USE OF THE NITROBLUE TETRAZOLIUM TEST IN VETERINARY HEMATOLOGY

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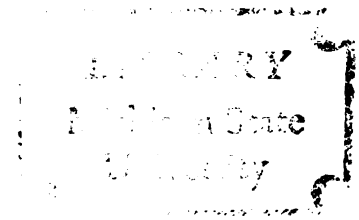

ABSTRACT

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By

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In human patients, the occurrence of bacterial infection is reflected in an increase in the percentage of nitroblue tetrazolium (NBT) reducing neutrophils above the levels observed in healthy controls and in individuals with nonbacterial conditions.

In this study, a modified technique was employed to perform the NBT test on blood samples of 4 species of domestic animals. The study utilized blood samples from 139 dogs, 39 cats, 62 horses and 34 cattle.

Average scores obtained in healthy control animals were as follows: dog - 3.0%, cat - 3.5%, horse - 3.2%, and cow - 1.5%. Bacterial infections reflected in the systemic circulation resulted in an increase in NBT reduction in all 4 species similar to that reported in human patients. Most of the positive scores ranged from 15 to 35%. A few values over 70% were obtained in dogs and horses. The highest scores observed in cats and cattle were approximately 35%, but fewer individuals of these 2 species were sampled.

Clumping of cells and dense deposits of formazan which obliterated cell nuclei and ruptured cytoplasmic membranes were often seen in animals exhibiting high percentages of positive cells.

Although the incidence of false-negative and false-positive scores was low, false scores did occur in all 4 species. In many cases, explanation of these results was extremely difficult. Moreover, since in some cases treatment histories were incomplete and/or cultures could not be performed, it is possible that there were some additional false results which were unrecognized. In view of the rather high incidence of false results in human patients, it is likely that several of the conditions resulting in false scores in human patients are duplicated in animal species and could be detected by controlled studies.

The results obtained in this study indicate that the NBT test is useful in differentiating bacterial infection from nonbacterial conditions in all 4 of the species sampled. The NBT test would appear to be especially useful: 1) in monitoring high-risk patients for early bacterial infection, and 2) in serial tests to determine the efficacy of antibacterial therapy in animals with severe infections.

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INTRODUCTION

Tetrazolium salts are widely used in histochemical studies of various enzymes (Thompson, 1966). At the site of enzyme activity, colorless tetrazolium salts are reduced to water-insoluble formazan, which can easily be recognized as a deep blue deposit. Only in recent years has nitroblue tetrazolium (NBT) been employed in a dye test to distinguish bacterial diseases from other clinically similar conditions (Park *et al.*, 1968). Although examination of the literature revealed only a few articles concerning the use of this test in animals, this procedure is gaining some prominence in the clinical diagnosis of human disease. The study described herein was undertaken in order to study the potential of the NBT dye test as a diagnostic aid in veterinary medicine.

LITERATURE REVIEW

Use of the NBT Test in Chronic Granulomatous Disease of Children

In an early study, Baehner and Nathan (1967) reported that, *in vitro*, a low percentage of neutrophils of normal individuals were able to reduce the clear pale yellow nitroblue tetrazolium salt to opaque, blue-black formazan crystals which were deposited within the cytoplasm of the cells. These investigators subsequently discovered that there was a decreased reduction of the tetrazolium salt in the neutrophils of children with chronic granulomatous disease (CGD). This disease is an X-linked genetic trait in which the phagocytes of the individual are able to engulf bacteria in the normal manner but are unable to kill the microorganisms. This condition results in recurrent granulomatous infections due to pyogenic pathogens of low virulence and eventual death due to chronic suppurative infections. The susceptibility to infection is due to a functional defect of the phagocytic cells (Holmes *et al.*, 1966). The neutrophils of children with CGD are able to take up the NBT dye but are completely unable to reduce it to the characteristic formazan deposits.

Other investigators have expanded the use of the nitroblue tetrazolium test in detecting chronic granulomatous disease of children (Holmes *et al.*, 1967; Windhorst *et al.*, 1967; Quie *et al.*,

1967; Nathan *et al.*, 1969; Gifford and Malawista, 1969; Baehner *et al.*, 1970; Cooper *et al.*, 1970; Ochs *et al.*, 1972; Johnston, 1969).

Baehner and Nathan (1968), using a quantitative spectrophotometric NBT test, were able to demonstrate that the rate of reduction of NBT by normal leukocytes was stimulated by phagocytosis of latex particles. The rate of reduction was also dependent on cell number, pH, and temperature.

These investigators also demonstrated that granulocytes of CGD-affected male patients failed to reduce NBT dye even when stimulated by phagocytosis. The leukocytes of carrier females, however, demonstrated intermediate dye reduction. In addition to the lack of NBT reduction, the cells of affected children had low values for oxygen consumption and diminished reduced nicotinamide adenine dinucleotide (NADH) oxidase.

In 1969, Park and his associates performed 24 histochemical NBT slide tests on 8 children affected with CGD. They reported no formazan deposits in neutrophils, monocytes, or platelet clumps from these children using this simplified method. These investigators concluded that the NBT test was a simple and useful screening test for this fatal disease.

Use of the NBT Test in Bacterial Infections

As mentioned previously, a small proportion of neutrophils of normal individuals reduce nitroblue tetrazolium dye spontaneously, but the *in vitro* phagocytosis of latex particles greatly increases this proportion. During the course of a natural infection, the

infecting organisms undergo phagocytosis by neutrophils *in vivo*. Park, Fikrig and Smithwick (1968) hypothesized that such a challenge would induce metabolic changes in these neutrophils sufficient to cause spontaneous *in vitro* reduction of NBT dye by an increased proportion of neutrophils as compared with healthy individuals or those with nonbacterial disease.

Using a supravital staining slide test, Park and his associates reported that healthy controls had between 145 and 720 NBT positive neutrophils per cubic millimeter, or 5.8 to 9.5% (mean 8.5%). Similar values were obtained in children affected with rheumatoid arthritis, systemic lupus erythematosus, primary tuberculosis or a variety of viral diseases.

However, they reported that both the absolute number and percentage of NBT positive neutrophils were markedly increased in children affected with *Candida albicans* septicemia, bacterial meningitis or other acute bacterial infections. The percentage of NBT positive neutrophils ranged from 29 to 47%.

Followup studies of all patients with acute bacterial infections revealed that the absolute number and percentage of NBT positive neutrophils eventually returned to normal after recovery from the septic episode.

Park and his colleagues concluded that the nitroblue tetrazolium test was a simple and useful method of differentiating certain types of bacterial infection from nonbacterial diseases.

Matula and Paterson (1971) subsequently investigated the usefulness of the NBT test in the differentiation of febrile illnesses in adults. They found that increased reduction of NBT was

an early discriminating event in adult patients with bacterial infections due to a wide variety of pyogenic microorganisms, with infections due to *Nocardia* species, or with malarial parasitism. They also reported that elevated NBT responses rapidly returned to the normal range after initiation of effective therapy.

These investigators reported NBT responses of 10% or less (mean 3.0%) in patients in whom bacterial infection was absent. Nitroblue tetrazolium responses of 3 to 59% (mean 23.8%) were reported in patients with acute pyogenic bacterial infection.

Humbert and his associates (1971) also confirmed the original hypothesis presented by Park. They reported that acute bacterial infections were most often associated with a high percentage of NBT positive neutrophils while low values predominated in viral infections regardless of the patient's total leukocyte count. They also reported that, among the patients with noninfectious diseases, those patients affected with osteogenesis imperfecta, relatives of these patients, and patients affected with hemophilia frequently displayed high NBT responses. They also reported that the test was falsely negative in 17% of patients with bacterial infection and positive in 4% of their "controlled" population.

Feigin and his associates (1971) subsequently extended the observations of these investigators by classifying patients into 4 groups utilizing only the percentage and absolute number of NBT positive neutrophils. These 4 groups were: 1) normal subjects, 2) individuals with viral infections, noninfectious fevers or partially treated bacterial infections, 3) patients with untreated

bacterial infections, and 4) those with bacterial infections unresponsive to the therapy provided. Discriminant analysis of this information was used to prepare a nomogram which permitted classification of patients into one of the 4 groups on a prospective basis, provided that the percentage and absolute number of NBT positive cells were known.

In a later study, Feigin, Shackelford and Choi (1971) tested the reliability of the previously derived nomogram. After using the nomogram to predict correct patient classification, the results were compared with classification based on the final diagnosis. The frequency of *serious* misclassification proved to be small. They found that 11% of patients with bacterial infection had false-negative tests and 20% of the controlled population known not to have bacterial infection had false-positive tests.

These investigators (Feigin *et al.*, 1971) also reported no correlation between the percentage of NBT-positive cells and either white blood cell counts or body temperature recorded at the time blood for the NBT test was obtained.

Hallett and Wilson (1973) utilized dogs in their study of NBT reduction in experimental shock. The purpose of their study was to determine whether the qualitative NBT test, as described by Park *et al.* (1969), was a reliable aid to rapid detection of systemic bacterial infection and endotoxemia after severe hemorrhage with blood replacement. They reported a baseline value of $9.6 \pm 3.8\%$. Nitroblue tetrazolium reduction by neutrophils was not significantly altered after 2 hours of hemorrhagic hypotension

(score 10.0 $\pm$ 1.8%). However, a moderate increase in NBT-positive neutrophils was observed following the reinfusion of shed blood (16.5 $\pm$ 5.3%). This was believed to be caused by increased NBT metabolism in phagocytic leukocytes due to either systemic bacterial invasion or neutrophil damage secondary to blood storage and *in vivo* metabolic derangements associated with shock. The authors concluded that the NBT test was not a reliable indicator of bacteremia or endotoxemia after resuscitation with stored blood.

