# STANDARDIZATION OF THE QUALITATIVE NITROBLUE TETRAZOLIUM DYE REDUCTION TEST AND ITS CLINICAL USEFULNESS

by

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# INTRODUCTION

## A. STATEMENT OF THE PROBLEM

The qualitative nitroblue tetrazolium dye reduction test was originally developed as a screening test for phagocyte defects in children suffering from Chronic Granulomatous Disease (CGD) (5). It was then translated into a procedure to aid in distinguishing bacterial from non-bacterial infections (59) and to monitor treatment of infectious disease processes (62). In the few years since its inception, the NBT test has suffered through many variations of technique, making difficult correlation of results from different laboratories. Many workers have addressed themselves to the confusions and problems resulting from the many variations as well as from the inherent mechanics of the procedure itself (2, 13, 25). They have emphasized the necessity for a uniform and standardized procedure that would allow for comparison among investigators.

It was the intention of this study to develop and standardize the qualitative, spontaneous (non stimulated) NBT dye reduction test on neutrophils to a procedure easily performed in the routine clinical laboratory and then to evaluate the method's application in various clinical situations.

## B. HISTORY OF PHAGOCYTOSIS

Elie Metchnikoff in 1883 (50) described phagocytosis and suggested that it functioned as the host's first line of defense against invading

microbes. Up to 1959 when Sbarra and Karnovsky (65) described the biochemical concomitants of phagocytosis, understanding was confined to morphologic events, i.e. recognition, ingestion, primary phagosome formation and degranulation.

The work of Sbarra and Karnovsky (65) and Iyer, Islam and Quastel (41) in 1961 confirmed that the burst of oxygen consumption noted during active phagocytosis was accompanied by increased hexose monophosphate shunt activity and increased hydrogen peroxide production by the cells. Selvaraj and Sbarra (68) in 1966 went on to separate the metabolic activities of phagocytosis into those required for actual ingestion (glycolytic) and to those required for bactericidal activity (oxygen dependent hexose monophosphate shunt).

The increased oxygen uptake was also discussed by Cagan and Karnovsky (11) who proposed that a cyanide insensitive NADH oxidase was responsible for the bulk of the hydrogen peroxide production during phagocytosis. On the contrary, Zatti and Rossi (86) proposed that granule bound NADPH oxidase served this function. Neither mechanism is yet proved (77).

Studies of the metabolic abnormalities of neutrophils from patients with Chronic Granulomatous Disease have shown that these cells do not demonstrate the normal post-phagocytic increase in hydrogen peroxide production (38).

Klebanoff (44) demonstrated that myeloperoxidase in the presence of catalytic amounts of hydrogen peroxide and a halide was a potent bactericidal mechanism for the destruction of non hydrogen peroxide producing microorganisms. Iodination of bacteria occurred in intact

neutrophils after the ingestion of bacteria, and the fixed iodide could be localized radioautographically in the cytoplasm of the cell in association with the ingested bacteria (45, 46).

It has also been shown that superoxide is produced during phagocytosis (4). By inhibiting superoxide with, for example, superoxide dismutase, bactericidal activity was demonstrated to be reduced. Therefore it was reasoned that superoxide production probably contributed to phagocyte bactericidal capacity.

Because of a disease associated with its deficiency, the bactericidal role of hydrogen peroxide production has been well studied. Other systems may be in effect, but are not as well investigated. Stossel's recent review (76, 77, 78) of the phagocytic process stated in summary that as a result of increased glycolysis during phagocytosis, there is an accumulation of lactic acid, therefore lowering the pH of the phagosome to less than 5.0. This is a bactericidal level for most species. Within the phagolysosomes have been discharged the contents of the primary and secondary neutrophil granules, including lysozyme, hydrolases and myeloperoxidase which are employed in subsequent bactericidal events.

## C. HISTORY OF NITROBLUE TETRAZOLIUM DYE REDUCTION

### 1. Introduction of NBT as a histochemical dye

In 1957, Nachlas et al (52) reported their use of a new p-nitrophenyl substituted ditetrazole (nitroblue tetrazolium) for histochemical purposes. NBT produced the following characteristics:

1. ready reduction to the insoluble formazan and resistance to oxidation by molecular oxygen back to the tetrazole,

2. strong attraction for the hydrogens or electrons liberated from the substrate so that atmospheric oxygen would not act as a significant competitor,

3. reduction appeared in tissues as a good quality pigment.

Other histochemists continue to report their use of nitroblue tetrazolium to demonstrate diaphorase activities in tissue sections (11, 63, 80).

2. Use of NBT reduction as screening device for CGD

Baehner and Nathan (5) first reported in an abstract in 1966 their use of nitroblue tetrazolium to confirm a deficiency of hydrogen peroxide production by the neutrophils from children suffering from Chronic Granulomatous Disease. Phagocytes separated from peripheral blood were allowed to phagocytose polystyrene spheres. To the media containing these intact leukocytes was added 0.2% nitroblue tetrazolium. During phagocytosis, NBT was reduced within five minutes to a deep blue precipitate within normal cells. In a resting state, they required up to 30 minutes to bring about an equal degree of dye reduction.

The response of the leukocytes from patients with CGD was abnormal. Although the spheres were ingested, the cells did not reduce NBT at a normal rate. The lack of NBT reduction was related to the lack of hydrogen peroxide production during phagocytosis. They concluded that this qualitative NBT test could provide a sensitive, diagnostic screening test for CGD (6).

This observation that intact leukocytes would reduce NBT during phagocytosis was expanded to a more quantitative procedure (7). Leukocytes isolated by fibrinogen sedimentation and differential

centrifugation were washed with Krebs-Henseleit bicarbonate buffer to which glucose had been added. The cells were adjusted to a concentration of 25,000 leukocytes per cubic millimeter. To tubes designated for phagocytosis was added a dilution of polystyrene spheres. Duplicate tubes designated as "resting" for comparison received no spheres.

The addition of 0.1% nitroblue tetrazolium in saline and 0.01M potassium cyanide to the reaction mixture resulted in reduction of the dye to a deep blue color. After extraction with pyridine, this blue color was quantitated as a change in optical density at 515nm, based on comparison of the resting and phagocytosing tubes.

3. Adaptation of NBT reduction for differentiation of febrile disorders

In 1968 Park, Fikrig, and Smithwick (59) described qualitative NBT reduction without phagocytosis. A plastic syringe was used to draw 0.5 to 1.0 ml of blood which was transferred to a plastic tube containing 75 to 100 units of heparin per ml of blood. An aliquot of this heparinized blood was added to an equal aliquot of working NBT solution for the reaction which was carried out in plastic tubes. The working solution was made up of equal parts of 0.2% NBT in physiologic saline and 0.15M phosphate buffered saline at a pH of 7.2. After incubation at 37°C for 15 minutes and a further incubation at room temperature for another 15 minutes, a cover slip smear of the blood was dried and stained with Wright's stain. One hundred neutrophils were evaluated under oil immersion for the presence of a large black deposit which would qualify them for the designation of "NBT positive".

The investigators reasoned that circulating phagocytes in patients with bacterial infections would be stimulated to produce hydrogen

peroxide. In vitro this would be reflected by an increase in reduction of NBT without phagocytosis. They reported a mean proportion of NBT positive neutrophils of 8.5% in healthy controls, a combined group of 30 children and adults. The NBT positive range of scores was 5.8% to 9.5% in a pediatric patient group with nonbacterial illnesses including rheumatoid arthritis, systemic lupus erythematosis, and viral infections. Acute bacterial infections gave a range of 29% to 47%.

4. Variations in the technical aspects of the qualitative NBT dye reduction test

Numerous modifications have been proposed and just as many technical difficulties have been cited, resulting in varied and contradictory interpretations. The following tables present the array of variations introduced into the original NBT reduction procedure, the references noted representing only the first literature reference to this alteration in technique.

Table 1 lists the variation of types of syringes, amount of blood required at phlebotomy, type of glassware, and the designated time lag between phlebotomy and the commencement of the procedure as employed by various investigators.

Table 2 identifies the anticoagulants employed and the variations in concentration per ml of blood.

Table 3 outlines the varying concentrations of NBT dye, the initial solvent, any working diluent added and its pH, as well as additional treatment of the solution deemed necessary.

Table 4 lists the varieties of incubation times and temperatures,

TABLE 1. NBT TEST SPECIFICATION VARIABLES

| Syringe | Ref. | Phlebotomy | Ref. | Reaction Tube | Ref. | Time Lag | Ref. |
|---|---|---|---|---|---|---|---|
| Plastic | 58 | 8-20 ml | 83 | Plastic | 58 | A few minutes | 16 |
| Siliconized | 41 | 0.5-1.0 ml | 58 | | | Within 30 minutes | 14 |
| Vacutainer (glass) | 37 | 5-10 ml | 41 | Capped plastic | 16 | 30 minutes | 38 |
| Plastic capillary tubes | 8 | 1-2 ml | 20 | | | Up to 2 hours | 37 |
| Glass | 26 | 1.5 ml | 37 | Glass | 26 | Immediate | 8 |
| | | 45 lambda | 8 | | | Immediate to 1 hour | 66 |
| | | 1.0 ml | 26 | Siliconized | 78 | 15-30 minutes | 9 |
| | | 3-4 drops | 78 | | | If not immediate, store at 4°C up to 12 hours | 29 |

TABLE 2. NBT TEST SPECIFICATION VARIABLES

| Type of anticoagulant | Concentration per ml of blood | Ref. |
|---|---|---|
| Heparin | 5-12.5 units | 83 |
| Heparin | 75-100 units | 58 |
| Heparin | not given | 41 |
| Heparin | 50 units | 16 |
| Heparinized saline | 5 units | 38 |
| Sodium citrate, 3.8% | 3 ml | 37 |
| Heparin | 1 unit | 8 |
| Heparinized vacutainer | 30 units | 84 |
| Heparinized saline | 10 units | 35 |
| Heparin | 1 unit/ drop of blood | 78 |
| EDTA transferred to heparin capillary tube | not given | 63 |
| EDTA | 2.4 mg | 66 |
| Heparin | 10 units | 9 |

