Standardisation of the Nitro-blue Tetrazolium Test and Factors Affecting its Clinical Application

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SUMMARY

Attempting to standardise the nitro-blue tetrazolium (NBT) test, the following modifications of previously described procedures were introduced. Leucocyte-rich plasma obtained by simple gravitation at 37°C was mixed with NBT reagent which had previously been centrifuged to remove undissolved particles. Thick smears were made and stained with dilute haematoxylin, and physical manipulation during the various procedures was reduced to a minimum. Reconstitution of the buffy coat in fresh normal serum did not alter the NBT result.

Normal NBT readings were obtained in pregnant females, and women on contraceptive hormonal preparations.

S. Afr. Med. J., **48**, 209 (1974).

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Date received: 27 August 1972.

The nitro-blue tetrazolium (NBT) test has been shown by a number of workers to be useful in the diagnosis of chronic granulomatous disease[1-5] and to monitor the progress of bacterial infections.[6] The test has also been shown to be positive in mycoplasma,[7] fungal, and protozoal infections such as malaria, and in various parasitic infestations.[8,9] The qualitative NBT test is now used as a routine in many hospital laboratories.[2,3,10,11]

Since the original description by Park *et al.*,[10] however, contradictory reports have appeared in the literature with regard to the normal NBT test values,[12,14] the effect of pregnancy on the results,[15] and the findings in viral meningo-encephalitis.[16] Because of the contradictions and the problems which were encountered in our laboratories, such as when neutropenia was present, and differentiating the formazan particle from heavily stained polymorphonuclear neutrophilic (PMN) nuclei using Romanowsky stain, the method of Park *et al.*[10] was critically examined. It was found necessary to introduce minor modifications mainly with a view to minimising undue stimulation of PMN cells during the laboratory procedures. Alterations to Park *et al.*'s[10] original method were also advocated by Freeman and King.[12,17] We are in agreement with most of their modifications. Our study was carried out indepen-

dently and our findings show that the test could be further improved.

MATERIAL

Blood samples were obtained from 100 healthy male and female laboratory workers, from 100 patients with acute bacterial and viral infections, from a group of normal pregnant women in the first trimester, and from pregnant women in the third trimester, hospitalised for a variety of conditions. In addition, specimens were taken from women attending a family-planning clinic, who were receiving various combinations of contraceptive hormonal preparations.

Preparation of the NBT Solution

The NBT reagent was prepared by dissolving 1 mg of NBT in 0,5 ml phosphate-buffered saline (pH 7,2), to which was added 0,5 ml physiological saline. The NBT reagent should be completely dissolved. This 0,1% solution was used as such in one group of experiments and was centrifuged at 3 000 *g* for 10 min, and the supernatant employed in a further set of experiments. In yet another group of tests, the unspun NBT reagent was gently warmed to 37°C for 30 min and used immediately as described by Freeman and King.[18]

METHOD

By using a plastic syringe with a wide-bore needle, 2 ml of blood was withdrawn from the antecubital fossa and placed in plastic test tubes, after removing the needle. This was gently mixed with 50 units of preservative-free heparin. The blood was allowed to sediment at 37°C for 20 - 40 min after which time 0,1 ml buffy coat was removed, placed in a plastic tube and an equal amount of NBT solution added. The mixture was incubated at 37°C for 15 min and then allowed to stand at room temperature for a further 15 min. The tube was then shaken gently and thick smears, the size of the surface area of a coverslip, were made, allowed to dry, fixed in methanol for 1 min, and then stained with a 1 in 10 dilution of a 1% solution of well-filtered haematoxylin stain for 2 - 4 min.

In addition, NBT tests were performed using whole blood instead of buffy coat, according to the method of Park *et al.*[1]

Furthermore, with a number of specimens, reconstitution of the buffy coat was carried out by adding an equal amount of fresh normal AB serum and pre-incubating for 30 min at 37°C before the NBT was added.

Cell Count

Smears were examined under oil emersion and 100 PMN cells were counted. Neutrophils with a large bluish-black precipitate were counted as positive and recorded as a percentage.

RESULTS

By using the buffy coat only, lower readings were obtained than when whole blood was used (Table I). The buffy coat preparations, moreover, were easier and less time-consuming to read, and clumping of PMN cells was minimal. When the buffy coat was reconstituted with normal, fresh AB serum, no significant change was present (Table II).