Although the results of most investigators have indicated that the NBT test is a useful diagnostic aid, 2 research teams strongly disagree and suggest that many of the affirmatory studies exaggerate the discriminatory ability of the test. Segal, Trustey and Levi (1973), using 2 different methods, reevaluated the NBT test in 223 individuals, including healthy controls, and patients with a wide spectrum of disease. They reported wide overlap between the results obtained from controls, patients with pyogenic infection and patients with other diseases. They also reported poor agreement between the results obtained from the 2 methods used for the test. In addition, significant observer error was found in interpretation of the results, and this was related to observer experience. These investigators concluded that the NBT test had no diagnostic relevance and that it had not upheld its early promise as a simple, accurate method of distinguishing bacterial infection from other disease processes.

Bittner *et al.* (1973), in their reassessment of the unstimulated nitroblue tetrazolium test as a routine screening test for

bacterial infection in an adult population, used a simplified version of the test potentially adaptable for routine hospital use. In 141 subjects in 4 population groups (healthy adults, patients with bacterial infections, patients with nonbacterial infections, and patients with noninfectious illness) there was no distinct segregation of patients with bacterial infections from patients with other disease processes. However, there were some average differences between groups which were significantly different. In particular, the average count for the group with bacterial infections was significantly higher than the average counts for the population with nonbacterial infection and the normal controls.

The NBT test failed to correlate with the underlying diagnosis in 27% of the subjects studied. These investigators simultaneously used the method of Park *et al.* (1969) in 45 subjects and the 2 methods were found to have comparable accuracy of discrimination. The NBT test was also compared with the erythrocyte sedimentation rate, pyrexia and neutrophilia as indices for discriminating bacterial infection from other diseases and found to be the least discriminatory test.

They concluded that the unstimulated NBT test might be somewhat useful as an adjunct to other tests in diagnosing bacterial infection, but it was not appropriate for the routine laboratories of general hospitals.

Use of the NBT Test in Newborn and Premature Infants

Park and his colleagues (1969) reported that, in all cord blood samples of newborn infants which they had tested, both the absolute number and the proportion of NBT-positive neutrophils were greatly increased (average 38.1%). However, matched maternal

blood samples had NBT values which fell within the normal range despite the presence of leukocytosis. These investigators also reported that the deposits in the cord blood were slightly larger and more diffuse than those observed in blood samples from older persons. In all cases, the number and percentage of NBT-positive neutrophils returned to normal limits within 7 to 20 days after birth.

Humbert, Kurtz, and Hathaway (1970) also reported significantly increased reduction of NBT by the phagocytes of normal newborn infants without infection. The cord blood samples of these infants were tested by both the histochemical method and the quantitative spectrophotometric method. They also reported that stimulation of the phagocytes by latex particles produced a rise in the NBT reduction that was significantly greater than they had expected.

Since these high values did not indicate an infection, Park and his associates (1969) considered them to be "false-positive" values due to the increased metabolism of leukocytes in newborn infants. The neutrophils of the newborn are thought to be activated in a manner similar to that which occurs during phagocytosis. Oxygen consumption, hexose monophosphate pathway metabolism and NBT dye reduction are all increased in the neutrophils of the newborn. The neutrophils also show the normal response to phagocytosis and normal staphylocidal capacity, thus indicating functional maturity.

In studies done with premature infants (Wehinger, Kim and Pringsheim, 1972; Cocchi, Mori and Becattini, 1969), healthy

premature infants and premature infants with nonbacterial diseases exhibited the same increased reduction of NBT as full-term newborn infants. However, premature infants with bacterial disease exhibited leukocytosis with depressed NBT reduction. These investigators considered the results to be similar to those reported in children with CGD and possibly to be due to a non-functioning phagocytosis system in the neutrophils. After 2 weeks of life, the premature infants showed the same increase of NBT-positive cells during bacterial infection as that reported for full-term infants.

False-Negative and False-Positive Results of the NBT Test (Table 1)

Negative results to the NBT test (less than 10% of the neutrophils contain formazan deposits) in the presence of bacterial infection could indicate a metabolic defect, either cellular or humoral, preventing the reduction of nitroblue tetrazolium dye. While chronic granulomatous disease of children is considered to be the classic example, this defect has also been reported with myeloperoxidase deficiency (Lehrer, 1970) and glucose-6-phosphate dehydrogenase deficiency of neutrophils (Cooper *et al.*, 1970). Congenital and acquired agammaglobulinemia has also been reported to give falsely negative results in the face of bacterial infection (Park, South, and Barrett, 1970), which suggests that the antibody production by the host may be a determinant in the generation of positive NBT responses.

Park *et al.* (1969b) also reported falsely negative results in nephrosis with pneumococcal peritonitis, in sickle cell disease

Table 1. Accumulated misdiagnoses by NBT test from the literature:* false-negatives

Streptococcal cellulitis
Chronic granulomatous disease
Myeloperoxidase deficiency
G-6-PD deficiency of neutrophils
Congenital and acquired agammaglobulinemia
Mixed cryoglobulinemia
Lipochrome histiocytosis with rheumatoid arthritis
Nephrotic syndrome with pneumococcal peritonitis and pneumococcal bacteremia
Sickle-cell anemia with pneumococcal meningitis or *Salmonella* osteomyelitis
Local infection
Histiocytosis-X
Shunt infection
Alcoholism with abscesses
Burn infection
Pneumonia with influenza infection
Ineffective antibiotic therapy
Corticosteroid therapy (conflicting reports)
Phenylbutazone therapy (in guinea pigs)
Osteomyelitis in diabetes mellitus
Uncomplicated urinary tract infection
Endometritis
Infection in debilitated persons: meningitis, pneumonia, abdominal abscess, carcinomatosis with septicemia, *Pseudomonas* endocarditis
Tertiary syphilis
Urinary tract infection and septicemia in a patient with syringomyelia
Leprosy
Systemic lupus erythematosus
Chronic myeloid leukemia

*Bittner *et al.* (1973).

with pneumococcal meningitis, and in lipochrome histiocytosis (Niemann-Pick disease) in association with rheumatoid arthritis and pneumococcal pneumonia. Goihman, Conert, and Rodriguez-Ochoa (1973) reported that leprosy gives false-negative results. Systemic lupus erythematosus (Wenger and Bole, 1972; Douwes, 1972) and chronic myeloid leukemia with concomitant bacterial infection have both resulted in false-negative responses. Any localized infection that is not reflected in the systemic circulation may also be responsible for false results (Park *et al.*, 1969b). Bittner *et al.* (1973) reported false-negatives in severely debilitated patients with bacterial infections. Individuals with diabetes mellitus also have abnormal NBT-reducing capacity due to certain plasma factors (De La Vega, Freyre-Horta and Benitez-Bibriesca, 1973). Diabetic patients are able to increase the nitroblue-tetrazolium reducing activity of their neutrophils under the influence of noninfectious diseases, but fail to do so in response to infection (Pujol-Moix, 1973).

It has been reported that the administration of certain antibiotics or other drugs before testing may obscure otherwise positive responses. Corticosteroids, phenylbutazone and cytotoxic drugs such as vinblastine and nitrogen mustard have also been reported to have this effect (Miller and Kaplan, 1970; Straus, Paul and Sbarra, 1968; Wollman *et al.*, 1972; Chretien and Garagusi, 1972; Matula and Paterson, 1971). However, with steroids, differing opinions concerning the method of testing and the time of the test in relation to the time of steroid administration made the conclusions questionable. After 12 hours,

intravenous steroids seemed to have no effect on the NBT test, whereas, prior to 12 hours, depression of the NBT-positive neutrophils was observed. Oral steroids were reported to give inconsistent results.

From the study of Wollman and his colleagues (1972) concerning the use of the NBT test in immunosuppressed renal transplant patients and uremic individuals, the following conclusions were drawn.

1. Noninfected uremic individuals have normal baseline NBT values.

2. Extracorporeal hemodialysis does not change the NBT values from baseline in uninfected uremics.

3. Bacterially infected uremic patients are able to mount a normal, positive NBT response to their infections. Viral infections in uremic patients, as in normal individuals, are not associated with significant increases in NBT positivity.

4. NBT test values fall within normal range in acute renal transplant rejection, permitting a differentiation between infection and rejection.

5. Despite the administration of large doses of steroids, patients undergoing renal transplantation are capable of responding to bacterial infections with a marked rise in the percentage of NBT-positive cells.

