

Technique for the performance of the nitro-blue tetrazolium (NBT) test

R. FREEMAN AND B. KING *From the Department of Bacteriology, University of Leeds*

The nitro-blue tetrazolium (NBT) test is rapidly assuming importance as a method of diagnosing bacterial infection (*Lancet*, 1971), and of investigating phagocytic defects (Johnston and Baehner, 1971). Various observations have led to modifications of the technique (Freeman and King, 1971). We have performed many of these tests in recent months, and have evolved a standard technique which has been suitable for most occasions.

Performance of Direct NBT Test

REQUIREMENTS

Nitro-blue tetrazolium dye

This is available commercially, and is made up as a 0.2% solution in saline. It may require gentle heating to dissolve fully. This solution is stable at room temperature for several weeks. Discard if turbidity or discoloration appears.

Phosphate-buffered saline, pH 7.2

Plastic haemagglutination tray

Pipettes to deliver 0.1 ml

Slides and coverslips (cleaned)

Methanol

Pappenheim's stain (methyl green-pyronine) is prepared from the powder supplied commercially, as described in 'Clinical Diagnosis by Laboratory Methods', page 938. Once made up, the stain is stable for months, but will tend to intensify with time.

METHOD

Blood is obtained in glass tubes, using heparin as anticoagulant (the concentration of heparin should be 75-100 units/ml of blood). The usual amounts of blood required from the patient is never more than 1.0 ml.

Pipette 0.1 ml of the blood into a well of the plastic tray. Make the 'working' NBT solution by mixing equal volumes of the stock 0.2% solution and the phosphate-buffered saline. This solution must be made freshly for each batch of tests. Pipette 0.1 ml of the 'working' NBT solution into each well containing blood and mix the contents.

Received for publication 21 April 1972.

Cover the tray with another tray to ensure humidity, and incubate at 37°C for 15 minutes. Follow this with an equal period at room temperature.

Using a Pasteur pipette, or a large bacteriological loop, with which to transfer the blood-NBT mixture, make careful coverslip smears and allow them to dry in air.

Fix the smears in methanol for three minutes.

Stain with Pappenheim's stain for three to five minutes. (The actual time is best decided by trial, the desired result being to stain the granulocyte nuclei green and to leave most of the erythrocytes as 'ghosts').

Air dry and mount in DePeX.

Examine under the 40× objective and assess the number of neutrophils containing the formazan deposit as a percentage.

RECOGNITION OF THE FORMAZAN DEPOSIT

Formazan deposits occur in two forms: (1) A single large black deposit in the cytoplasm of the affected cell. This represents the phagocytic vacuole (phagosome). Cells having this appearance are counted as positive. (2) Multiple black speckles, randomly distributed in the cytoplasm. There is some difference of opinion as to whether such cells should be included as positive. At least one group does include them (Matula and Paterson, 1971), but most workers appear not to do so. We recommend that they are not included as positive, and we find that our results have not suffered. Indeed, on several occasions we would have produced a falsely high value had we done this (as judged by parallel clinical and cultural assessments).

Formazan can also be found in two other situations: *Monocytes* will reduce the dye to formazan and produce a speckled appearance. Great care must be taken to differentiate monocytes from neutrophils, and monocytes must not be included as positive cells. *Platelets and neutrophil clumps* will cause extracellular masses of formazan to be deposited. These should be ignored.

Figures 1 and 2 show the appearance of the types of deposit associated with the neutrophils.

INTERPRETATION

Normal persons will have a value in the direct test within the range 1-11%. Values greater than 11% will be obtained in cases of bacterial infection. There are, however, circumstances in which the test is unreliable, or in which abnormal results might reflect a previously undetected underlying disease. Two excellent reviews on this topic can be found in the *Lancet* (1971), and the *Journal of Pediatrics* (Feigin, Shackelford, Choi, Flake, Franklin, and Eisenberg, 1971.)