TABLE I. NBT TESTS ON 20 NORMAL VOLUNTEERS USING WHOLE BLOOD AND BUFFY COAT PREPARATIONS

	Average	Range
Whole blood	7,3	0 - 11
Buffy layer	2,4	0 - 8

TABLE II. COMPARISON OF BUFFY LAYER AND RECONSTITUTED SERIES OF 20 NORMAL AND INFECTED CASES

	Average	Range
Buffy layer only	9,2	0 - 44
Buffy layer + 0,1 ml of normal AB serum	7,5	0 - 33

To concentrate the PMN cells more rapidly, dextran was added to a number of specimens. This produced extremely high readings and was considered unsatisfactory. The buffy coat was also centrifuged briefly to further concentrate the neutrophils. Slightly higher readings were obtained than in the unspun specimens (Table III). Excessive clumping was observed in these spun specimens.

TABLE III. EFFECTS OF ADDING DEXTRAN (MW 160 000) AND SPINNING OF BUFFY LAYER ON 20 NBT READING

	Average	Range
Buffy layer only	1,4	0 - 8
Dextran	25,5	17 - 39
Centrifugation at 200 *g*/2,5 min	3,4	0 - 12

By using both uncentrifuged and warmed NBT reagent, higher values were obtained than when reagent cleared by centrifugation was used (Table IV).

TABLE IV. RESULTS OF 20 NORMAL NBT TESTS USING CENTRIFUGED, WARMED AND UNCENTRIFUGED NBT SOLUTION

	Average	Range
Centrifuged	0,8	0 - 2
Warmed	3,4	0 - 8
Uncentrifuged	5,0	0 - 10

A group of 100 normal volunteers gave NBT results varying from 0 to 10%, with a mean of 2,1% (Fig. 1).

Blood taken from pregnant women and women on hormonal contraceptive preparations showed normal NBT results (Tables V and VI, respectively).

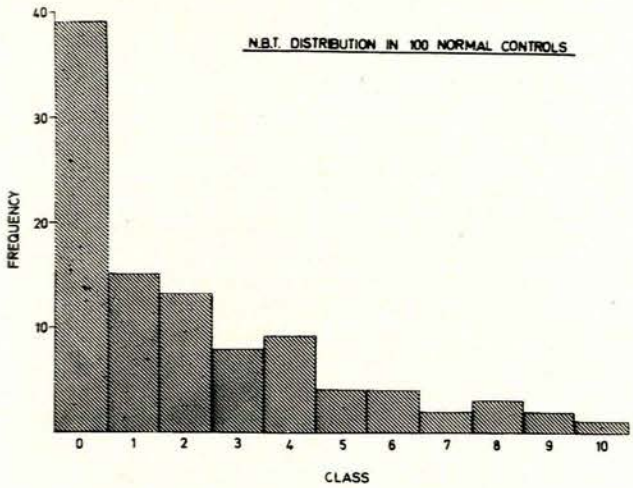

Fig. 1. NBT distribution in 100 normal controls.

TABLE V. NBT TESTS ON PREGNANT WOMEN

Duration of pregnancy (weeks)	Complications	NBT result %
11	—	4
18	—	0
8	—	2
11	—	6
11	—	0
22	—	0
13	—	1
13	—	7
15	—	0
7	—	1
36	Postmaturity	0
32	Antepartum haemorrhage	0
37	Hypertension	3
37	Hypertension	0
33	Antepartum haemorrhage	1
31	Pre-eclamptic toxaemia	1
30	Twins	6
34	Diabetes	2
39	—	0

TABLE VI. EFFECT OF CONTRACEPTIVE HORMONAL PREPARATIONS ON NBT READINGS

Preparation	No. of volunteers	Average (%)	Range
Oestrogen/progesterone	33	1	0 - 8
Oestrogen alone	3	1,5	0 - 3
Progesterone injections	12	2,5	0 - 9
Oral progesterone	4	2	0 - 8

The data appearing in Tables V and VI were tested by means of a χ^2-test and gave P values which do not indicate a significant difference between individuals in the normal and in these two groups.

We confirmed the results of other authors, that PMN cells from patients with a variety of bacterial infections gave increased reduction of NBT (Fig. 2).