In addition to the previously mentioned false-positive results observed in newborn infants and in individuals affected with osteogenesis imperfecta or hemophilia (Humbert *et al.*, 1971),

a number of other conditions have been reported to give falsely positive responses to the NBT test (Table 2). High values for the NBT test were reported in patients receiving typhoid-paratyphoid vaccines and in individuals with Chediak-Higashi disease (Grush and Mauer, 1969; Ng, Chan and Todd, 1972), both of which conditions are believed to be associated with increased lysosomal lability. Patients affected with parasitic diseases, including malaria, loiasis, trichinosis and amebic liver abscesses (Anderson, 1971; Chretien and Garagusi, 1971; Pujol-Moix, 1971) also produce high NBT results. Other conditions which have been reported to cause false-positive NBT tests include cases of viral meningitis which have been complicated by orchitis, pancreatitis or encephalitis (with no evidence of bacterial involvement) (Elgefors and Olling, 1972), *Mycoplasma pneumoniae* infections (Freeman and King, 1972) and 2 cases of idiopathic myelofibrosis without infection (Ng, Chan and Todd, 1972). Catovsky and Galton (1972) reported positive results in patients with Hodgkin's disease associated with fever and leukocytosis, although the leukocytes of chronic granulocytic leukemia seem to be unassociated with increased numbers of NBT positive cells. Silverman and Reed (1973) reported that the NBT test could be positive in lymphoma uncomplicated by infection and might be suppressed by chemotherapy or corticosteroids. Nocardiosis and systemic fungal infections, including histoplasmosis and candidiasis, have produced positive test results according to several investigators (Feigin *et al.*, 1971; Park *et al.*, 1968; Matula and Paterson, 1971).

Table 2. Accumulated misdiagnoses by NBT test from the literature:* false-positives

Malaria	Loiasis
Candidiasis	Trichinosis
Aseptic meningitis	Amebic hepatic abscess
ECHO virus infection	Normal controls
Herpes simplex and zoster infections	Ulcerative colitis
	Psoriasis
Nonbacterial pneumonitis	Asthmatic attack
Acute rheumatic fever	Penicillin allergy
Acute juvenile rheumatoid arthritis	Transfusion reaction
Neonates	Polymyalgia rheumatica
Congestive heart failure	Allergic granulomatous vasculitis
Chediak-Higashi syndrome	Rheumatoid arthritis in an adult
Osteogenesis imperfecta	*Mycoplasma pneumoniae* infection
Hemophilia	Idiopathic myelofibrosis
Multiple drug overdosages	Malignant lymphoma
Typhoid-paratyphoid vaccine	Histoplasmosis
Hodgkin's disease	Nocardiosis
Oral contraceptive use	

*Bittner *et al.* (1973).

Reduction of NBT Dye by Monocytes

In addition to the reduction of nitroblue tetrazolium dye by polymorphonuclear leukocytes, it is also reduced by monocytes and clumps of platelets (Park *et al.*, 1972). In neutropenic patients, a sharp, prompt rise in monocytes with an increased number of NBT-positive monocytes was noted with each episode of bacterial infection. During infection, the absolute number of NBT-positive monocytes was greater than 500 per cubic millimeter, and in excess of 15% of the total monocytes. Park and his associates (1972) concluded that the NBT test was useful for identifying neutropenic patients with bacterial infection who have a corresponding adequate compensatory monocytosis.

Other authors (Humbert *et al.*, 1971) have advocated the combined use of both neutrophils and monocytes as a single group of "phagocytes" in the NBT test. However, there is no real advantage in using this except the elimination of the necessity of differentiating monocytes from neutrophils when the nuclear details are obscured by formazan deposits. On the other hand, Allen (1973) stated that the results of the NBT test in monocytes were inconsistent in patients with normal or increased numbers of neutrophils.

During the course of a study of nitroblue tetrazolium reduction by neutrophils from patients with acute myeloid leukemia, Goodman and Catovsky (1972) discovered that a proportion of immature and mature monocytes from these cases could also reduce NBT. Catovsky and Galton (1973) subsequently used this test to determine if it would be helpful in the cytochemical analysis of acute myeloid leukemia. In myeloblastic leukemia, the majority

of the blasts were negative. In myelomonocytic leukemia, variable proportions of NBT-positive monocytes were present and appeared more plentiful in the type of disease which had a more conspicuous monocytic component. In acute and chronic monocytic leukemia, approximately 25% of the leukemic cells were positive. These investigators concluded that the NBT test could be used as an indication of monocytic differentiation in these 3 types of acute myeloid leukemia.

Mechanism of the NBT Test

Nitroblue tetrazolium (NBT) is chemically (2,2'-dinitro-5,5'-diphenyl-3,3'-[3,3'-dimethyl-4,4'-biphenylene]) ditetrazolium chloride. According to Thompson (1966), the reduction of NBT to formazan occurs as illustrated in Figure 1. However, the actual enzymatic mechanism resulting in the reduction of nitroblue tetrazolium to formazan within the cytoplasm of the phagocytic leukocyte remains unclear. The reduction of NBT can take place within phagocytic vacuoles of neutrophils, monocytes, or eosinophils. Since the resting unstimulated membrane is not permeable to the dye (Park, 1971), alteration to the cell membrane is necessary and may be induced either by phagocytosis of bacteria or liberation of their products. According to Matula and Paterson (1970), there is a bacterial filtrate factor responsible which is stable at temperatures ranging from 70 to 100 C. This does not appear to be an endotoxin and has induced varied NBT responses from different subjects.

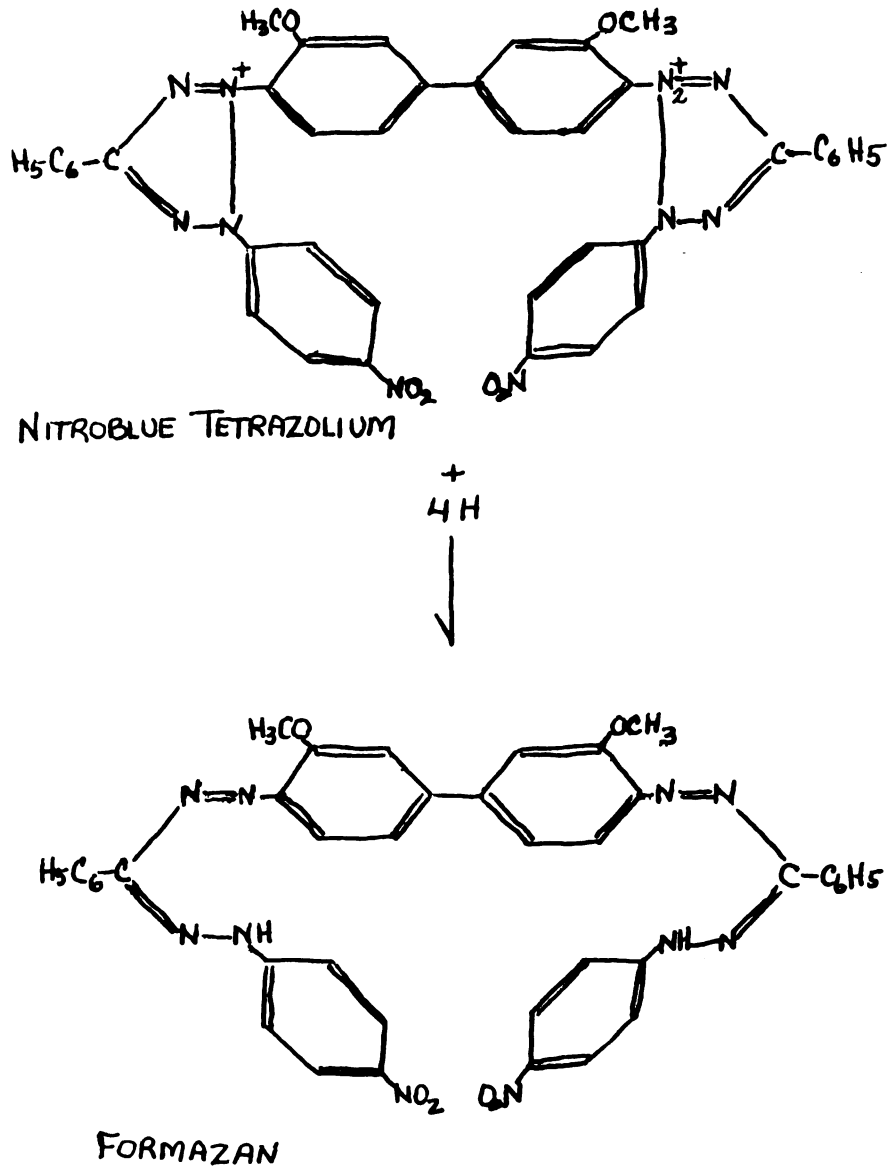

Figure 1. Structure of nitroblue tetrazolium and reduction to formazan (Thompson, 1966).

Although there is no certainty about the precise conditions under which NBT enters the cell, intracellular metabolic changes as well as membrane changes are anticipated. Gifford and Malawista (1972) stressed the importance of prior contact with a foreign surface resulting in an increase in cellular adhesiveness and membrane permeability. Neuwirtova and Setkova (1973) and Graham (1973) noted that, in highly positive NBT blood samples, clumps of neutrophils, monocytes, and platelets are found. They hypothesized that NBT might form molecular aggregates or tiny crystals that are ingested especially by activated leukocytes so that, in fact, phagocytosis was one of the possible mechanisms of entrance of NBT into the cell. They believed that the adhesiveness of the blood elements and the changes of the cellular membrane were vital for the entrance of NBT into the cell. However, in the case of aggregated platelets, they were uncertain whether the formazan was adsorbed onto the surfaces of these aggregates or actually present inside the thrombocytes. If the formazan was only adsorbed, the question remained as to what mechanism reduced the NBT on the surfaces of the thrombocytes and also on contact sites of leukocytes. Leventhal and Soulsby (1972), in their study of NBT as an indicator of lysosomal activity at a larval surface, assumed that lysosomal activity did occur at the cellular surface.