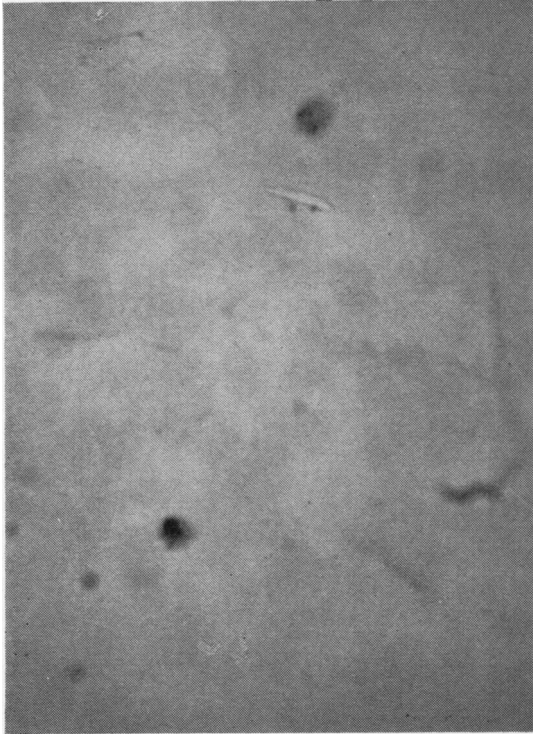


Fig. 1.

Fig. 1 Two neutrophils are clearly seen. The cell at the bottom shows a typical phagosome type of deposit. The cell at the top shows the multiple speckled type of appearance (Pappenheim's stain $\times 1000$).

Fig. 2 Several neutrophils are seen, most of which are positive. The patient had a Gram-negative septicaemia, and his NBT level was 52%.

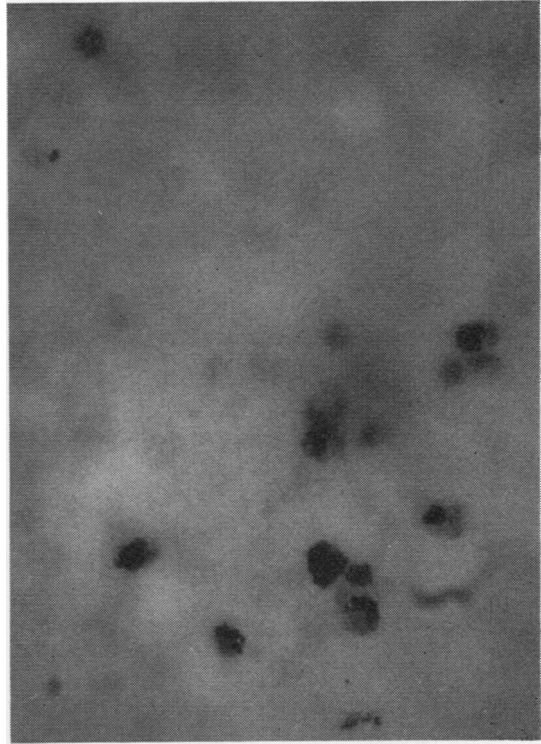


Fig. 2.

COMMON SOURCES OF TECHNICAL ERROR

It cannot be sufficiently emphasized that this test depends on many factors, many of which are unknown. Of the known factors, two merit particular attention.

1 The NBT response is low or absent in cases of hypogammaglobulinaemia (Park, 1971). Subsequent replacement of the gamma globulin may induce a normal response in the polymorph (Freeman and King, unpublished observation).

2 If EDTA (sequestrene) is used as anticoagulant instead of heparin, the result may again be falsely low (Park, 1970). Thus, it is likely that both immunoglobulins and complement are necessary for the test. For these reasons it is unwise to apply such procedures as dextran-sedimentation, since the proportions of these and other substances may be altered to an unknown extent.

It occasionally happens that the test is required to be performed on a patient with a neutropenia.

It is in such situations that the temptation to resort to techniques such as dextran-sedimentation is greatest. We have found that even in cases with neutrophil counts as low as 300/c mm it is still possible to obtain reliable results by the techniques described above, by making the coverslip smears on very large (histology) coverslips. The counting will only take a little longer than normal.

Performance of the Stimulated Test

In certain situations (usually when a very low or completely negative result is obtained by the direct method), it is necessary to perform a stimulated test in order to differentiate a transient lack of responsiveness from a congenital phagocytic defect. A good example of the former might be a patient on high doses of corticosteroids, and of the latter the classical example is chronic granulomatous disease of childhood (Johnston and Baehner, 1971).