TABLE 3. NBT TEST SPECIFICATION VARIABLES

| NBT Dye | | Working Diluent | Handling Notes | Ref. |
|---|---|---|---|---|
| Percent | Solvent | | | |
| 0.2 | Not given | Not given | None | 5 |
| 0.13 | Phosphate buffer, pH 7.0 | Not given | None | 83 |
| 0.2 | Physiologic saline | 0.15M phosphate buffered saline, pH 7.2 | None | 58 |
| 0.2 | Physiologic saline | Not given | Filter, Whatman #1; store at 4° C in opaque bottle | 42 |
| 0.1 | Isotonic saline | Kreb's phosphate buffer, pH 7.4 | 200 mg glucose/100 ml buffer | 38 |
| 0.2 | Distilled water | Not given | 6 parts dye solution to 1 part blood | 37 |
| 0.2 | Not given | Not given | 1 unit heparin/ml dye solution | 8 |
| 0.2 | Saline | Phosphate buffered saline, pH 7.2 | None | 26 |
| 0.2 | Phosphate buffer, pH 7.21 | Not given | 200 mg "Ficoll"/ml solution | 35 |
| 0.2 | Not given | Phosphate buffer, pH 7.4 | 100 mg glucose/100 ml buffer | 78 |
| 0.1 | Phosphate buffered saline, pH 7.2 | | None | 66 |
| 0.02 | Physiologic saline | 0.15M phosphate buffered saline, pH 7.2 | Filtered; aliquots frozen | 70 |
| "NBT mixture from General Biochemicals" | | | | 14 |

TABLE 4. NBT TEST SPECIFICATION VARIABLES

| FIRST INCUBATION | | | SECOND INCUBATION | | Ref. |
|---|---|---|---|---|---|
| Time in mins. | Temperature | Location if given | Time in mins. | Temperature | |
| 1.5-2 | Room temp. | Not given | 15 | 37° C | 83 |
| 15 | 37° C | Not given | 15 | Room temp. | 58 |
| 25 | 37° C | Waterbath or incubator | Not done | | 41 |
| 15 | 37° C | Waterbath | Not done | | 38 |
| 60 | 37° C | Not given | Not done | | 14 |
| 15 | 37° C | Drying oven on tilting mixer | 15 | Room temp. | 37 |
| 25 | 37° C | Not given | Not done | | 47 |
| Not given | 37° C | "Fibrometer" thermal block | Not given | | 84 |
| 30 | 37° C | Waterbath | 15 | Room temp. | 31 |
| 25 | 37° C | Not given | 15 | Room temp. | 69 |
| 10 (before addition of NBT solution) | 37° C | | | | 66 |
| 15 (after addition of NBT solution) | 37° C | | 15 | Room temp. | |

TABLE 5. NBT TEST SPECIFICATION VARIABLES

| Smear Mode | Ref. | Leukocyte Dye | Ref. | No. Evaluated | Ref. |
|---|---|---|---|---|---|
| Coverslip | 58 | Methyl green | 83 | 100 | 58 |
| Large coverslip | 23 | Wright's | 58 | | |
| Glass slides | 31 | Wright-Giemsa | 14 | 300 | 35 |
| Thin slide smears | 35 | 0.1% Neutral red | 37 | 1000 | 78 |
| Object glass | 78 | Pappenheim's | 22 | | |
| Siliconized Boerner serologic slides | 9 | 0.5% Aqueous safranin | 31 | 200 | 74 |
| | | May Grunwald-Giemsa | 35 | | |
| | | Giemsa | 78 | | |
| | | 5% Aqueous methyl green | 9 | | |
| | | "Diff-Quik" | 74 | | |

TABLE 6. NBT TEST SPECIFICATION VARIABLES

| Leukocytes Evaluated | Formazan Description | Ref. |
|---|---|---|
| Not given | Deep blue precipitate | 5 |
| Neutrophils or mononuclear cells with at least 10 phagocytized latex particles | Deep blue somewhat granular precipitate present with particles | 83 |
| Not given | A large black deposit | 58 |
| Neutrophils | Definite mass of formazan | 38 |
| Not given | Black deposits | 14 |
| Neutrophils | Large black deposit | 20 |
| Neutrophils | Graded: 0 = no reduction<br>1 = 1-4 small formazan granules<br>2 = 1 medium-sized deposit or several granules<br>3 = very heavy deposition of reduced dye | 37 |
| Neutrophils and monocytes | Definite deposit of reduced NBT | 39 |
| Neutrophils; clumps ignored | Stippled cytoplasmic distribution and single dense cytoplasmic deposit | 47 |
| Not given | A single large black deposit | 26 |
| Not given | Discrete particulate cytoplasmic distribution as well as very dense deposits | 31 |
| Not given | Clump or stippled | 9 |
| No ruptured cells included | Solid deposits or stippled cytoplasmic granules | 35 |
| Not given | Both large and stippled | 69 |
| Not given; clumps neglected | Any deposit larger than granules of neutrophils | 78 |
| Any phagocytes | Not given | 12 |
| Not given | Blue-black formazan deposits | 74 |

TABLE 7. NBT TEST SPECIFICATION VARIABLES

| Further Innovations | Ref. |
|---|---|
| 0.16 mg glucose in 0.05 ml water added to reaction tube to ensure adequate oxidative substrate | 83 |
| 1 ml 6% dextran in saline added to aid in sedimentation and collection of leukocyte rich plasma | 41 |
| 30% bovine albumin added at time of making smear to resolve clumping and produce minimal disruption of membranes | 25 |
| Buffy coat prepared | 31 |
| Capillary tube centrifugation of reaction mixture for buffy coat | 78 |
| 37$^{o}$C incubation phase done in 5% $CO_2$ incubator | 74 |

and equipment employed when designated.

Table 5 gives the various modes of smear preparations, types of dyes used to stain the leukocytes and the number of leukocytes evaluated.

Table 6 quotes the descriptive terms employed in the cellular interpretations to qualify a cell as "NBT positive".

Table 7 lists the further innovations introduced by a few investigators.

The majority of investigators reported the results uniformly, i.e. as percent positive with perhaps an added absolute value calculated from the total leukocyte count and differential. Hicks and Bennett (37) developed a scoring system by which results were reported as a calculated value out of a possible 300. Feigen and coworkers (18, 19, 20) expanded the values of percent positive combined with absolute positive to develop a nomogram with the result that reports could be classified into four groups; i.e. Group A = control, normal population; Group B = viral infections, and non infectious febrile illnesses; Group C = untreated bacterial infections; and Group D = ineffectively treated bacterial infections. With subsequent patient data, the percentage of NBT positive cells was plotted on the abscissa and the absolute number of NBT positive cells on the ordinate and a prospective diagnosis obtained.

5. <u>Controversies associated with technical aspects of the NBT test</u>

A review of the literature surrounding the NBT test thus reveals variables in nearly every aspect of the technical procedure, illustrated in the above charts. Many of these variations have led to confusion and controversy on the relative value of the influences of these modi-

fications on the formazan formation in neutrophils. In addition they have made comparison of published results difficult. The following influences have been discussed: glass surfaces (28, 34), anticoagulants (8, 34, 35, 36, 37, 49, 60, 64, 66), time elapsed before initiation of the test and storage of blood specimens (29), incubation times and temperatures (13, 71, 84).

Segal, Trustey, and Levi (67) noted the poor agreement between results obtained in the comparison of methods and Charette and Komp (13), Freeman and King (25), and Arrowsmith and Morin (2) emphasized the need for standardization of technique.

In addition to these controversies, the technical problem of interpreting the final product, the blood smear, has also been discussed. Windhorst, Holmes and Good (84) noted that the technical aspects of the test were still to be refined. These were no doubt responsible for the interpretive problems resulting from a tendency for the neutrophils most active in dye reduction to form large clusters. The clumps made counting difficult and tended to skew the results if they were ignored. Others have also noted these interpretive problems (32, 34, 54, 67, 81).

An addition problem of ruptured neutrophil cell membranes was noted by Hawkins (34) and Will and Grauman (81). The investigations reported by Gordon et al (31) apparently remedied this problem. These workers adapted into the NBT test the results of the trials by Stuart and Simpson (80). They reported that a 40% buffered concentration of an inert, synthetic, sucrose polymer (Ficoll) was found to have a protective effect on leukocytes during incubation for cytochemical reactions at 37$^{O}$C. This permitted enzyme cytochemistry to be performed

on unfixed bone marrow and peripheral blood smears, and on leukocytes cultured in vitro. The technique was suitable for the demonstration of both cytoplasmic and intramitochondrial dehydrogenase enzyme activity. The mechanism whereby the Ficoll apparently stabilized the membranes is yet unexplained.

The mechanism of the entry of the dye into a phagocyte had continued to elude investigators. Nathan and Baehner (53) measuring NBT reduction during phagocytosis of latex spheres reasoned that the NBT was ingested along with the spheres.

That ingestion of particulate matter was not a prerequisite of NBT reduction, however, was postulated by Gifford and Malawista (28). They felt their studies provided evidence that under certain conditions NBT could penetrate granulocytes in the absence of exogenous substrates that stimulate phagocytosis. The observation was made, however, that the neutrophils studied did have a large part of their surface area in direct contact with and adherent to a glass surface. The suggestion was therefore made that the NBT could have gained access to the interior of the cell as the cell tried to phagocytose the glass surface.

Segal and Levi (66) reported extensive experiments and observations in 1973 that have apparently resolved this riddle of how the dye gets into the phagocyte. It was demonstrated that NBT complexed with and then precipitated heparin and/or fibrinogen from solution, following which phagocytosis of the macromolecular complex occurred. This macro complex of dye and heparin was demonstrated by electron microscopy to be within the membrane bound phagocytic vacuole.

