced by superoxide (Nathan, 1974). In patients with systemic bacterial infection there is an increase in phagocytic action, in nucleotide oxidase activity, and in reduction of NBT (Park *et al*, 1968; Feigin *et al*, 1971; Matula and Paterson, 1971; Gordon *et al*, 1973). The present study has confirmed, and defined more closely, technical variables which influence NBT reduction and may give rise to a false high score. The final method adopted has either eliminated major variables in the original method of Park *et al* (1968) or reduced their effect to a minimum.

The concentration of heparin used in previous studies has usually been 75 units/ml or above. False high scores with concentrations above 50 units/ml were reported by Björkstén (1974), and the present study has also demonstrated elevated scores with concentrations above 40 units/ml. At these relatively high concentrations any alteration in the relative proportion of heparin to blood becomes an important variable, as in Vacutainer blood samples which have given unreliable results (Wollman *et al*, 1972; Bittner *et al*, 1973; Steigbigel *et al*, 1974). Vacutainer samples are also prone to false high scores owing to the inevitable glass contact and should therefore not be used. A reduction in heparin concentration to 20 units/ml in the present study was found to be a less critical concentration for NBT reduction while providing adequate anti-coagulation.

The concentration of NBT itself is a less important variable although high concentrations may increase the percentage NBT score and give rise to solubility difficulties requiring filtration of the working solution (Björkstén, 1973). A reduction in concentration to 0.075% ensured ready solubility and provided clear separation between resting and endotoxin stimulated polymorphs.

The duration and temperature of the incubation reaction has varied in previous studies but most methods have adopted a two-level incubation temperature. This stems from the original method of Park *et al* (1968) which recommended incubation for 15 minutes at 37°C followed by 15 minutes at room temperature. It is unlikely, however, that the reagents are maintained at 37°C for longer than 7 minutes during the whole period (Björkstén, 1974). More precise control of the incubation temperature can be gained by the use of pre-warmed reagents and a thermostatically controlled waterbath. Since NBT continues to be reduced intracellularly at room temperature it is clearly important to terminate the reaction at a predefined point, and this can be achieved by formalin fixation.

Conventional NBT preparations show a tendency to neutrophil clumping and disruption (Ridgway and Johnson, 1973). Cells containing large formazan deposits are mechanically fragile and prone to cytoplasmic membrane disruption when subjected to the lateral shear involved in smearing on to glass; thus false low NBT scores may result. The cytocentrifuge technique avoids these difficulties, and the shorter incubation period plus post-fixation of the cells also preserve neutrophil morphology.

Previous workers have considered a cell to be NBT positive when it contains a block of formazan deposit of unspecified size (Park *et al*, 1968) or of a size equivalent to a neutrophil granule (Björkstén,

1973) or a nuclear lobe (Segal *et al*, 1973). Others have scored as positive a polymorph containing a stippled deposit (Matula and Paterson, 1971) or any type of deposit (Gordon *et al*, 1973). Semiquantitative assessment of the type of formazan deposit increases observer error, which has been shown to be considerable (Segal *et al*, 1973). The distribution of intracellular tetrazole-formazan deposits is influenced by extraneous factors such as lipid solubility and protein affinity (Pearse, 1972) and is not primarily dependent on the kinetics of oxidase enzymes within the cell. The incubation period should therefore be as short as possible to preserve cell morphology and minimize clumping while generating a visible formazan deposit; any deposit of formazan should then be regarded as positive.

An important application of the NBT test is the monitoring of patients at risk of developing systemic infection. In particular, this includes the neutropenic patient, and it is therefore desirable to be able to concentrate the few available neutrophils for study. The centrifugation involved in preparing a buffy coat preparation before incubation should be avoided since this is a potential source of neutrophil membrane stimulation. In the cyto-centrifuge technique the concentration of neutrophils is delayed until the NBT-formazan reaction has been completed and the cells fixed. In the severely neutropenic patient it is of value to increase the volume of the original blood sample and of NBT to 0.2 ml, or halve the volume of distilled water and hypertonic saline in the haemolysis step, and then prepare several slides simultaneously in the cyto-centrifuge.

An NBT test performed on capillary blood is of particular value in children and in the daily monitoring of neutropenic patients when the number of venepunctures should be limited. A previous report (Björkstén, 1973) suggested that capillary NBT results are unreliable on account of haemolysis. There was no macroscopic evidence of haemolysis in the present study, and the control values for capillary samples were almost identical with venous specimens, provided that glass contact was avoided. This point is critically important, and glass-contact stimulation of the neutrophil must be avoided during the collecting and processing stages of the NBT test until the chemical reaction has terminated.

A satisfactory NBT test must give a low score in healthy controls and in ill patients who do not have systemic bacterial infection, and must show an elevated score in bacteraemia. Our preliminary clinical evaluation of the cyto-centrifuge NBT test has demonstrated elevated scores in patients with a positive blood culture. The upper limit of normal for uninfected patients is, however, an arbitrary level dependent on the concentrations of heparin

and NBT and the temperature and duration of incubation. With the present technique this level could be set at either 12% (mean + 2 SD for 177 uninfected patients) or at 18% (extreme upper limit for uninfected patients and patients with viraemia). Two of the nine septicaemic patients gave scores of 15 and 18% but since a serial study was not performed in these patients it is unclear whether these borderline scores resulted from the timing of sampling.