PROCEDURE

Of the test blood, 0.1 ml is mixed with an equal volume of an endotoxin solution. The original method (Park and Good, 1970) recommends the commercial preparations, and these will give the best results; we have found, however, that a crude filtrate of a broth culture of *Escherichia coli* or *Pseudomonas pyocyanea* will work sufficiently well. It is vital to perform the procedure in parallel on a known normal blood sample. The blood-endotoxin mixtures are incubated at 37°C for 30 minutes, and then, using 0.1 ml aliquots the direct test is performed, exactly as described earlier. The normal control will be stimulated to a level well above the normal range, ie, will rise from less than 11 to 25-30%, and the test sample is assessed against this. A temporarily suppressed neutrophil population will reveal some stimulation, though it may not be of the magnitude of the normal control, but the neutrophils in chronic granulomatous disease will remain completely unreactive.

Comment

If the early promise of the NBT test is realized, it will quickly and justifiably become a standard test in many of our hospitals. Our concern is that it should not suffer any unjustified setbacks simply because it uses techniques which are unfamiliar to many. We hope that this guide, culled from our short experience and that of others, will help to promote an important addition to laboratory investigation.

We thank those of our clinical colleagues who allowed us such generous access to their records. This has been invaluable in trying to evaluate our techniques.

References

- Davidsohn, I., and Wells, B. B. (Eds.) (1962). *Todd-Sanford Clinical Diagnosis by Laboratory Methods*, 13th ed., p. 938. Saunders, Philadelphia.
- 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., and King, B. (1971). A modification to the N.B.T. test. *Lancet*, **2**, 1154.
- Johnston, R. B., Jr., and Baehner, R. L. (1971). Chronic granulomatous disease: correlation between pathogenesis and clinical findings. *Pediatrics*, **48**, 730-739.
- Lancet* (1971). Nitroblue tetrazolium: a routine test? (Editorial). *Lancet*, **2**, 909-910.
- 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.
- Park, B. H. (1971). The use and limitations of the nitroblue tetrazolium test as a diagnostic aid. *J. Pediat.*, **78**, 376-378.
- Park, B. H., and Good, R. A. (1970). N.B.T. test stimulated. *Lancet*, **2**, 616.

Reprocessing of wax-embedded tissue blocks for the electron microscope applied to Crohn's disease

T. WATSON, M. J. FULKER, AND M. G. COOK *From the Department of Experimental Pathology and Cancer Research, Leeds University, and the University Department of Surgery, The General Infirmary at Leeds*

The reprocessing of tissue from wax blocks for electron microscopy provides a means of observing ultrastructural detail at the expense of some resolution (Kobernick and Thomas, 1970; Hallowes and Streek, 1970). Much ultrastructural information can, however, be obtained at medium magnifications. This is of considerable value in certain pathological situations where the sparsity of significant lesions makes conventional electron microscopy uneconomical. In this case we have applied the technique to the study of giant cells in Crohn's disease (Albot, Parturier-Albot, Camilleri, and Diebold, 1970).

Materials and Method

Tissue blocks (approx $2 \times 2 \times 0.5$ cm) were removed from fresh proctocolectomy specimens. The specimens were fixed for four hours in 4% glutaraldehyde in sodium cacodylate buffer (pH 7) and subsequently dehydrated with graded alcohols, immersed in chloroform, and embedded in paraffin wax following the conventional histological procedure. Sections, 3-4 μ m, were cut and stained with haematoxylin and eosin and the areas thought worthy of further study with the electron microscope were selected by light microscopy. The required parts of the wax block were then cut out, to a size of approximately $2 \times 3 \times 2$ mm and immersed in benzene at 45°C for one hour and rehydrated through graded alcohols. The tissue was washed for one hour in two changes of sodium cacodylate buffer, postfixed for one hour in sodium cacodylate-buffered 1% osmium tetroxide. This was followed by dehydration through graded alcohols to propylene oxide and embedding in Araldite. Sections, 0.5 μ m, were stained with toluidine blue. Thin sections approximately 80 nm thick cut with an LKB Ultratome III, mounted on plain copper grids, and stained with lead tartrate, were examined on a Philips EM 300 electron micro-

Received for publication 27 April 1972.