6. Use of the NBT test as a diagnostic aid

During the last few years the medical literature has reflected the great interest that has been focused upon the potential diagnostic aid of the NBT test in febrile disorders (57, 58). Several investigators have reported its use in infectious disease states (19, 20, 21, 27, 40, 48, 73) and others have reported its use in detection and management of infection in patients with a high risk of infection (24, 62, 83, 85). The use of the test to evaluate febrile states other than infection has also been reported (3, 47, 70, 74).

The terms "false positive" and "false negative" with reference to the reduction score of the NBT test being able to differentiate bacterial inflammatory processes or not began appearing immediately after the institution of the original procedure (15, 39, 61). These terms must of course be evaluated with reference to the individual workers' technique employed as well as the normal ranges obtained with the technique. If the minutiae of the test procedures are not comparable, then the numerical results of the NBT tests cannot be compared fairly.

In this investigation of the NBT test, both standardization of methodology and correlation with bacterial infections and other disease states have been considered.

# MATERIALS AND METHODS

## A. SELECTION OF CONTROL AND PATIENT SUBJECTS

As the medical, medical technology, and dental hygiene students were entering the University of Oregon Medical and Dental Schools during Fall Term, 1973, routine interviews were conducted by the Student Health Service to establish baseline health service records. At this time blood samples were taken for complete blood counts with differentials and then further processed for the NBT test. Of those with no current signs or symptoms of underlying systemic infection, 100 were selected as control subjects. This group was made up of 55 males and 45 females ranging in ages from 19 to 36. Of the females, 16 were currently receiving oral contraceptive medications.

From a nearby senior citizen community, 32 volunteers in good health with ages ranging from 67 to 86 were used as a further control group.

Through the cooperation of the Emergency Room at the University of Oregon Medical School Hospitals and Clinics, patients presenting with signs of infection and/or fevers of undetermined origin were referred for complete blood counts and NBT test. Selected to be included in the final statistical evaluation of results were those patients with no given history of current antibiotic use at the time of phlebotomy. Cultures obtained at the time were used to confirm the diagnosis of any bacterial involvement. Twenty adults made up the resultant patient group of bacterial infections.

It was observed that some of the febrile cases possibly reflected

underlying tissue necrosis in patients with either hepatic cirrhosis or neoplasms. This study was then expanded to include groups of patients with these disorders to see whether tissue necrosis or the underlying disease was responsible for increased NBT reduction.

Of the six patients studied with alcoholic cirrhosis, two were designated by the attending physician as also having alcoholic hepatitis. This "necrosis" was established on the basis of clinical impression, enzyme batteries and/or biopsy. Fifteen patients being seen in Chemotherapy Clinic with metastatic neoplasms were also evaluated. The presence of necrosis was established on clinical and radiologic grounds by the physician caring for the patient.

## B. DEVELOPMENT OF STANDARDIZED PROCEDURE FOR QUALITATIVE NBT DYE REDUCTION

### 1. Determination of optimal incubation equipment

When a reaction mixture is incubated in a laboratory procedure, it is desirable that the reaction mixture itself quickly approach and maintain a predetermined temperature. The recordable temperature of the air or water surrounding the outside of a tube containing the reaction mixture is not necessarily an indication of temperature changes within the tube. In order to determine the ability of available incubating equipment to produce an internal reaction temperature equivalent to the desired incubation temperature, simulated test solutions with thermometers immersed in the solution were placed in the various incubators representative of dry air incubators, water bath incubators and dry heating thermal blocks. Time and temperature points were noted until the reaction mixture attained the desired temperature. On the basis of

these results the dry heating block system was selected for use in this investigation. A Temp-Blok Module Heater (Scientific Products) was purchased.

2. <u>Determination of optimal anticoagulant and reaction medium</u>

The cell morphology resulting from the use of the five ml capacity "Vacutainers" (Becton-Dickinson) containing 7.5 mg of the anticoagulant disodium edetate (EDTA) was evaluated against the morphology from those containing 143 USP units of sodium heparin. Before 10 different samples of blood could be compared using the two different anticoagulated glass vacutainers, the heparin tubes were abandoned as the resulting cells were generally impossible to evaluate. They presented either as large indeterminate clumps or as ruptured cellular debris. With the EDTA vacutainer specimens, many cell membranes were ruptured also and only a few neutrophils demonstrated the presence of formazan deposits.

The same samples were tested simultaneously with the addition of the sucrose polymer "Ficoll" to the NBT working solution as suggested in the work of Gordon et al (31). Buffy coat smears were also attempted as reported in the technique, but resulted in greater cell clumping and distortion. The buffy coat variation was not pursued.

The next approach was to add sodium heparin to an EDTA anticoagulated specimen of blood just prior to incubation with the NBT/Ficoll dye solution and then compare the cell morphology and NBT reduction with those of the specimen untreated with heparin. The heparin vacutainers contained approximately 29 units per ml of blood. A lesser amount of heparin, 10 units per ml of blood, was experimentally added to an EDTA anticoagulated specimen and the blood processed with the NBT/Ficoll

dye solution. The resultant cell morphology was good.

3. Determination of optimal leukocyte counterstain

The use of various counterstains to demonstrate the blood cells proved to be an exercise in relearning identification of cell morphology with each stain. Therefore it was decided to employ the stain currently in general use in the hematology laboratory where the NBT test, if established as a routine procedure, would have to be done, a stain producing neutrophil morphology already familiar to the hematology technologists. A modified Romanowsky stain similar to Wright's stain, a polychrome methylene blue stain was therefore employed in this investigation. This is the stain contained in the Hema-Tek Slide Stainer (Ames Company) currently used in the Hematology Division of the Department of Clinical Pathology.

4. Preparation of stock reagents

a. 0.2% NBT solution in saline

Nitroblue Tetrazolium, Crystalline, Grade III was obtained from the Sigma Chemical Company and 200 mg of the yellow crystals was dissolved in and made up to 100 ml volume with physiologic saline (0.85%). The solution was then filtered through Whatman No. 1 paper before storage in an opaque bottle at $4^{o}C$.

b. 0.15M phosphate buffered saline, pH 7.2

Reagent grade chemicals were obtained from current laboratory supplies. A 0.15M solution was prepared by weighing 6.798 g sodium chloride (M.W. 58.44), 1.478 g sodium phosphate dibasic, anhydrous (M.W. 141.95), and 0.43 g potassium phosphate, monobasic (M.W. 136.09). These were dissolved in and made up to one liter with demin-

eralized water and the pH adjusted to 7.2.

c. 40% Ficoll solution

The sucrose polymer "Ficoll" was obtained from the Sigma Chemical Company and 2.0 g was dissolved in five ml of phosphate buffered saline by allowing it to stand overnight at room temperature. The solution was stored at 4°C.

d. Sodium heparin

Vials containing 10 ml of sodium heparin in a concentration of 1,000 USP units per ml were obtained from Grand Island Biologic.

5. Preparation of working NBT dye solution

Preparation of a working NBT dye solution involved allowing stock reagents to warm to room temperature, followed by the mixing of equal aliquots of the 0.2% NBT in saline and the 40% Ficoll in phosphate buffered saline. The working solution was made up just prior to initiation of the procedure on a sample of blood.

6. Comparison of EDTA versus EDTA/heparin with 100 student subjects

Phlebotomy was accomplished on the 100 student subjects with a 21 gauge needle attached to a 5 ml capacity vacutainer containing 7.5 mg EDTA. Once the aliquot for the Model S Coulter Counter (Coulter Electronics) determinations was removed and routine differential slide made, the remaining sample of blood was split into two, one aliquot of which had 10 units of sodium heparin per ml of blood added using a tuberculin syringe. If the aliquot to receive the heparin was 2 ml in volume, for example, the 10 units per ml of blood was equivalent to 20 units for the 2 ml, or 0.02 ml from a 1,000 unit per ml vial of heparin. The other aliquot received no heparin.

The dye reduction test was commenced immediately on each aliquot using plastic disposable tubes. Each tube received equal volumes of the anticoagulated blood (either EDTA or EDTA/heparin) and the working NBT dye solution. The tube labelled, for example, "E" received 0.2 ml EDTA blood and 0.2 ml working NBT dye solution. The tube labelled "H" received 0.2 ml EDTA/heparin blood in addition to the 0.2 ml dye.

After gently mixing, these tubes were placed in the heating block set at 37°C for 15 minutes, then allowed to stand at room temperature for a further 15 minutes. A thin smear was made on glass microscope slides and processed as a routine differential slide in the Hema-Tek Slide Stainer.

In evaluating the completed smears, 100 consecutive neutrophils, segmented and banded forms, were observed under oil immersion for the presence of distinctive formazan deposits, no stippled cells included. The percentage obtained was noted and used to calculate the absolute NBT positive value from the total leukocyte count and differential previously completed, i.e.

1. TOTAL LEUKOCYTE/cu mm X (% SEGMENTED + % BANDED NEUTROPHILS) = ABSOLUTE NEUTROPHILS/cu mm
2. ABSOLUTE NEUTROPHILS/cu mm X % POSITIVE NBT CELLS = ABSOLUTE POSITIVE NBT CELLS/cu mm.

The decision was made to continue with only the EDTA/heparin method of anticoagulation in evaluating the subsequent 32 senior citizen control subjects and the patient subjects.

Hematology profiles and differentials of these control groups as well as patient groups were also evaluated.