In regard to the actual reduction of NBT, Park (1971) reported that the mechanism was due to an NBT diaphorase which was responsible for the transfer of hydrogen atoms from reduced pyridine nucleotide to NBT dye. It seemed to be localized in the

granules of the cytoplasm. He also reported that the alteration of the cell membrane and release of diaphorase were probably induced by phagocytosis either of bacteria or of latex particles. Since endotoxin, staphylococcal protein-A and streptolysin-O induced an increased reduction of NBT dye when they were added to whole blood, Park assumed that any bacterial product or bacterial component might also be responsible for the increased reduction of the dye during natural infection or after vaccination.

The mechanism of NBT reduction appears to be dependent on the activity of reduced nicotinamide adenine dinucleotide (NADPH) oxidase within the neutrophil since it has been shown by Baehner, Nathan and Karnovsky (1970) that this enzyme was absent in chronic granulomatous disease, a situation in which dye reduction does not occur. In addition, several authors (Hicks and Bennett, 1971; Cocchi, Mori and Ravina, 1971) reported a burst of respiratory activity with increased cyanide-insensitive oxygen consumption and increased activity of the hexose monophosphate shunt associated with the precipitation of formazan salt.

Humbert and his associates (1973) used methylene blue to study the mechanism of NBT reduction. Methylene blue, which is known to stimulate the hexose monophosphate shunt-linked oxidative metabolism in neutrophils, caused a marked increase in nitroblue tetrazolium reduction by these cells in normal adults and in a child having chronic granulomatous disease. They felt that this confirmed that the phagocytosis of particulate matter was *not* essential for the reduction of NBT by neutrophils or for the entry

of tetrazolium dye into these cells. In experiments with cell-free systems, these investigators showed that methylene blue can act as an electron carrier between NBT and 2 pyridine nucleotides important in the oxidative pathway of neutrophils, namely NADH and NADPH. They also observed the localization of formazan by both light and electron microscopy in leukocytes stimulated by methylene blue, in leukocytes of a newborn infant, and in those of a child with acute bacterial infection. They reported that formazan was seen most often in the cytoplasm on or near the smooth endoplasmic reticulum of neutrophils and monocytes.

Other investigators (McCall, Cooper and DeChatelet, 1971) believed that the common denominator for metabolic alterations in the leukocytes was the age of the circulating leukocyte population rather than the presence of active inflammation. Young leukocytes may be more active metabolically or may have a higher complement of cellular enzymes. It was their view that this could explain the increase in respiration, hexose monophosphate activity and NBT dye reduction reported in the leukocytes of newborn infants and pregnant women in the last trimester, both of which have a "shift to the left."

In contrast to the opinions of other investigators, Grush and Mauer (1969) reported that during NBT dye reduction, activated neutrophils showed *no* change in intracellular bactericidal capacity, the hexose monophosphate shunt was *not* increased and, furthermore, there was *no* evidence that the cells were hyperactive functionally or metabolically despite increased numbers of cells reducing NBT. They concluded that the dye reduction might depend

on hydrogen peroxide production or the subsequent stimulation of other enzymes such as glutathione peroxidase.

Methods for Performing the NBT Test

Several different methods have been proposed for performing the nitroblue tetrazolium test. One of the earliest was proposed by Baehner and Nathan (1968). It involved a spectrophotometric system requiring 25 ml of blood, which is often too great an amount to be taken from an infant. Moreover, it was a quantitative test requiring leukocyte isolation. Thus, it required a great deal of specialized equipment.

The method of Park *et al.* (1968) is the one most often used. It requires 0.5 to 1.0 ml of heparinized blood, a 0.2% NBT solution with a phosphate buffer. After the blood and NBT solution are incubated together, blood smears are made on coverslips or microscopic slides and stained with Wright's stain. Although the method is comparatively simple and requires a minimum of equipment, cell preservation is poor. Therefore, morphologic examination is difficult and inaccurate for semiquantitation. Several authors (Matula and Paterson, 1971; Freeman and King, 1971; Wollman *et al.*, 1972) have described modifications of Park's method involving changes in staining and in incubation time.

Hicks and Bennett (1971) developed a simple, rapid test tube system for the semiquantitative NBT test. Their method involved the use of 3.8% sodium citrate as an anticoagulant, neutral red dye as a differential stain, and latex particles to stimulate

phagocytosis. The principal advantage of this technique was reported to be excellent morphologic preservation.

Freeman and King (1972a) stated that, if the "whole blood" method was not employed, the results would be unreliable. However, several authors (Gordon *et al.*, 1973; Humbert *et al.*, 1971; Will and Grauman, 1973) have developed methods using buffy coat preparations and have reported excellent results.

Freeman and King (1972a) also reported that, using EDTA as an anticoagulant, inconsistent and low results would be obtained. However, Gordon *et al.* (1973) reported that, in an experiment comparing EDTA with heparin as an anticoagulant, staining of formazan was satisfactory with heparin but cell definition was relatively poor and clumping rendered counting difficult. On the other hand, EDTA alleviated these difficulties.

Stuart and Simpson (1970) discovered that the inert synthetic sucrose polymer Ficoll had a protective effect on leukocytes during incubation at 37 C. The protection appeared to be due to preservation of the cytoplasmic membrane. Subsequently, Gordon *et al.* (1973) also advocated the use of Ficoll with the NBT reagent to prevent "extravasation of formazan" from the neutrophils, to preserve cellular integrity, and to prevent the loss of sensitivity resulting from the use of EDTA.

Several investigators have developed "stimulated" *in vitro* NBT tests to distinguish falsely negative results from true negative results. The tests induce phagocytosis by the use of latex particles (Baehner and Nathan, 1968; Hicks and Bennett,

1971), the use of endotoxin-coated slides (Park and Good, 1970), allowing the neutrophils to adhere to a glass coverslip (Gifford and Malawista, 1970) or the use of bacterial filtrates (Matula and Paterson, 1970). Several simple and rapid screening tests for chronic granulomatous disease of children have also been developed utilizing some of these techniques (Gifford and Malawista, 1970; Windhorst, Holmes *et al.*, 1966; Ochs *et al.*, 1972; Johnston, 1969).

Conclusions

The NBT test has shown a remarkable capacity to distinguish human patients with active bacterial infection from those with nonbacterial inflammatory disease (Matula and Paterson, 1971). It appears to be a more accurate test than such traditional indices as body temperature and white blood cell counts. Positive NBT responses are induced by a wide range of bacterial species. The major clinical advantage of the NBT response is its capacity to alert the physician to the possibility of bacterial infection before more definitive cultural data are available. Conversely, a negative NBT response is strong evidence against the presence of bacterial infection, or rarely, evidence of a neutrophil abnormality, and should be encourage a more intense search for another type of disease.

However, it appears that early claims for the general application of the NBT test as a routine laboratory screening procedure for systemic bacterial infection in human beings may have been overoptimistic, largely because of technical difficulties and the production of a large number of false results (Neuwirtova

and Setkova, 1974). Nonetheless, the test remains useful as a screening procedure for patients with chronic granulomatous disease (CGD) and to identify carriers of CGD. False-negatives in this condition can be eliminated by using the "stimulated" NBT test (Park and Good, 1970). The test is also useful in the early detection of bacterial disease in patients unusually susceptible to infection, particularly those on immunosuppressive therapy or undergoing intensive care (Miller and Kaplan, 1970; Wollman *et al.*, 1973; Freeman and King, 1972), in uremic patients, including those on maintenance hemodialysis, and in differentiating rejection from infection in immunosuppressed renal transplant recipients (Wollman *et al.*, 1973).

MATERIALS AND METHODS

This study involved the application of the nitroblue tetrazolium test to blood samples obtained from 4 species of domestic animals.

Blood Samples

Control blood samples were obtained from clinically healthy dogs (25), cats (12), horses (20), and cattle (10). Test blood samples were obtained randomly from animals presented at Michigan State University Veterinary Clinic for various therapeutic and surgical procedures.

Venous blood was collected in Vacutainers[a] using the disodium salt of ethylenediamminetetraacetic acid (EDTA) as the anticoagulant. Blood samples were processed soon after collection and always within the first 4 hours (Hicks and Bennett, 1972; Gordon and Rowan, 1973).

Standard Solution

A standard solution of NBT was prepared by dissolving 100 mg NBT[b] in 50 ml distilled water at room temperature (Hicks and

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[a]Reg TM, Becton, Dickinson and Company, Rutherford, New Jersey, #3206W.

[b]Sigma Chemical Company, Grade III Reagent (N6876), St. Louis, Missouri.

Bennett, 1972). The solution was stored at 4 C and fresh solution was prepared every 3 months.

Staining Procedure

A modified Romanowsky type (Wright's) stain was used, and the entire staining procedure was accomplished by an automatic slide-staining processor.[a]

Test Tubes

The tubes used for incubation of the blood and NBT mixture were 5 ml disposable capped polypropylene test tubes.[b]

Procedure and Slide Preparation

One-half milliliter of the whole blood sample was placed within a polypropylene test tube. One-tenth milliliter of NBT solution was added for a total of .6 ml of incubate. The tubes were tightly capped, mixed by inversion for 5 seconds and incubated in a constant temperature water bath at 37 C for 15 minutes. The test tubes were then removed from the water bath, the caps were loosened, and incubation was continued at room temperature for an additional 15 minutes in a test tube rack. Blood smears were made on microscopic slides and air dried. The slides were then positioned in the rack of the automatic stainer and processed.

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[a]Hematek-Ames Company, Division of Miles Laboratories, Elkhart, Indiana.

[b]Falcon Plastics Company, Los Angeles, California.