Further clinical evaluation of the cyto-centrifuge NBT test is required in patients with a wider spectrum of disease in order to establish a more precise upper limit of normal. The NBT test is, however, a non-specific cytochemical test of neutrophil cytoplasmic membrane function, and membrane changes may be induced *in vivo* not only by endotoxin but also by tissue breakdown products (Lauter *et al*, 1973) and acute-phase proteins (Segal, 1974). The subsequent *in vitro* reduction of NBT cannot therefore be expected to distinguish the primary membrane stimulant. Future clinical studies should therefore distinguish between patients with pyrexia but without gross metabolic upset and patients with a severe metabolic upset in which the differential diagnosis includes septic shock. The diagnostic limitations of blood culture and the *Limulus* lysate test in patients with transient bacteraemia or endotoxaemia add to the difficulties of clinical evaluation.

The cyto-centrifuge NBT test reduces the risk of non-specific stimulation of the neutrophil membrane *in vitro*, and a score above 18% indicates that previous membrane stimulation has occurred *in vivo*. The resulting differential diagnosis must then include systemic bacterial infection, and more specific investigations should be initiated. At the present time a score between 12 and 18% is equivocal and an indication for follow-up investigations.

We are indebted to the Central Birmingham Health District Trust Funds for financial support, to Dr A. M. Geddes for allowing access to his patients, and to Mr A. M. Picken for technical help. Part of this study was presented to the British Society for Haematology, London 1975.

References

- Bittner, S. J., Kieff, E., Windhorst, D., and Meier, P. (1973). The use of the unstimulated nitroblue tetrazolium test as a routine screening test for bacterial infection in an adult population. *Amer. J. clin. Path.*, **60**, 843-853.
- Björkstén, B. (1973). The NBT test using venous and capillary blood. *Scand. J. Haemat.*, **11**, 270-274.
- Björkstén, B. (1974). The influence of technical factors on the NBT test. *Scand. J. Haemat.*, **12**, 46-50.

- Feigin, R. D., Shackelford, P. G., Choi, S. C., Flake, K. K., Franklin, F. A., Jr., and Eisenberg, C. S. (1971). Nitroblue tetrazolium dye test as an aid in the differential diagnosis of febrile disorders. *J. Pediat.*, **78**, 230-237.
- Freeman, R., King, B., and Kite, P. (1973). Serial nitroblue tetrazolium tests in the management of infection. *J. clin. Path.*, **26**, 57-59.
- Fridovich, I. (1974). Superoxide radical and the bactericidal action of phagocytes. *New Engl. J. Med.*, **290**, 624-625.
- Gordon, A. M., Rowan, R. M., Brown, T., and Carson, H. G. (1973). Routine application of the nitroblue tetrazolium test in the clinical laboratory. *J. clin. Path.*, **26**, 52-56.
- Lancet* (1971). Leading article. Nitroblue tetrazolium: a routine test? *Lancet*, **2**, 909-910.
- Lauter, C. B., el Khatib, M. R., Rising, J. A., and Robin, E. (1973). The nitroblue tetrazolium test and acute myocardial infarction. *Ann. intern. Med.*, **79**, 59-62.
- Matula, G. and Paterson, P. Y. (1971). Spontaneous in vitro reduction of nitroblue tetrazolium by neutrophils of adult patients with bacterial infection. *New Engl. J. Med.*, **285**, 311-317.
- Nathan, D. G. (1974). NBT reduction by human phagocytes. *New Engl. J. Med.*, **290**, 280-281.
- Nelken, D., Gilboa-Garber, N., and Gurevitch, J. (1960). A method for the simultaneous separation of human thrombocytes and leucocytes. *J. clin. Path.*, **13**, 266.
- Park, B. H., Fikrig, S. M., and Smithwick, E. M. (1968). Infection and nitroblue-tetrazolium reduction by neutrophils; a diagnostic aid. *Lancet*, **2**, 532-534.
- Pearse, A. G. E. (1972). *Histochemistry, Theoretical and Applied*, 3rd ed., vol. 2, p. 887. Churchill Livingstone, Edinburgh and London.
- Ridgway, G. L. and Johnson, W. (1973). The NBT test in systemic bacterial infections. *Lancet*, **1**, 1180.
- Segal, A. W. (1974). Nitroblue-tetrazolium tests. *Lancet*, **2**, 1248-1252.
- Segal, A. W., and Levi, A. J. (1973). The mechanism of the entry of dye into neutrophils in the nitroblue tetrazolium (NBT) test. *Clin. Sci. mol. Med.*, **45**, 817-826.
- Segal, A. W., Trustey, S. F., and Levi, A. J. (1973). Re-evaluation of nitroblue tetrazolium test. *Lancet*, **2**, 879-883.
- Steigbigel, R. T., Johnson, P. K., and Remington, J. S. (1974). The nitroblue tetrazolium reduction test versus conventional hematology in the diagnosis of bacterial infection. *New Engl. J. Med.*, **290**, 235-238.
- Wollman, M. R., David, D. S., Brennan, B. L., Lewy, J. E., Stenzel, K. H., Rubin, A. L., and Miller, D. R. (1972). The nitroblue-tetrazolium test: usefulness in detecting bacterial infections in uraemic and immunosuppressed renal transplant patients. *Lancet*, **2**, 289-291.