# RESULTS

## A. DETERMINATION OF OPTIMAL INCUBATION EQUIPMENT

Results of the evaluation of available laboratory incubation equipment are given in Figure 1. In a dry air oven whose internal suspended quality control thermometer consistently read at 37.5°C, the reaction mixture was unable to attain this level after one hour, having levelled off at 36.1°C in 45 minutes. A water bath set at 34°C was able to produce an equivalent temperature in the reaction tube at six minutes. A dry heating block of shallow depth was unable to attain its set temperature of 37°C after 20 minutes, having levelled at 36.4°C in 14 minutes. The dry heating thermal blocks of full tube depth, e.g. Fibrometer and Temp-Blok, were able to establish and maintain desired temperatures of 37°C within four minutes.

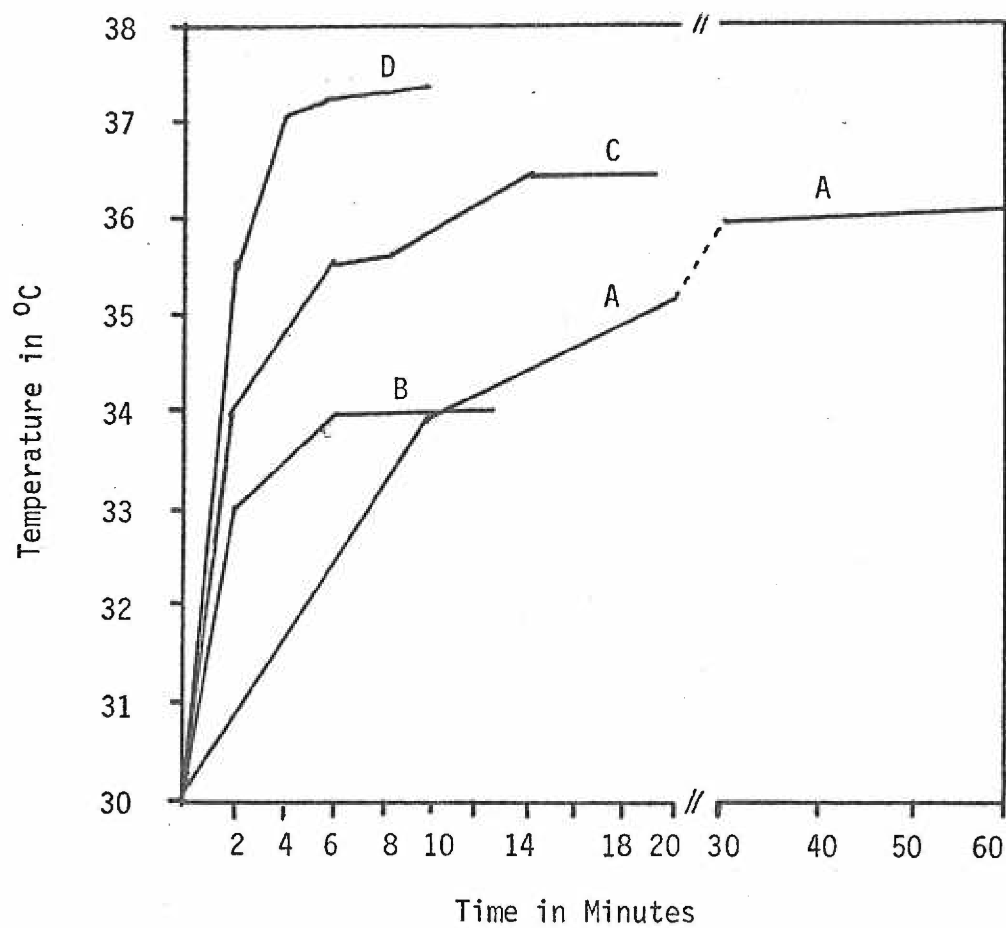
## B. DETERMINATION OF OPTIMAL ANTICOAGULANT AND REACTION MEDIUM

Ten units of sodium heparin per ml of blood was experimentally added to an EDTA anticoagulated specimen of blood and the specimen processed with the NBT/Ficoll dye solution. The resultant cell morphology was good; the cell membranes were stable and formazan deposition was readily seen.

## C. COMPARISON OF EDTA VERSUS EDTA/HEPARIN WITH 100 STUDENT SUBJECTS

On a specimen of blood from each subject in the control group of students, the NBT dye reduction test was performed with two variations in the method of anticoagulation. The remainder of the technique was identical for each sample. The results are summarized in Table 8.

FIGURE 1. DETERMINATION OF OPTIMAL INCUBATION EQUIPMENT




A = Dry air oven

B = Water bath

C = Shallow dry heating block

D = Deep dry heating block

TABLE 8. NBT DYE REDUCTION SCORES

EDTA vs. EDTA/Heparin With 100 Student Subjects

| Sex | No. | NBT Positive PMN, Mean ± 1 S.D. | | | |
|---|---|---|---|---|---|
| | | EDTA | | EDTA/Heparin | |
| | | Percent | Absolute/cu mm | Percent | Absolute/cu mm |
| Female + | 16 | 3.6 ± 3.7 | 125 ± 115 | 8.9 ± 5.9 | 331 ± 224 |
| Female - | 29 | 3.2 ± 3.8 | 125 ± 181 | 7.2 ± 5.6 | 276 ± 281 |
| Total Female | 45 | 3.3 ± 3.8 | 125 ± 161 | 7.8 ± 5.7 | 295 ± 264 |
| Male | 55 | 2.0 ± 2.0 | 66 ± 69 | 5.7 ± 3.8 | 177 ± 115 |
| Total Student | 100 | 2.6 ± 3.0 | 93 ± 123 | 6.6 ± 4.9 | 230 ± 205 |

Female + = on oral contraceptives
- = not on oral contraceptives

By standard score analysis (17) of the means of the results of the two procedures, it was demonstrated that the two groups were statistically different ($P<0.0001$). It was observed that 24 of the 100 specimens of blood anticoagulated with EDTA alone produced NBT reduction scores of "zero".

It was concluded that the use of Ficoll had resulted in better cell preservation. Of the two methods of anticoagulation, it was concluded that the EDTA/heparin combination provided more easily definable formazan precipitate with minimal artifact interference. The final procedure was thus established.

D. FINAL PROCEDURE

1. Stock reagents

a. 0.2% NBT in saline

| | |
|---|---|
| Nitroblue tetrazolium (Sigma) | 200 mg |
| Saline, 0.85% | to 100 ml |

Filter, store at $4^{o}C$.

b. 0.15M phosphate buffered saline, pH 7.2

| | |
|---|---|
| Sodium chloride | 6.798 g |
| Sodium phosphate, dibasic, anhydrous | 1.478 g |
| Potassium phosphate, monobasic | 0.430 g |
| Demineralized water | to 1000 ml |

c. 40% Ficoll

| | |
|---|---|
| Ficoll (Sigma) | 2.0 g |
| Phosphate buffered saline | 5.0 ml |

Store at $4^{o}C$.

2. Working NBT dye solution

| | |
|---|---|
| 0.2% NBT in saline | 1 part |
| 40% Ficoll in phosphate buffered saline | 1 part |

Make up just prior to use.

3. Additional materials

"Vacutainers" (Becton-Dickinson), disodium edetate (EDTA), 5 ml

Sodium heparin, 1,000 USP units per ml

Temp-Blok module heater

Hema-Tek slide stainer

Pipettes, test tubes, microscope slides as needed

4. Method

Dye reduction is initiated immediately after specimens for Coulter Counter and differential have been removed to avoid possible interference of heparin in other hematology parameters.

a. Add 10 units sodium heparin per ml of blood to EDTA anticoagulated blood specimen, and mix gently.

b. Place equal aliquots of EDTA/heparin blood and working NBT dye solution in a disposable test tube, e.g. 0.2 ml each. Mix gently.

c. Incubate tube in Temp-Blok module heater at 37$^{o}$C for 15 minutes. Mix gently.

d. Incubate tube at room temperature for 15 minutes. Mix gently.

e. Make push slide and process as a routine differential slide in the Hema-Tek Slide Stainer.

5. Neutrophil evaluation

Photographic reproductions of these cells and their interpretations are given in Figures 2 through 10.

a. Under oil immersion evaluate 100 consecutive neutrophils, segmented and banded forms, for distinctive formazan deposits. Ignore ruptured cells, stippled deposits of formazan, cells other than neutrophils.

b. Determine percent of NBT positive cells observed.

c. Calculate absolute value of NBT positive cells.

d. Report NBT reduction score as "Percent NBT positive cells" and "Absolute NBT positive cells per cubic millimeter of blood".

6. Technical comments

The stock NBT solution remains stable at 4°C for at least nine months although periodic filtration is necessary.

The 40% Ficoll solution remains stable for at least one month at 4°C if contaminant growth does not develop. It is necessary to warm the viscous solution to room temperature before pipetting.

The NBT reduction test must be completed within two hours of phlebotomy to avoid disintegration and fragmentation of neutrophils.

E. DETERMINATION OF NORMAL VALUES FOR THE NBT POSITIVE CELLS IN CONTROL SUBJECTS

Using the standard score and t test (17), the results of the EDTA/heparin technique in the three subdivisions within the student controls were analyzed. Comparing the males and females in this group, there was a slight difference ($P>0.01,<0.05$). No significant difference was

FIGURE 2. Segmented Neutrophil. NBT negative.

FIGURE 3. Segmented Neutrophil. NBT negative.
(Stippled formazan precipitate)

FIGURE 4. Segmented Neutrophil. NBT negative.
(Stippled formazan precipitate)

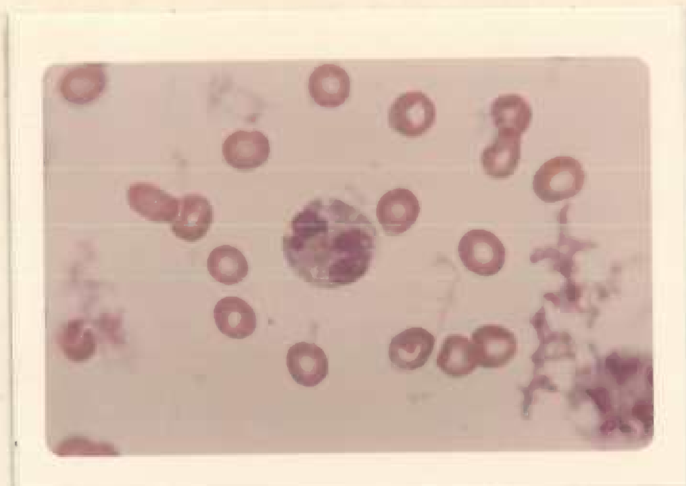


Figure 2.