Scoring Procedure

The blood smears were examined by light microscopy (40X and 100X-oil immersion). One hundred neutrophils (mature and juvenile) were examined on each of the stained slides and the number of positive neutrophils was determined. Positive neutrophils included only those cells containing at least 1 heavy deposit of formazan. These cells were designated "NBT-positive." Formazan deposits could be identified most readily by rapidly focusing up and down on the cell because the edges of the formazan deposits tend to be somewhat refractile. The percentage of NBT-positive neutrophils was then recorded. Notations of NBT-positive monocytes were made but not included in the final tabulations. Leukocytes of equivocal type were excluded. Each count was repeated twice more and the final result was determined by averaging the 3 counts.

Supportive Data

In all blood samples examined, hemoglobin, packed cell volume and white cell counts were determined. Also, a white blood cell differential count was performed. Total plasma protein concentration was determined and, in addition, a fibrinogen determination (Schalm, 1965) was made on all cattle blood samples. The purpose of measuring fibrinogen levels in cattle was to determine more accurately the presence of inflammatory disease. Schalm has stated that fibrinogen levels are more reliable indicators of inflammatory disease in cattle than are total or differential white blood cell counts (Schalm, 1972).

According to the clinical signs of each animal, various other laboratory diagnostic tests were performed. These included serum enzyme determinations, electrolyte determinations, blood urea nitrogen levels, serum creatinine levels, and serum protein electrophoresis when indicated. In addition, fecal examinations for intestinal parasites and blood examination for microfilaria were performed when indicated. In cases of suspected bacterial infection, cultures of the affected tissues were made whenever possible or, in some cases, bacteria were demonstrated on smears or in histopathologic sections. Blood cultures and attempted virus isolations were performed when deemed necessary. Necropsies and biopsies were also performed as required.

Final Diagnosis

The final diagnosis for each animal was made by the veterinarian responsible for the case after consideration of the physical examination and all pertinent laboratory data.

RESULTS

Dog

Nitroblue tetrazolium scores for dogs are given in Tables 3, 4, 5, 6, 7 and 8 and Figure 2. Examples of NBT positive cells can be found in Figures 3 and 4.

Scores in healthy control dogs ranged from 0 to 10% with an average score of 3%. Dogs diagnosed as having nonbacterial conditions also had scores averaging 3%. However, dogs with confirmed bacterial disease had values ranging from 6 to 71%. The average score was 21%. Three dogs with confirmed systemic fungal disease or infection by higher bacteria had scores ranging from 15 to 24% (average 20%). For the distribution of scores, see Figure 2.

Diagnoses in dogs with positive NBT scores (scores over 10%) included septicemias, pneumonia, metritis and osteomyelitis (confirmed by either culture of biopsy or necropsy specimens) among others. Highest scores occurred in dogs with frank septicemias confirmed by positive blood cultures. Pneumonia, metritis, and osteomyelitis also had fairly high scores. Lower positive scores were seen in dogs with cystitis, generalized pyoderma, tetanus, peritonitis (treated), cellulitis and enteritis (Table 4).

Table 3. Summary of NBT scores: dogs

Diagnosis	No.	Mean (%)	Range (%)
Clinically healthy	25	3.0 ± 2.2	(0-10)
Bacterial disease	26	20.7 ± 6.9	(6-71)
Nonbacterial conditions	85	3.0 ± 1.9	(0-13)
Fungal and higher bacterial diseases	3	20.0 ± 3.3	(15-24)

Table 4. Diagnoses and NBT scores in dogs with bacterial diseases

Diagnosis	No.	Mean (%)	Range (%)
Cellulitis	1	11.0	---
Cystitis	5	13.8	(6-21)
Enteritis	2	15.0	(13-17)
Osteomyelitis	2	21.5	(17-26)
Pneumonia	2	22.5	(22-23)
Pyoderma	3	14.7	(14-15)
Peritonitis	1	11.0	---
Septicemia	6	31.8	(13-71)
Tetanus	1	12.0	---
Metritis	4	19.2	(13-25)

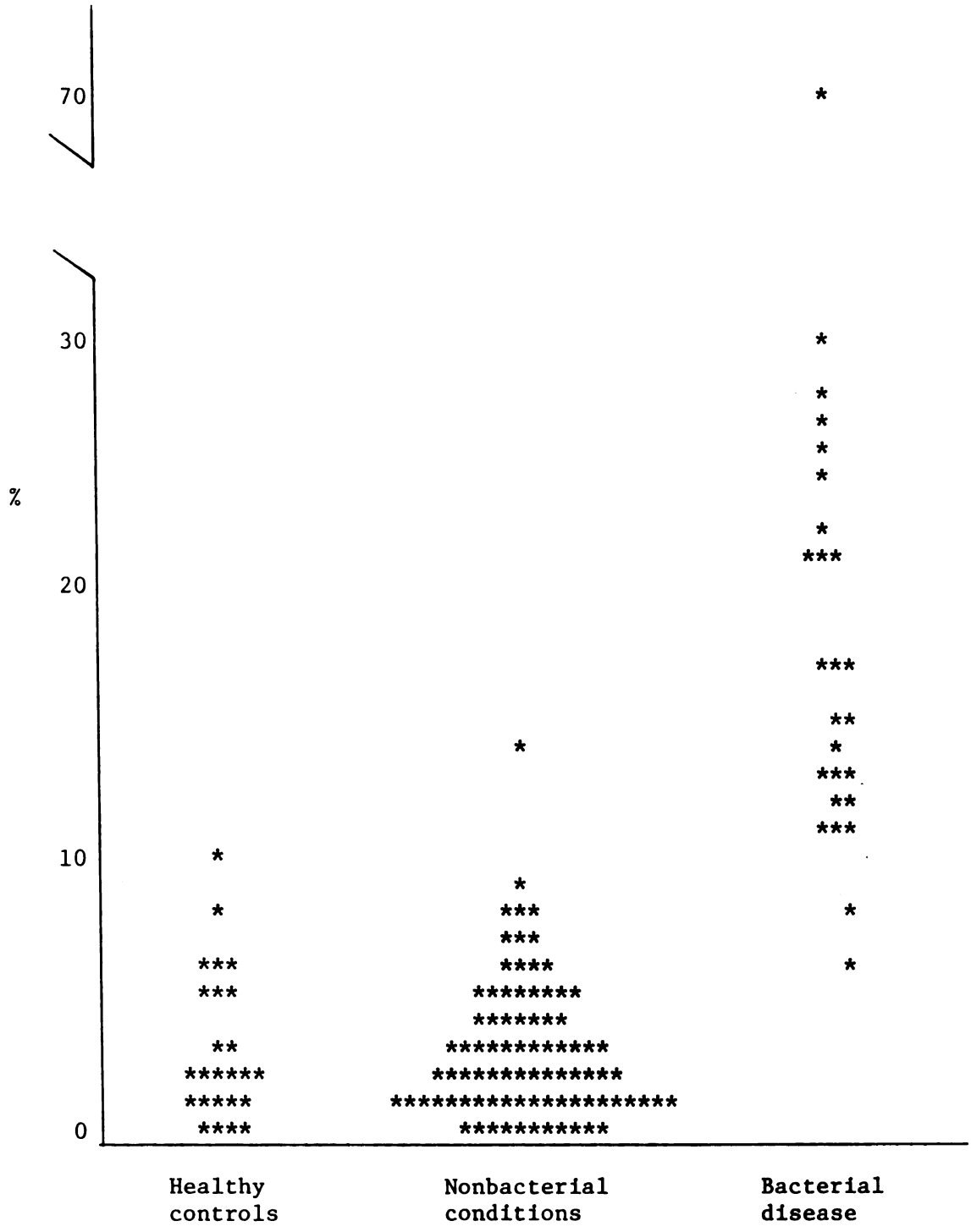

Figure 2. Distribution of NBT scores: dogs.

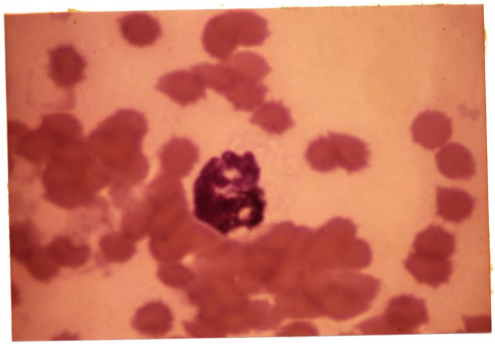

Figure 3. Canine neutrophil with dense formazan deposit in the perinuclear area. Wright's stain; x 1200.

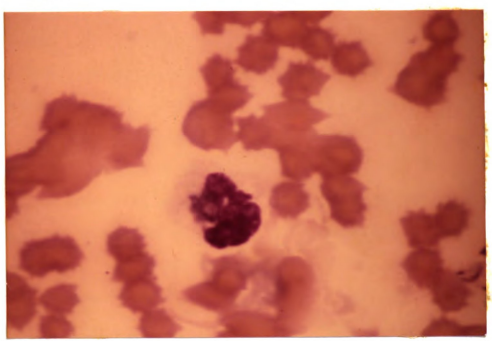

Figure 4. Canine neutrophil with a formazan deposit (reduced nitroblue tetrazolium). Wright's stain; x 1200.

Of the organisms cultured from dogs with positive scores, 50% were gram negative, 33.3% were gram positive and 16.66% were mixed infections. The highest scores were obtained in dogs with septicemia caused by gram negative organisms (proven by positive blood culture).