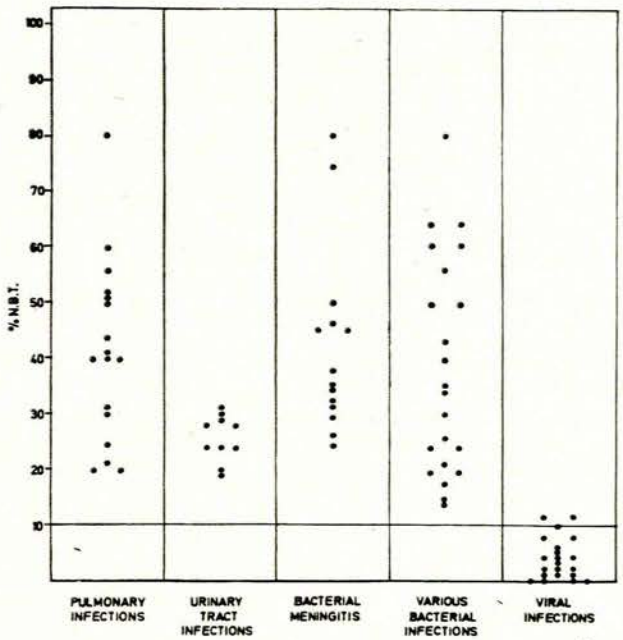

Fig. 2. NBT results in infected patients.

DISCUSSION

The qualitative slide NBT test consists essentially of an assessment of the resting NADH oxidase activity of PMN leucocytes. The test depends upon the *in vitro* reduction of soluble colourless nitro-blue tetrazolium to form an insoluble blue-black precipitate referred to as the formazan particle. The mechanism of this reduction is not fully understood, but an intact intracellular enzyme system is mandatory, as the reduction will not occur in chronic granulomatous disease where enzyme defects are present.

Since an intact cell membrane is not permeable to the dye, it also appears that some change must occur in this membrane to allow the NBT to pass into the cell. The mechanism is not yet known.

In attempting to reduce any stimulation of the PMN cells, it was considered advisable to remove the needle from the syringe when transferring blood to tubes containing heparin. It was found that by allowing the leucocytes to settle spontaneously, and using the leucocyte-rich plasma, less stimulation was observed than when whole blood was used. The degree of clumping of PMN cells seen in the whole blood specimens was considerably reduced in the buffy layer preparations. When attempts were made to hasten cell sedimentation by adding dextran, marked stimulation of the PMN cells was found. To facilitate counting, it is recommended that a nuclear stain be employed instead of a Romanowsky stain. By using haematoxylin, the red cells are not stained, but

the white cell nuclei are clearly visible and are not too dark to disguise the formazan precipitates.

To reduce non-specific stimulation further and to avoid possible artefact, the NBT solution was centrifuged to remove any insoluble particles or debris. Lower readings were obtained than when this step was excluded. By warming the NBT solution to 37°C, although it showed a moderate decrease over uncentrifuged NBT, it gave higher readings than the centrifuged NBT. Too vigorous mixing of the NBT solution and the buffy coat should be avoided. The gentlest shaking will suffice. The varying results found by the different authors may be due to excessive laboratory manipulations. Delays in performing the test after collection of the blood should be avoided.

There are certain host factors which have been reported to interfere with the NBT test. They are age, pregnancy and the concomitant use of certain drugs such as steroids.[14,17,18] The high percentage of positives in the first 2 months of life is documented by Cocchi *et al.*[19] and Humbert *et al.*[20]

It has been reported that high readings were obtained in pregnancy.[15] Our results on pregnant women indicate no abnormality in the NBT result.

Controversy also exists as to the effect of contraceptive hormonal preparations (oestrogens and progesterones, either in combination or alone) on the NBT result.[21,22] Our results are in agreement with those of Björkstein and Solheim,[23] who showed normal NBT results in patients on various steroid contraceptive preparations.

Park[24] reported that the NBT response is low or absent in cases of hypogammaglobulinaemia. In order to show that the buffy coat separation technique did not give false low results due to separation of PMN cells from the normal plasma concentrations of immunoglobulin and complement, reconstitution experiments were carried out. These showed no significant difference from the control group.

The NBT test is a useful addition to our tests available for diagnosis and monitoring of infectious diseases.[25-27] We are convinced from our work that the many controversies associated with this test are due to technical factors. Undue stimulation of the PMN cells during laboratory manipulation gives unduly high readings, and if this is avoided and the test accordingly standardised, these controversies would be resolved and the efficacy of the test established.

ADDENDUM

Since submitting this paper, a critical assessment of the NBT test has appeared.[28] The authors used a modification of Park's original method and in addition a method employing EDTA and Ficoll. They found both methods to be unreliable in distinguishing between bacterial and viral infection. We did not compare our modified technique with their latter method, but we are convinced that using our modification, the NBT test is an extremely useful indicator of bacterial infection.

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