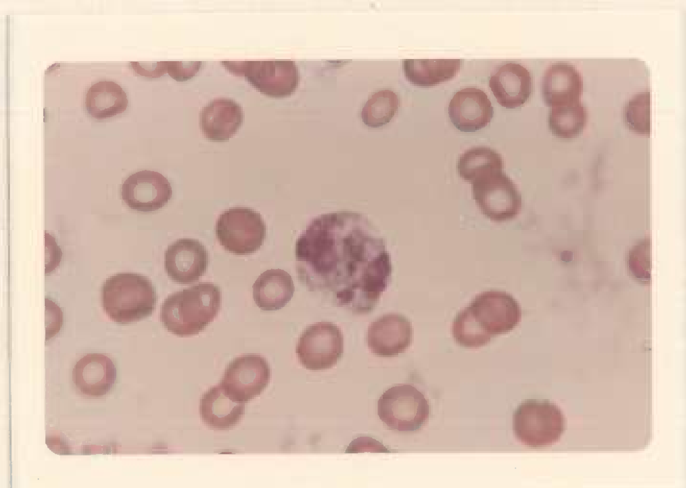


Figure 3.

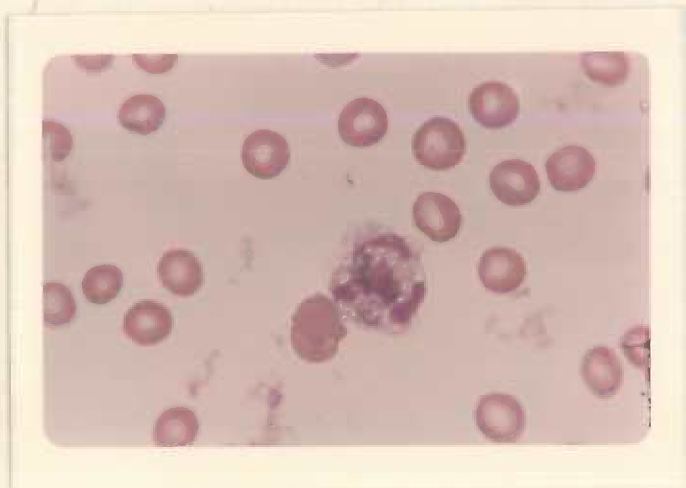


Figure 4.

FIGURE 5. Segmented Neutrophil. NBT positive.

FIGURE 6. Segmented Neutrophils. Upper: NBT positive,
Lower: NBT negative.

FIGURE 7. Segmented Neutrophils. Upper: NBT positive,
Lower: NBT negative. (Lymphocyte not included).

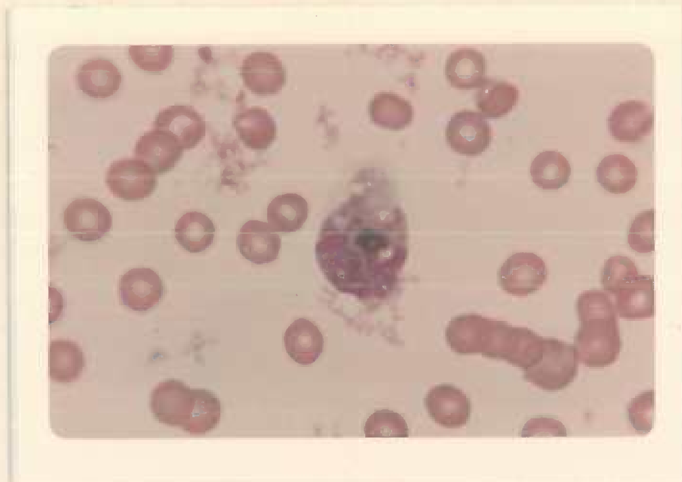


Figure 5.

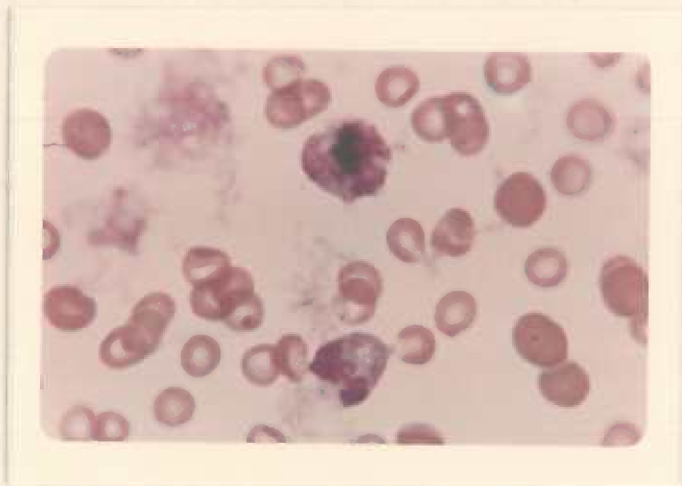


Figure 6.

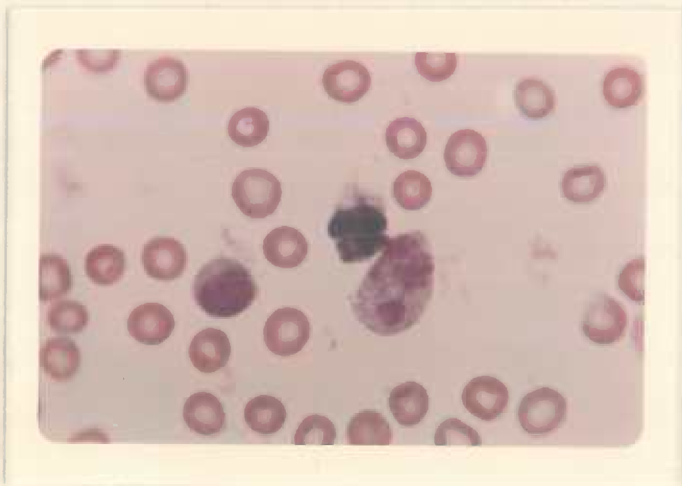


Figure 7.

FIGURE 8. Segmented Neutrophil with ruptured membrane. (Not included).

FIGURE 9. Unidentified Cell. (Not included).

FIGURE 10. Monocyte. (Not included).

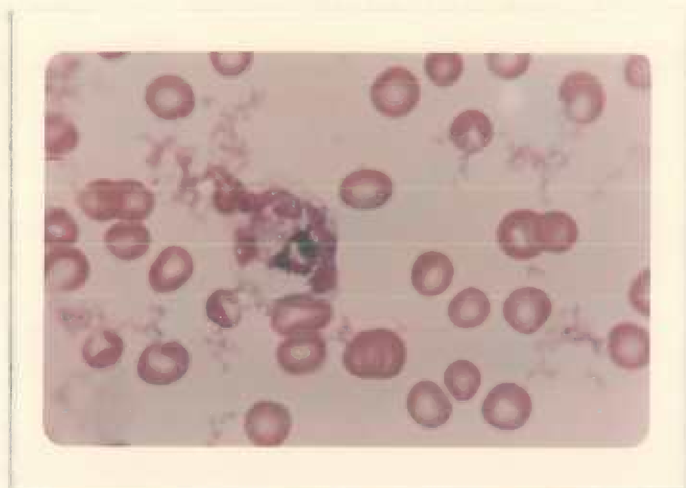


Figure 8.

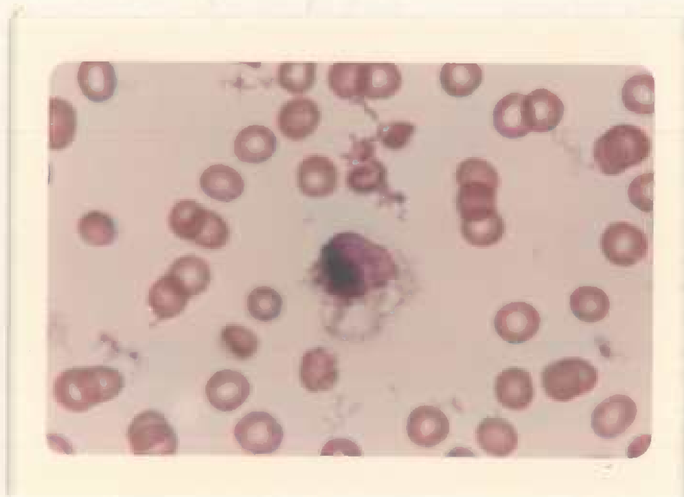


Figure 9.

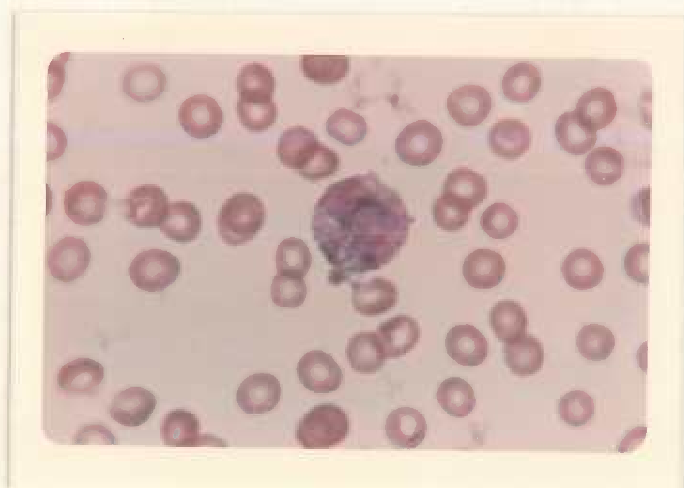


Figure 10.

observed between the females receiving oral contraceptive medication and those not (P>0.05). Table 9 summarizes these results.