Positive NBT scores were seen in dogs with leukocyte counts ranging from 5000 WBC/cmm to 55,300 WBC/cmm, a greater than 10-fold distribution ranging from leukopenia to leukocytosis, as well as including normal values (Table 5).

Diagnoses in dogs with nonbacterial disease are listed in Table 6. Of this group, dogs with malignant lymphoma had the highest scores with occasional positive scores. Some of the conditions which resulted in scores within the normal range included dermatitides from a variety of causes, distemper (with no secondary bacterial infection), neoplasia, demodectic mange, microfilariasis, intestinal parasitism, viral pneumonia and chronic pericarditis.

Organisms cultured from dogs with negative NBT scores were all gram positive and were found in conditions in which the infections were localized rather than systemic (Table 7).

In addition, Table 8 lists the NBT scores recorded in 2 cases of systemic blastomycosis and 1 case of nocardiosis.

In dogs, the formazan deposits were usually sharp and clear except in cases where the dog had been on drug therapy for several days. In those cases, the deposits were somewhat paler with indistinct borders. In dogs with severe systemic bacterial disease, the deposits were often so large that they completely filled the cell, obscuring the nucleus and often resulting in

Table 5. Examples of leukocyte counts and culture results in dogs with positive NBT scores

Diagnosis	NBT Score	WBC Count	Culture Result
Septicemia	71	12,000	*Pseudomonas* sp.
Septicemia	30	23,200	*Escherichia coli*
Septicemia	27	39,400	*Streptococcus* sp.
Septicemia	22	15,400	*E. coli*
Pyometritis	26	50,000	*Proteus* sp., *E. coli*
Pyometritis	22	30,400	*Proteus* sp.
Metritis	17	37,700	*E. coli*
Metritis	13	28,400	*Klebsiella* sp.
Pneumonia	23	14,900	*Streptococcus* sp., *Moraxella* sp., *Pasteurella* sp.
Osteomyelitis	26	55,300	*Pseudomonas* sp.
Bronchitis	22	49,300	*Streptococcus* sp., *Staphylococcus* sp.
Cystitis	21	13,200	*Streptococcus* sp., *Proteus* sp.
Cystitis	11	10,000	*E. coli*
Cystitis	12	8,900	*E. coli*
Cystitis	12	36,900	*Streptococcus* sp.
Enteritis	13	10,300	*Proteus* sp., *Klebsiella* sp.
Otitis media	12	29,900	*Micrococcus* sp., *Staphylococcus* sp., *E. coli*
Pyoderma	15	19,000	*Staphylocccus* sp.
Pyoderma	14	14,700	*Staphylococcus* sp., *Streptococcus* sp.
Peritonitis	11	26,800	*Klebsiella* sp., *E. coli*
Peritonitis	28	12,900	*Streptococcus* sp.
Cellulitis	11	9,100	*Streptococcus* sp., *Staphylococcus* sp.

Table 6. Diagnoses and NBT scores in dogs with nonbacterial conditions

Diagnosis	No.	Mean (%)	Range (%)
Dermatitis	7	2.0	(0-4)
Gastroenteritis	10	1.9	(0-5)
Epilepsy	6	3.5	(0-9)
Distemper (no secondary infection)	4	2.2	(0-4)
Otitis	2	3.5	(2-5)
Orthopedic conditions	6	2.0	(0-4)
Malignant lymphoma	4	6.0	(1-13)
Other neoplasms	14	3.5	(1-7)
Pneumonia (viral)	2	2.0	(0-4)
Chronic pericarditis	2	3.5	(2-5)
Demodectic mange	4	1.5	(0-3)
Heartworm infestation	7	2.1	(1-5)
Pancreatitis (no organisms cultured)	3	4.0	(1-8)
Lead toxicosis	2	1.5	(1-2)
Polyradiculoneuritis	2	4.0	(1-7)
Other	9	3.2	(0-8)

Table 7. Examples of leukocyte counts and culture results in dogs with negative NBT scores

Diagnosis	NBT Score	WBC Count	Culture Result
Pneumonia	0	15,000	no growth
Microfilariasis	1	11,700	no growth
Microfilariasis	1	8,200	no growth
Microfilariasis	5	22,000	no growth
Otitis externa	5	7,400	*Staphylococcus* sp.
Pericarditis	5	13,500	no growth
Canine distemper	4	17,800	no growth, FA positive
Neoplasia	1	22,600	no growth
Malignant lymphoma	2	4,200	no growth
Enteritis	3	13,400	no growth
Nematodiasis	0	10,500	---
Nematodiasis	2	8,700	---
Nephritis	2	11,300	no growth
Demodectic mange	0	12,400	no growth
Demodectic mange	3	6,400	*Staphylococcus* sp.
Demodectic mange	0	19,100	*Staphylococcus* sp.
Dermatitis	3	5,400	*Micrococcus* sp., *Streptococcus* sp.

Note: not all cases having negative NBT scores were cultured.

Table 8. Diagnoses and NBT scores in dogs with systemic fungal and higher bacterial infections

Diagnosis	No.	Mean (%)	Range (%)
Nocardiosis	1	15.0	---
Blastomycosis	2	22.5	(21-24)

disruption of the cellular membrane (Figures 3 and 4).

In dogs with bacterial infections, definite decreases in the percentages of positive neutrophils were observed over a period of days following effective antibiotic therapy. In dogs in which treatment of the infection was ineffective, the scores remained approximately the same. If effective drug therapy was then instituted, there was a decrease in the NBT positive neutrophils within 2 days.

There appeared to be very little overlap between NBT scores in dogs with bacterial disease and those without bacterial disease. However, culture specimens from all dogs and, in many instances, the prior history regarding antibiotic therapy was not known. Dogs with malignant lymphoma tended towards positive NBT scores. This may have been due to concomitant low grade bacterial infection or may have been a result of the lymphoma process itself.

Cat

The data on cats are presented in Tables 9, 10, 11, 12 and 13 and Figures 5, 6 and 7.

Table 9. Summary of NBT scores: cat

Diagnosis	No.	Mean (%)	Range (%)
Clinically healthy	12	3.5 ± 1.8	(0-8)
Bacterial disease	7	21.0 ± 4.6	(12-30)
Nonbacterial conditions	20	4.8 ± 3.1	(0-15)

Table 10. Diagnoses and NBT scores in cats with bacterial diseases

Diagnosis	No.	Mean (%)	Range (%)
Bacterial gastroenteritis	2	13.5	(12-15)
Suspected systemic bacterial infection	2	23.5	(23-24)
Pneumonia and metritis	1	23.0	---
Septicemia	2	25.0	(20-30)
Chronic nephritis	1	6.0	---

Table 11. Examples of leukocyte counts and culture results in cats with positive NBT scores

Diagnosis	NBT Score	WBC Count	Culture Result
Undetermined	23	12,100	---
Undetermined	24	13,700	---
Bacterial enteritis	15	7,800	*E. coli*, *Proteus* sp., *Klebsiella* sp.
Feline viral rhinotracheitis	15	8,600	*Streptococcus* sp., *Staphylococcus* sp.
Pneumonia, metritis	23	6,900	*Proteus* sp., *Pasteurella* sp.
Septicemia	20	12,000	*Klebsiella* sp., *E. coli*
Septicemia	30	18,000	*E. coli*

Table 12. Diagnoses and NBT scores in cats with nonbacterial conditions

Diagnosis	No.	Mean (%)	Range (%)
Malignant lymphoma	9	5.1	(0-9)
Feline viral rhinotracheitis	4	7.8	(3-15)
Urolithiasis	1	1.0	---
Chronic sinusitis	2	2.5	(2-3)
Hemobartonellosis	4	3.2	(1-7)

Table 13. Examples of leukocyte counts and culture results in cats with negative NBT scores

Diagnosis	NBT Score	WBC Count	Culture Result
Feline viral rhinotracheitis	8	10,300	*Streptococcus* sp., *Pasteurella* sp.
Feline viral rhinotracheitis	5	8,000	no growth
Chronic nephritis	6	29,400	*E. coli*
Urolithiasis	1	2,700	no growth
Chronic sinusitis	2	12,000	no growth
Chronic sinusitis	3	18,900	no growth
Hemobartonellosis	7	18,100	---

Note: not all cases having negative NBT scores were cultured.