The results of the bloods analyzed from the 32 senior citizens are also given in Table 9. No statistical difference was observed between their values and those of the student population (P>0.05). These were therefore combined into one set of normal ranges giving values for percent NBT positive of 6.6% (±4.8) and for absolute NBT positive per cubic millimeter of 226 (±198), mean ±one standard deviation.

To determine whether any abnormal individuals had been included in the control group, the subjects whose NBT reduction scores exceeded the mean with two standard deviations were examined. The upper limits of NBT reduction were set as 16% or 622 per cubic millimeter. Of the 132 control subjects, seven were found to lie above 16% Two of these seven however had absolute values less than 622 per cubic millimeter. The results of these individuals are tabulated in Table 10.

Three of the seven gave no contributory history to possibly account for the elevated score. Subject No. 1 later gave a history of recurrent bladder infections, and No. 5 of active acne. Subject No. 3 was later demonstrated to have a positive homogeneous "Antinuclear Antibody Titer" of 1:80 and No. 7 three weeks later developed infectious mononucleosis.

With the results of these seven individuals eliminated, the NBT reduction values of the remaining 125 control subjects were re-examined to see if the normal values were different. This gave new mean values (±one standard deviation) for percent NBT positive of 5.8% (±3.6) and for absolute NBT positive per cubic millimeter of 196 (±126). By

TABLE 9. NBT DYE REDUCTION SCORES

EDTA/Heparin With Control Population

| Subjects | No. | NBT Positive PMN, Mean ± 1 S.D. | |
|---|---|---|---|
| | | Percent | Absolute/cu mm |
| Students | 100 | 6.6 ± 4.9 | 230 ± 205 |
| Senior Citizens | 32 | 6.5 ± 4.7 | 213 ± 170 |
| Total Control Population | 132 | 6.6 ± 4.8 | 226 ± 198 |

TABLE 10. CONTROL SUBJECTS ABOVE NORMAL RANGE OF NBT SCORES

| Subject No. | Sex | HEMATOLOGY PARAMETERS | | | NBT POSITIVE PMN | |
|---|---|---|---|---|---|---|
| | | Total WBC x $10^3$/cu mm | Percent Bands | Absolute Neutrophils x $10^3$/cu mm | Percent | Absolute/ cu mm |
| 1 | Female | 4.0 | 3 | 1.7 | 20 | 344 |
| 2 | Female | 7.0 | 3 | 5.1 | 19 | 971 |
| 3 | Female | 6.4 | 8 | 4.9 | 18 | 876 |
| 4 | Female | 6.9 | 5 | 3.7 | 20 | 731 |
| 5 | Female | 7.1 | 11 | 5.6 | 24 | 1346 |
| 6 | Female | 6.3 | 3 | 4.0 | 21 | 833 |
| 7 | Male | 4.4 | 2 | 1.8 | 18 | 333 |

standard score analysis these figures did not differ from the values obtained with the seven included ($P>0.05$).

## F. DETERMINATION OF NORMAL VALUES OF RELEVANT HEMATOLOGY PARAMETERS IN CONTROL SUBJECTS

Hematology profiles provided by the Model S Coulter Counter measured the parameters of the total leukocyte and erythrocyte counts per cubic millimeter, the hemoglobin in grams per 100 milliliters and mean cell volume with the resultant calculated hematocrit and other cell indices.

Leukocyte differential smears were evaluated using the criteria for differentiating banded and segmented cells as currently given by Miale (51). A neutrophil was not classified as a segmented form unless a typical threadlike filament was visible.

The traditional indices of inflammatory involvement have been body temperature and an elevated leukocyte count although often the only indication of infection will be an increase in banded forms of neutrophils. The parameters from the hematology profile and differential used in this investigation in assessing correlations with potential disease states were the total leukocyte count, percent of banded neutrophils and the absolute neutrophil count. The statistical calculations of the mean (±one standard deviation) of these parameters for the 100 subjects of the students and the 32 senior citizens used as control subjects are given in Table 11.

No statistical difference was observed between the student female group on oral contraceptives and the one not ($P>0.05$). Nor was there

TABLE 11. RELEVANT HEMATOLOGY PARAMETERS FOR THE ASSESSMENT OF THE NBT TEST

Normal Values of Control Population

| Subject | No. | Age ($\overline{x}$) | Total WBC x $10^3$/cu mm ($\overline{x}$ ± 1 S.D.) | Percent Bands ($\overline{x}$ ± 1 S.D.) | Absolute Neutrophils x $10^3$/cu mm ($\overline{x}$ ± 1 S.D.) |
|---|---|---|---|---|---|
| Students | | | | | |
| Female + | 16 | 23.0 | 6.4 ± 1.7 | 7.8 ± 5.9 | 4.1 ± 1.4 |
| Female - | 29 | 23.9 | 5.8 ± 1.5 | 7.4 ± 6.3 | 3.7 ± 1.5 |
| Total Female | 45 | 23.6 | 6.0 ± 1.6 | 7.5 ± 6.2 | 3.9 ± 1.4 |
| Male | 55 | 25.7 | 5.7 ± 1.5 | 6.5 ± 5.4 | 3.4 ± 1.3 |
| Total Students | 100 | 24.7 | 5.8 ± 1.5 | 7.0 ± 5.8 | 3.6 ± 1.4 |
| Senior Citizens | | | | | |
| Total | 32 | 80.6 | 6.0 ± 1.2 | 4.4 ± 3.0 | 3.3 ± 0.8 |
| Total Control Population | 132 | 38.2 | 5.8 ± 1.6 | 6.4 ± 5.3 | 3.5 ± 1.4 |

Female + = on oral contraceptives
- = not on oral contraceptives

a difference observed between the male students and the combined student female subjects ($P>0.05$).

For the purposes of this investigation, the hematology parameters of the student subjects and those of the senior citizens were accepted as representing the same adult group. This gave a final summary for the 132 control subjects having a mean age of 38.2 years as follows in mean (±one standard deviation): total leukocyte count, $5.8 \times 10^3$ (±1.6) per cubic millimeter; percent bands, 6.4% (±5.3); and absolute neutrophils, $3.5 \times 10^3$ (±1.4) per cubic millimeter.

G. EVALUATION OF PATIENT SUBJECTS

1. Bacterial infections

Of the patients from the Emergency Room, 20 were identified through culture results as having bacterial infectious processes and were evaluated with the NBT test. Among these adults it was observed that 16 gave percent NBT reduction scores higher than the upper limit of 16%; 18 demonstrating absolute NBT reduction higher than the upper control level of 622 per cubic millimeter. This group had a mean age of 27.3 years and was represented by 15 females and five males. The mean percent NBT reduction was 32.2% and the mean absolute score, $3.94 \times 10^3$ per cubic millimeter. The individual results of these evaluations are given in Table 12.

Chi-square analysis (17) of the percent NBT score clearly differentiated between healthy controls and these patients with infectious states ($P<0.01$).

TABLE 12. PATIENT POPULATION OF INFECTIOUS DISEASES

| Subject No. | Sex | Age | Temp. °F | HEMATOLOGY PARAMETERS | | | NBT POSITIVE PMN | | Diagnosis |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Total WBC x $10^3$/cu mm | Percent Bands | Absolute Neutrophils x $10^3$/cu mm | Percent | Absolute x $10^3$/cu mm | |
| Normal Subjects | | | | 5.8 ± 1.6 | 6.4 ± 5.3 | 3.5 ± 1.4 | 6.6 ± 4.8 | 0.226 ± 0.198 | |
| 1 | F | 49 | 99.2 | 15.4 | 22 | 11.5 | 48 | 5.5 | Cholecystitis |
| 2 | F | 62 | 99.3 | 29.9 | 75 | 25.4 | 52 | 13.2 | Pneumonia; UTI |
| 3 | F | 26 | 100.3 | 14.6 | 22 | 12.1 | 18 | 2.2 | Endometritis |
| 4 | F | 36 | 100 | 5.9 | 24 | 4.4 | 17 | 0.75 | Pelvic cellulitis |
| 5 | F | 21 | 99.7 | 14.1 | 56 | 12.1 | 64 | 7.8 | UTI |
| 6 | F | 18 | 101.5 | 19.8 | 36 | 15.8 | 37 | 5.9 | Pyelonephritis |
| 7 | F | 35 | 98.9 | 7.4 | 9 | 4.8 | 11 | 0.53 | UTI |
| 8 | F | 18 | 99.1 | 10.8 | 37 | 7.7 | 34 | 2.6 | PID |
| 9 | F | 17 | 102.7 | 9.5 | 21 | 7.8 | 23 | 1.8 | UTI |
| 10 | F | 38 | 100 | 12.4 | 21 | 8.9 | 52 | 4.6 | PID |
| 11 | F | 35 | 102.1 | 22.3 | 34 | 18.5 | 16 | 3.0 | Breast abscess |
| 12 | F | 19 | 97.6 | 6.6 | 6 | 4.5 | 39 | 1.8 | Cervicitis; UTI |
| 13 | F | 15 | 98 | 14.3 | 42 | 11.0 | 58 | 6.4 | Pharyngitis |
| 14 | F | 20 | 99 | 13.1 | 13 | 9.7 | 48 | 4.7 | Pharyngitis |
| 15 | F | 18 | 102.1 | 10.6 | 14 | 8.5 | 2 | 0.17 | Mastitis |
| 16 | M | 23 | 102.3 | 11.9 | 43 | 9.8 | 25 | 2.4 | Pharyngitis |
| 17 | M | 24 | 100.3 | 11.9 | 26 | 8.2 | 25 | 2.0 | Pharyngitis |
| | | | | | | | | | Tonsillitis |
| 18 | M | 18 | 99.5 | 25.8 | 53 | 21.4 | 27 | 5.8 | Groin abscess |
| 19 | M | 31 | 97.6 | 29.8 | 28 | 28.9 | 12 | 3.5 | Pneumonia |
| 20 | M | 23 | 102.7 | 14.2 | 50 | 11.5 | 36 | 4.1 | Pharyngitis |
| Summary | | 27.3 | 100.1 | 15.0 | 31.6 | 12.1 | 32.2 | 3.9 | |

2. Liver disease

The six patients in this group of liver diseases evaluated with the NBT test were classified by the attending physician into the two general categories of alcoholic cirrhosis with and without alcoholic hepatitis (necrosis). The results are given in Table 13.

The number of patients seen was too few for adequate statistical comparison with normal subjects. However, the results appeared to have more similarity with the group of bacterial infections than with the normal subjects. Five of the six subjects had increased NBT reduction. All sedimentation rates done were elevated. Five of the six patients demonstrated a degree of fever.

3. Metastatic neoplasm

Tissue necrosis in malignant neoplasms was established by the attending physician on the basis of clinical appearance of the tumor and/or significant radiologic findings. Inflammatory breast carcinoma was not included in this category since necrosis is not a part of that process. Eight patients with necrosis associated with their neoplasms and seven without were evaluated with the NBT test and the results are given in Table 14.

There was no statistical difference between the mean of 37.6% NBT positive for the "non necrotic" group and the mean of 34.8% for the "necrotic" group ($P>0.05$). The mean sedimentation rate of the necrotic group was greater than that of the non necrotic, although the remainder of the hematology parameters appeared similar. Chi-square analysis clearly differentiated between these groups and the healthy controls ($P<0.01$).

TABLE 13. PATIENT POPULATION OF LIVER DISEASES

| Subject No. | Sex | Age | ESR | Temp. °F | Total WBC x $10^3$/cu mm | Percent Bands | Absolute Neutrophils x $10^3$/cu mm | NBT Positive PMN: Percent | NBT Positive PMN: Absolute x $10^3$/cu mm | Diagnosis |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | **A. Alcoholic Cirrhosis** — HEMATOLOGY PARAMETERS | | | | | |
| Normal Subjects | | | | | 5.8 ± 1.6 | 6.4 ± 5.3 | 3.5 ± 1.4 | 6.6 ± 4.8 | 0.226 ± 0.198 | |
| 21 | F | 65 | n.d. | 98.4 | 7.5 | 13 | 5.4 | 36 | 1.9 | Alcoholic Cirrhosis |
| 22 | M | 64 | n.d. | 99.0 | 5.3 | 7 | 3.1 | 21 | 0.7 | |
| 23 | M | 57 | 20 | 99.2 | 9.5 | 7 | 7.9 | 29 | 2.3 | |
| 24 | M | 63 | 47 | 99.6 | 5.7 | 22 | 4.3 | 31 | 1.3 | |
| Summary | | 62.3 | 33.5 | 99.1 | 7.0 | 12.3 | 5.2 | 29.3 | 1.6 | |
| | | | | | **B. Alcoholic Cirrhosis With Alcoholic Hepatitis** | | | | | |
| 25 | F | 36 | 45 | 101.5 | 13.9 | 38 | 11.3 | 11 | 1.2 | Alcoholic Cirrhosis With Alcoholic Hepatitis |
| 26 | M | 56 | 65 | 99.4 | 7.4 | 35 | 6.5 | 24 | 1.6 | |
| Summary | | 46 | 55 | 100.5 | 10.7 | 36.5 | 8.9 | 17.5 | 1.4 | |

TABLE 14. PATIENT POPULATION OF METASTATIC NEOPLASMS

| Subject No. | Sex | Age | ESR | A. Non-necrotic: HEMATOLOGY PARAMETERS: Total WBC x $10^3$/cu mm | Percent Bands | Absolute Neutrophils x $10^3$/cu mm | NBT POSITIVE PMN: Percent | Absolute x $10^3$/cu mm | Diagnosis |
|---|---|---|---|---|---|---|---|---|---|
| Normal Subjects | | | | 5.8 ± 1.6 | 6.4 ± 5.3 | 3.5 ± 1.4 | 6.6 ± 4.8 | 0.226 ± 0.198 | |
| 27 | M | 78 | n.d. | 11.9 | 33 | 10.3 | 7 | 0.7 | Carcinoma of lung |
| 28 | F | 25 | 37 | 8.3 | 16 | 7.5 | 72 | 5.4 | Carcinoma of ovary |
| 29 | F | 80 | 35 | 4.0 | 17 | 3.4 | 61 | 2.0 | Carcinoma of breast |
| 30 | F | 42 | 25 | 4.2 | 8 | 2.3 | 47 | 1.1 | Carcinoma of adrenals |
| 31 | M | 45 | 8 | 4.6 | 4 | 2.0 | 27 | 0.5 | Melanoma |
| 32 | M | 45 | 23 | 8.4 | 6 | 3.1 | 9 | 0.3 | Melanoma |
| 33 | F | 62 | 43 | 6.0 | 11 | 4.7 | 40 | 1.9 | Carcinoma of breast |
| Summary | | 53.9 | 28.5 | 6.8 | 13.8 | 4.8 | 37.6 | 1.7 | |
| B. Necrotic | | | | | | | | | |
| 34 | M | 72 | 68 | 3.1 | 35 | 2.9 | 47 | 1.4 | Myocardial infarction; Carcinoma of colon |
| 35 | F | 61 | 82 | 4.4 | 46 | 3.6 | 71 | 2.6 | Carcinoma of cervix |
| 36 | F | 32 | 65 | 7.9 | 50 | 7.0 | 46 | 3.2 | Melanoma |
| 37 | F | 47 | 119 | 8.7 | 26 | 8.0 | 7 | 0.6 | Carcinoma of ovary |
| 38 | M | 76 | 107 | 5.2 | 13 | 3.9 | 7 | 0.3 | Carcinoma of tonsil |
| 39 | F | 107 | 45 | 8.0 | 13 | 6.6 | 38 | 2.5 | Carcinoma of colon |
| 40 | F | 50 | 37 | 4.9 | 3 | 4.1 | 21 | 0.8 | Carcinoma of bronchus |
| 41 | F | 60 | 9 | 8.1 | 8 | 5.3 | 41 | 2.2 | Carcinoma of breast |
| Summary | | 63.1 | 66.5 | 6.3 | 24.3 | 5.2 | 34.8 | 1.7 | |

Summaries of the values of the control and patient subjects for the NBT reduction scores are given in Table 15.

TABLE 15. SUMMARY OF NBT SCORES FROM CONTROL AND PATIENT POPULATIONS

| Subjects | No. | Age | NBT Positive PMN, Mean ± 1 S.D. | |
|---|---|---|---|---|
| | | | Percent | Absolute/$10^3$ cu mm |
| Normals | 132 | 38.2 | 6.6 ± 4.8 | 0.226 ± 0.198 |
| Infectious diseases | 20 | 27.3 | 32.2 | 3.9 |
| Liver diseases | | | | |
| Cirrhosis | 4 | 62.3 | 29.3 | 1.6 |
| Cirrhosis with hepatitis | 2 | 46 | 17.5 | 1.4 |
| Metastatic neoplasms | | | | |
| Non-necrotic | 7 | 53.9 | 37.6 | 1.7 |
| Necrotic | 8 | 63.1 | 34.8 | 1.7 |

# DISCUSSION

The host response in a normal person to a bacterial infection is seen in the peripheral blood as leukocytosis. The presence of immature neutrophils or fever may sometimes be the only indication that infection is present. However, hematologists emphasize that leukocytosis occurs in many physiologic situations unrelated to inflammatory processes, e.g. emotional stress or the physical stresses of strenuous exercise, convulsive seizures, anesthesia, paroxysmal tachycardia, cold exposure, ultra-violet irradiation, obstetrical labor and menstruation (51, 82). Disease states other than infectious are also known to produce a fever or an elevated leukocyte count. A rapid and differential indicator of bacterial infection would therefore have important clinical use not only to avoid indiscriminate therapy, but also to direct the physician to further definitive investigations.

Park et al (59) developed the qualitative NBT test as a diagnostic tool hopefully useful in differentiation of bacterial infection from nonbacterial disease. Since its introduction, the NBT test has undergone numerous technical variations. The several NBT procedures have yielded results difficult to evaluate. Cell membranes were often ruptured. Neutrophils tended to aggregate, forming unrecognizable clumps. These were major criticisms noted by many investigators (32, 34, 54, 67, 81). Reliable evaluation of cells in the NBT test requires easily identifiable cells as well as clear cut distinction between NBT positive and NBT negative cells.

The initial purpose of this investigation was to adapt and standardize the qualitative NBT dye reduction test into a procedure easily performed in a routine clinical laboratory. Once satisfactorily established, the procedure was to be evaluated with normal healthy adult control subjects and adult patients.

Optimal incubation temperatures were obtainable with either the water bath system or the full tube depth dry well thermal blocks. Because of its small size and convenience, the thermal block was used in this investigation. Temperature studies using other incubation systems revealed that dry air incubators at 37°C do not significantly change tube contents from their previous room temperature within 60 minutes.

Trials with some of the current proposed procedures suggested that the heparin concentrations used were somehow partially responsible for the excessive clumping noted. The use of EDTA anticoagulant alone as suggested by Gordon et al (31), resulted in less clumping, but gave a significant number of "zero" results, nearly one quarter of the control subjects tested. The innovation of this investigation was to add a small amount of heparin (10 units/ml of blood) to an EDTA anticoagulated blood specimen, apparently stimulating the neutrophils to a measurable level of NBT reduction above "zero".