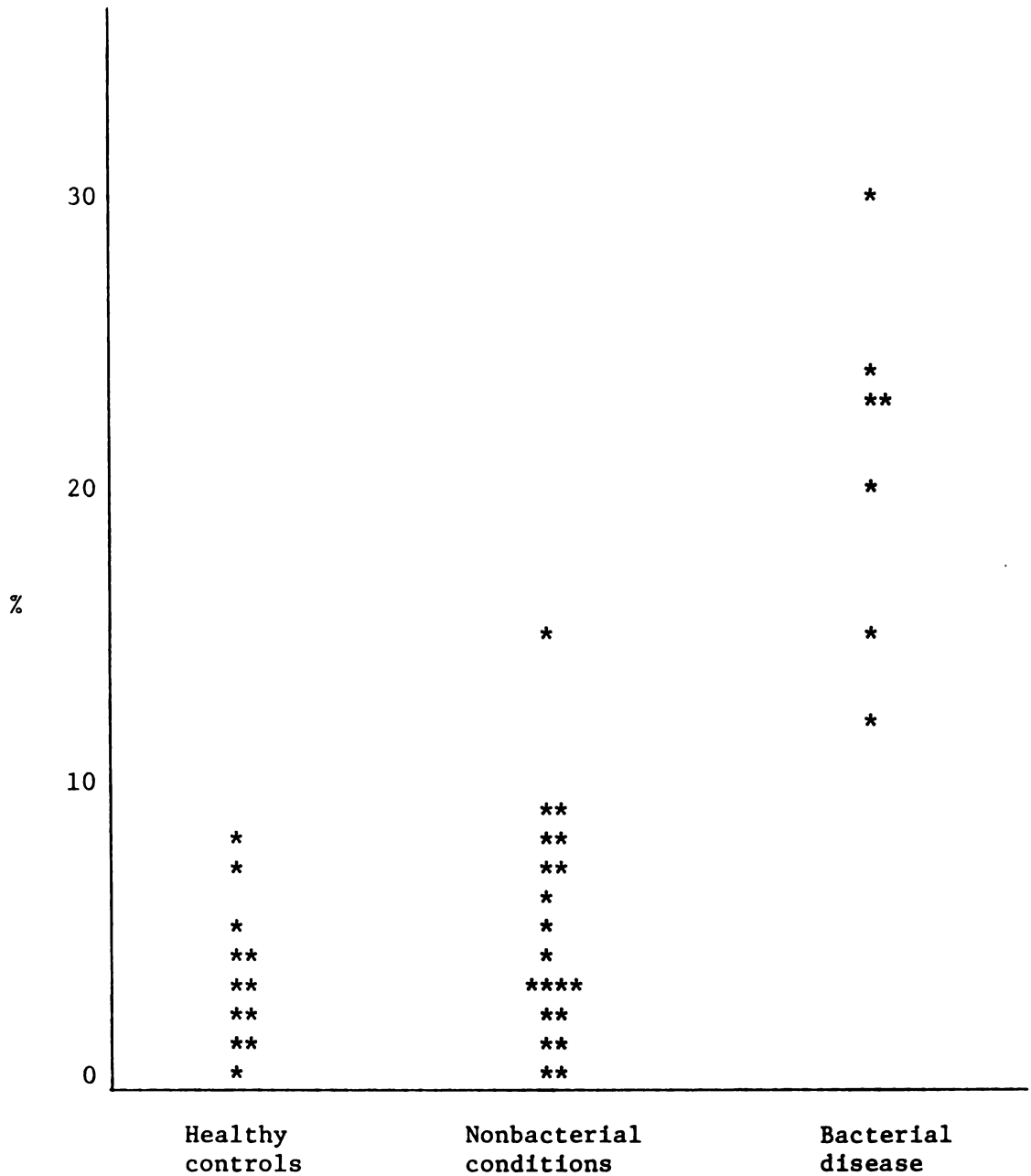

Figure 5. Distribution of NBT scores: cats.

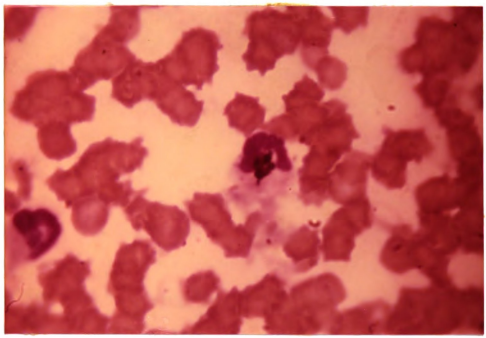

Figure 6. Feline neutrophil with a dense formazan deposit. Wright's stain; x 1200.

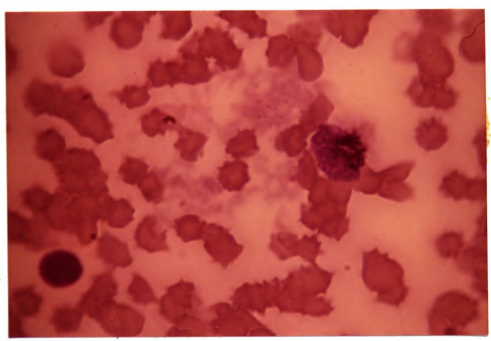

Figure 7. Formazan deposit in an "equivocal" leukocyte in a smear of cat blood. Wright's stain; x 1200.

In 12 clinically normal cats, scores ranging from 0 to 8% were obtained (average 3.5%). However, 7 cats with confirmed or highly suspected bacterial diseases had scores ranging from 12 to 30% (average 21%). Twenty cats with nonbacterial diseases had values of 1 to 15% (average 5%).

Deposits in cat neutrophils were generally quite distinct and dark. However, the cells were seldom so full that they ruptured. Responses of the cat to effective antibiotic therapy were dramatic with NBT scores returning to normal within 2 days.

Two cats tentatively diagnosed as having feline infectious peritonitis had scores of 23 and 24%, respectively, with heavy dark deposits. Both cats improved following antibiotic therapy although blood cultures were never performed. Both cats were eventually discharged in apparent good health with no definite diagnosis having been reached. Because of the high NBT scores and rapid response to antibiotic therapy, bacterial disease was considered likely.

Highest NBT scores were seen in cats with *E. coli* septicemia. In these animals, neutrophil clumping and slight leaking of formazan from ruptured cells was seen. There were several incidences of overlapping of scores from cats. A few of the cats diagnosed as having feline viral rhinotracheitis had values in the low positive range. This could be explained by concomitant low grade bacterial infection. As seen in Tables 12 and 13, throat cultures yielded *Pasteurella* sp., *Staphylococcus* sp., and *Streptococcus* sp. in these cats. One cat diagnosed as having

chronic nephritis from which *E. coli* was cultured had a score of 6%, which is within the normal range. However, the history was unknown and the animal may well have been previously treated to warrant the designation "chronic."

Leukocyte counts in positive bacterial disease ranged from 6900 WBC/cmm to 18,000 WBC/cmm and in nonbacterial conditions ranged from 2700 WBC/cmm to 18,900 WBC/cmm. Thus, the range was from leukopenia to leukocytosis in both instances.

Horse

The data on horses are presented in Tables 14, 15, 16, 17 and 18 and Figures 8, 9 and 10.

In 20 clinically healthy horses, NBT scores ranged from 1 to 9% (mean 3%). NBT scores in 28 horses diagnosed has having non-bacterial conditions were not strikingly different (mean 3%). However, in 15 horses with confirmed bacterial disease, scores ranged from 11 to 81% with a mean score of 28%.

The highest scores were obtained in Arabian foals having adenoviral pneumonia with secondary bacterial complications, and in horses with peritonitis due to gram negative organisms subsequent to abdominal surgery.

The deposits seen in neutrophils from horses were comparable with those seen in dogs. Extremely high NBT scores were accompanied by clumping of neutrophils and rupture of cell membranes with escape of formazan. The incidence of false-negative and false-positive scores in horses was very low.

Table 14. Summary of NBT scores: horse

Diagnosis	No.	Mean (%)	Range (%)
Clinically healthy	20	3.2 ± 1.5	(0-9)
Bacterial disease	15	28.2 ± 18.5	(11-81)
Nonbacterial conditions	28	3.1 ± 1.9	(0-9)

Table 15. Diagnoses and NBT scores in horses with bacterial diseases

Diagnosis	No.	Mean (%)	Range (%)
Septic arthritis	2	19.0	(17-21)
Septicemia	2	16.0	(11-21)
Bronchitis	2	12.5	(11-14)
Peritonitis	5	38.4	(13-68)
Osteomyelitis	1	14.0	---
Adenoviral pneumonia with secondary bacterial infection	3	38.3	(15-81)

Table 16. Examples of culture results and leukocyte counts in horses with positive NBT scores

Diagnosis	NBT Score	WBC Count	Culture Result
Septic arthritis	21	7,400	*E. coli*
Bronchitis	11	10,500	*Streptococcus* sp.
Bronchitis	14	12,100	*Streptococcus* sp., *Moraxella* sp., *Micrococcus* sp., *Pectobacterium* sp.
Adenoviral pneumonia	15	22,500	*Streptococcus* sp., Adenovirus
Adenoviral pneumonia	81	13,400	*Streptococcus* sp., Adenovirus
Adenoviral pneumonia	19	15,000	*Staphylococcus* sp., *Streptococcus* sp., Adenovirus
Peritonitis	20	9,600	*Klebsiella* sp.
Peritonitis	65	11,800	*E. coli*
Peritonitis	26	7,400	*Streptococcus* sp.
Peritonitis	68	7,500	*E. coli*
Peritonitis	13	7,700	*Corynebacterium* sp.
Osteomyelitis	14	13,100	*Bacillus* sp., *Streptococcus* sp., *E. coli*

Table 17. Diagnoses and NBT scores in horses with nonbacterial conditions

Diagnosis	No.	Mean (%)	Range (%)
Pharyngitis	2	2.0	---
Postsurgical	6	3.3	(1-8)
Chronic emphysema	1	9.0	---
Abdominal hernia	2	5.0	(3-7)
Allergic dermatitis	1	8.0	---
Verminous arteritis	1	3.0	---
Chronic bronchitis	2	2.0	(1-3)
Laryngeal hemiplegia	1	2.0	---
Laminitis	2	2.0	---
Colic	3	0.8	(0-2)
Laceration	1	4.0	---
Enteritis	2	5.5	(5-6)
Viral pneumonia (Herpes)	1	0.0	---
Orthopedic conditions	3	3.0	(1-4)

Table 18. Examples of culture results and leukocyte counts in horses with negative NBT scores

Diagnosis	NBT Score	WBC Count	Culture Result
Arthritis	2	9,100	no growth
Bronchitis	2	11,500	no growth, no virus
Pneumonia	0	10,600	virus-slow-Herpes
Pharyngitis	2	7,200	no growth
Strongylosis	3	6,400	no growth
Chronic emphysema	9	6,800	*Streptococcus* sp.
Allergic dermatitis	8	11,500	no growth
Arthritis	2	13,500	no growth
Laminitis	2	7,800	no growth
Postoperative	8	7,100	no growth (treated)
Enteritis	6	16,400	*Bacillus* sp., *Streptococcus* sp., *E. coli*, *Enterobacterium* sp.