Since the work of this current study, others have reported reaching the same conclusion. Hellum and Solberg (35, 36) have likewise determined 10 units of heparin per ml of blood to be a desirable concentration in the NBT test. They further demonstrated that chelating and calcium binding anticoagulants (e.g. EDTA, oxalates) caused a marked

inhibition of formazan formation.

The study of Segal and Levi (66) showed that the presence of heparin was necessary for dye reduction and that NBT had a dual action. NBT caused the precipitation of a macromolecular complex, the nature of which depended on the anticoagulant used. In the case of heparin, both heparin and fibrinogen were precipitated. In the presence of EDTA, fibrinogen alone was precipitated. Phagocytosis of the precipitated material resulted in concomitant phagocytosis of dye which turned dark blue after intracellular reduction. The blue colored formazan was easily seen under the light microscope and the cell containing it was labelled "NBT positive". Electron microscopy confirmed the presence of amorphous material, presumably complexed NBT, within the membrane bound phagosomes. Prior enhancement of phagocytic activity of neutrophils was necessary for production of elevated NBT reduction. It was therefore reasoned that circulating bacterial products could be the natural stimulus of phagocytosis that resulted in an increased NBT score in patients with bacterial infection.

The use of the EDTA blood specimen in the present investigation proved to be a convenience insofar as the EDTA specimen was often already submitted for routine hematology profiles and could simply be processed further for the NBT test. The addition of small amounts of heparin provided the media for measurable dye reduction.

The use of 40% Ficoll as suggested by Gordon et al (31) was noted to aid in the preservation of cell membranes and was therefore incorporated into this procedure.

The qualitative NBT dye reduction test as finally established in

this investigation satisfactorily meets the criteria for an easily performed procedure in which NBT positive and NBT negative cells are clearly distinguishable with minimal artifact interference.

Control subjects were students at the University of Oregon Medical and Dental Schools and volunteer senior citizens. These are not a random sample of the population. However, their hematology parameters compared well with the normal range, so they hopefully represented a healthy normal adult population. General agreement with current authorities is shown in Table 16 comparing values of this investigation with those of Miale (51) and Williams (82).

The standardized NBT test was performed on these 132 control subjects with the following results:

| | Mean ±1 standard deviation | Range |
|---|---|---|
| Percent NBT positive | 6.6 (±4.8) | 1 - 24 |
| Absolute NBT positive/cu mm | 226 (±198) | 25 - 1346 |

These values agree favorably with other published studies. Table 17 summarizes some of the published reports in comparison with the results of this investigation.

The female student control group was subdivided into those on oral contraceptives and those not. The use of oral contraceptives did not influence the values of the NBT scores in this study. In a study of seven females on oral contraceptives, Norden and Reese (56) noted a definite increase in NBT reduction in five. Follow up studies on one gave a normal NBT score one week after withdrawal from the medication. Their method could not be compared to that of this present study as no

TABLE 16. COMPARISON OF HEMATOLOGY PARAMETERS FROM CONTROL SUBJECTS WITH PUBLISHED TEXTS

| Age | Total WBC x $10^3$/cu mm ($\bar{x} \pm 1$ S.D.) | Percent Bands ($\bar{x} \pm 1$ S.D.) | Absolute Neutrophils x $10^3$/cu mm ($\bar{x} \pm 1$ S.D.) | Ref. |
|---|---|---|---|---|
| 38.2 | 5.8 ± 1.6 | 6.4 ± 5.3 | 3.5 ± 1.4 | Current Study |
| 21 | 7.4 ± 2 | 8.0 ± 3 | 4.4 | 51 |
| 21 | 7.4 | 3.0 | 4.4 | 82 |

TABLE 17. NBT DYE REDUCTION SCORES

Normal Values From Published Reports

| No. of Subjects | Age | NBT Positive PMN, Mean ± 1 S.D. | | Ref. |
|---|---|---|---|---|
| | | Percent | Absolute/cu mm | |
| 132 | 38.2 | 6.6 ± 4.8 | 226 ± 198 | Current Study |
| 24 | adults | 4.6 ± 4.3 (SE) | Not given | 10 |
| 76 | 28 | 8.0 | 600 | 12 |
| 20 | 3 mons to 15 yrs | 8.75 ± 5.25 | 420.5 ± 278.0 | 20 |
| 60 | 18-45 | 6.1 | Not given | 31 |
| 49 | adults | 7.7 ± 0.3 (SE) | Not given | 40 |
| 22 | 26 | 4.1 ± 4.0 | Not given | 73 |

reference was made to the technique employed for NBT reduction.

The "P" values given in the results of this study were derived in two ways. With groups numbering less than 25, the F test and Student's t test were used to compare the variances and means. The Medians Test (Chi-square) was used to compare those groups with unequal variances. Groups numbering more than 25 were analyzed by computing the standard score (z score) (17).

The intent of this study was to correlate NBT scores with presence or absence of bacterial infection in febrile patients seen in the Emergency Room. The results obtained with this modified procedure confirm the elevated NBT scores for bacterial infections reported by numerous investigators (19, 20, 21, 27, 40, 48, 73).

All five cases of streptococcal pharyngitis gave significantly elevated NBT scores (range, 25% to 58%) in this study. Three of the five demonstrated fevers above $100^{o}$F. These NBT results are in contrast to the findings recently reported by Shapera and Matsen (69) who used the original technique for NBT reduction of Park et al (59, 60). During peak clinical symptoms, their group of 17 patients with culture proven Group A streptococcal pharyngitis had less than 9% NBT positive neutrophils. They felt the NBT test was invalid in differentiating streptococcal from non streptococcal pharyngitis.

Other disorders referred from the Emergency Room were non infectious, e.g. cirrhosis and malignancies. It was reasoned that the elevated NBT scores might reflect underlying tissue necrosis. Small patient groups from the hospital and clinics were evaluated with NBT reduction to test this hypothesis. The physician relies on such

indications as fever, elevated sedimentation rate or signs of inflammation to make the diagnosis of tissue necrosis. The majority of subjects in these small groups of patients with alcoholic cirrhosis and metastatic neoplasms had elevated neutrophil NBT reduction, not correlated with the clinical impression of necrotic cirrhosis or tumor.

Others have since reported on the possible influence of necrosis on NBT reduction. Cell necrosis resulting from the infarcted myocardium has been noted to cause an increase in the activity of the hexose monophosphate shunt (72) possibly attributable to the scavenger activity of neutrophils and other phagocytes rushing to the scene (1). A rise in NBT score with acute myocardial infarction has since been reported (47) and seemed to correlate with the stages of maximal neutrophilic infiltration in the damaged areas. Similar elevations have recently been reported in neoplasms with fever (3).

Many writers have felt that the NBT response in neutrophils was a valuable adjunct to other diagnostic laboratory procedures in its capacity to distinguish adult patients with active bacterial infection from those with nonbacterial inflammatory disease (12, 18, 19, 20, 30, 31, 40, 48, 73).

However, increased levels of NBT reduction by neutrophils have also been reported in a number of clinical conditions without evidence of bacterial infection, e.g. lymphoma (70, 74), myelofibrosis (55), polycythemia vera (3), hemophilia (40), Chediak-Higashi syndrome (33), and neonates (15, 16, 61). From a study of 141 subjects, Bittner et al (10) recently reported that in severely ill adults, the NBT test did not discriminate well between a population of patien ts known to have

bacterial infection and a population of patients who had other illnesses. The use of conventional hematologic procedures appeared to be more sensitive than the NBT test in detection of bacterial infections. They concluded that its routine use was therefore not justified.

The proliferation of reports of conditions resulting in elevated NBT reduction scores dampens the original enthusiasm felt for the NBT test as a diagnostic screening procedure for systemic bacterial infection. The application of the test for distinguishing nonbacterial febrile states is made more complex by this addition of cirrhosis and metastic meoplasms without bacterial infections as causes of increased NBT reduction.

SUMMARY

In this study, technical modifications of the qualitative NBT dye reduction test were investigated and their effect on subsequent neutrophil morphology evaluated.

Optimal cell morphology was obtained by incorporating into the basic procedure several innovations. To the EDTA anticoagulated routine hematology blood specimen was added 10 units of sodium heparin per ml of blood. The 0.2% NBT dye solution was diluted with an equal aliquot of 40% Ficoll. Dry well thermal blocks gave optimal incubation. NBT dye solutions were filtered periodically.

Thus a standardized technique was established that resulted in good neutrophil morphology after NBT reduction, with intact cell membranes, easily definable formazan precipitate, and minimal artifact interference.

Normal hematology values were established for a control group of 100 student subjects and 32 senior citizens. The mean (±one standard deviation) NBT reduction scores for this control group were 6.6% (±4.8) or 226 (±198) per cubic millimeter.

Three groups of patients from the Emergency Room and hospitals and clinics were also evaluated. Twenty patient subjects with bacterial infections gave a mean NBT score of 32.2% (range, 2% to 64%) or 3900 per cubic millimeter (range, 170 to 13200).

Six patients with alcoholic cirrhosis were subdivided into those with alcoholic hepatitis (necrosis?) and those without in the hopes of

differentiating with the NBT test. The number studied was too small to statistically evaluate, but five of the six had definitely elevated NBT reduction (range, 11% to 36% or 700 to 2300 per cubic millimeter).

Fifteen patients with neoplastic disease were clinically divided into necrotic and non necrotic groups. The NBT test was likewise unable to differentiate tissue necrosis from non necrotic tumor, the mean of both groups being elevated above normal (range, 7% to 72% or 300 to 5400 per cubic millimeter).

The clinical application of the neutrophil NBT reduction procedure as standardized in this study as far as being a diagnostic aid in bacterial infections is questioned. It cannot discriminate between febrile disorders; neither can tissue necrosis be differentiated from non necrotic states.

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