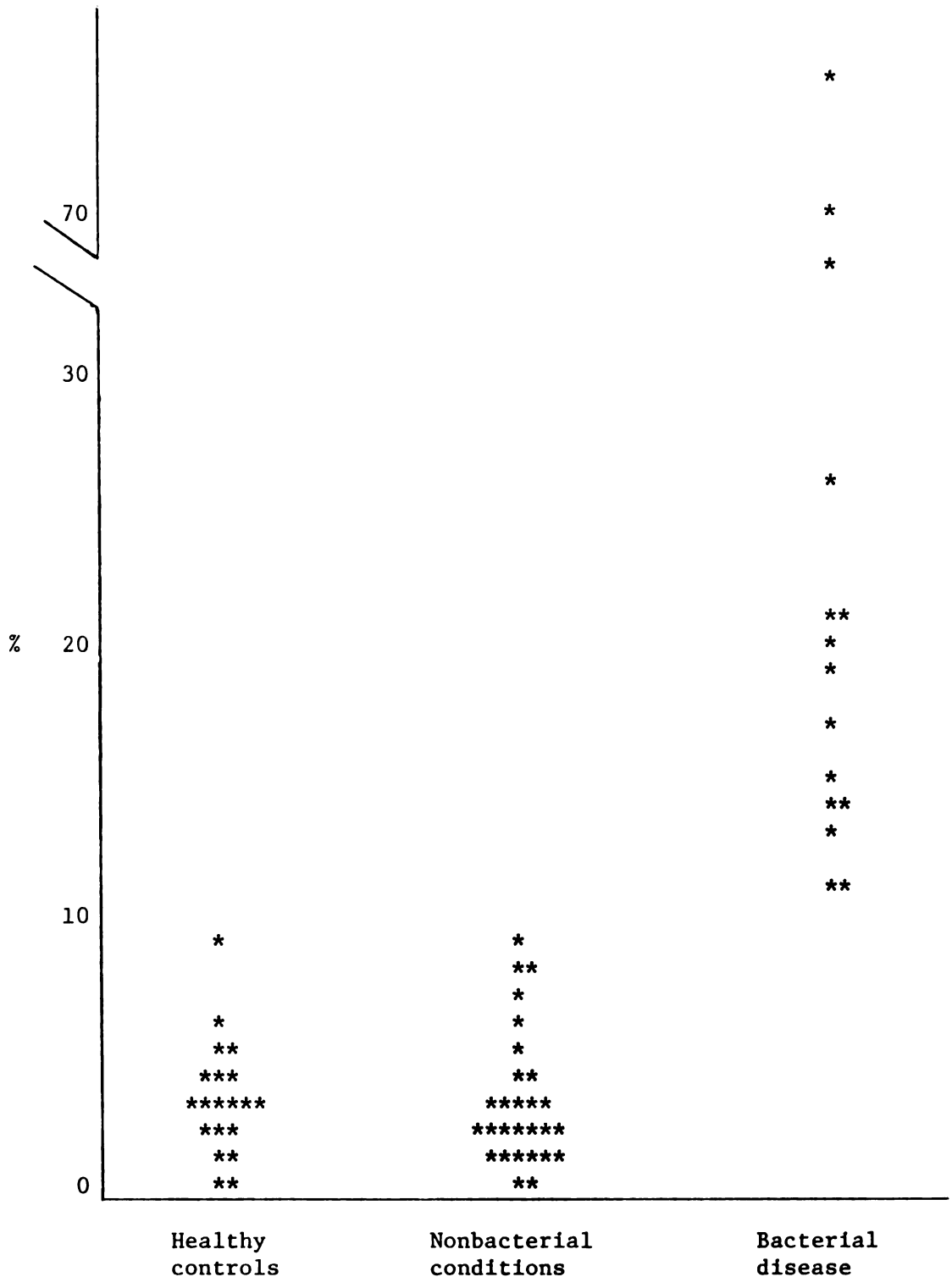

Figure 8. Distribution of NBT scores: horses.

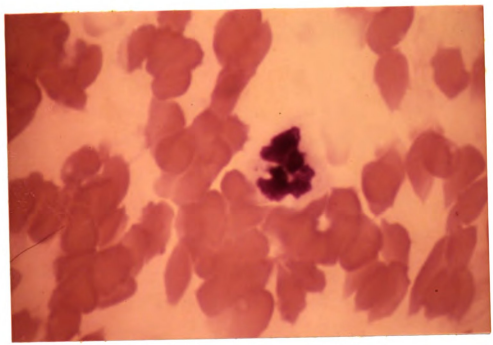

Figure 9. Equine neutrophil with dense formazan deposits. Wright's stain; x 1200.

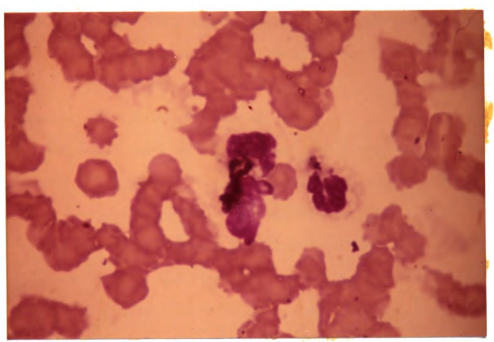

Figure 10. Neutrophil clumping in equine neutrophils containing formazan deposits. Wright's stain; x 1200.

Leukocyte counts in horses with positive NBT scores ranged from 7400 to 22,500 WBC/cmm. Horses with negative scores ranged from 6400 to 16,400 WBC/cmm.

Cow

The data on cattle are presented in Tables 19, 20, 21 and 22 and in Figures 11, 12 and 13.

The mean NBT score in 10 clinically healthy cattle was 1.5% (range 0 to 3%). This is considerably lower than the values obtained for the previous 3 species. Scores in 11 cattle with nonbacterial conditions ranged from 0 to 4% (mean 2%). However, 13 cattle with confirmed bacterial infections had values ranging from 0 to 34% with a mean score of 13.5%.

Cattle showed less ability to react to the NBT test than the previous 3 species. This may be related to the somewhat sluggish neutrophilic response to bacterial infection often observed in ruminants. Formazan deposits also tended to be less distinct, and some punctate deposition was evident.

The highest score was 34% in a cow with acute mastitis due to *Staphylococcus aureus*. At the time that the sample was drawn, the animal had already been treated twice with systemic antibiotics. The overlap of scores from bacterial and nonbacterial conditions was considerably more than that observed in the dog and horse, but was comparable to that observed in the cat. However, it was often difficult to determine whether or not cattle had been treated by the owners prior to presentation at the clinic. One calf which died of pneumonia had an NBT score of 0% at the time of admittance

Table 19. Summary of NBT scores: cow

Diagnosis	No.	Mean (%)	Range (%)
Clinically healthy	10	1.5 ± 0.9	(0-3)
Bacterial disease	13	13.5 ± 9.6	(0-34)
Nonbacterial conditions	11	2.0 ± 0.9	(0-4)

Table 20. Diagnoses and NBT scores in cattle with bacterial disease

Diagnosis	No.	Mean (%)	Range (%)
Metritis	5	12.0	(5-15)
Traumatic reticulitis	2	16.0	---
Acute mastitis	3	19.3	(10-34)
Calf pneumonia	2	5.0	(0-10)
Listeriosis	1	15.0	---

Table 21. Diagnoses and NBT scores in cattle with nonbacterial conditions

Diagnosis	No.	Mean (%)	Range (%)
Displaced abomasum	7	2.7	(0-4)
Toxemia-ketosis	2	1.0	(0-2)
Other	2	1.5	(1-2)

Table 22. Examples of leukocyte counts, culture results, and fibrinogen levels in cattle

Diagnosis	NBT Score	WBC Count	Fibrinogen (mg/100 ml)	Culture Result
Listeriosis with septicemia	15	23,700	2,000	*Listeria* sp., *Klebsiella* sp.
Traumatic reticulo-peritonitis	16	5,500	900	*Corynebacterium* sp.
Traumatic reticulo-peritonitis	16	15,000	100	*E. coli*
Metritis and pneumonia	5	4,400	400	---
Metritis	14	8,500	300	*E. coli*
Metritis	15	4,600	2,200	*E. coli*
Metritis	12	3,600	800	*E. coli*, *Proteus* sp., *Klebsiella* sp.
Metritis and gangrenous pneumonia	14	11,700	800	*E. coli*
Acute mastitis	34	14,400	800	*Staphylococcus* sp.
Mastitis	10	10,800	800	*Streptococcus* sp.
Mastitis	2	8,000	700	*Klebsiella* sp.
Calf pneumonia	0	11,100	1,100	*Pasteurella* sp., *Streptococcus* sp.
Pneumonia	10	9,000	1,700	*Pasteurella* sp.
Displaced abomasum	3	8,900	200	---
Displaced abomasum	3	6,500	300	---
Displaced abomasum	4	7,300	400	---

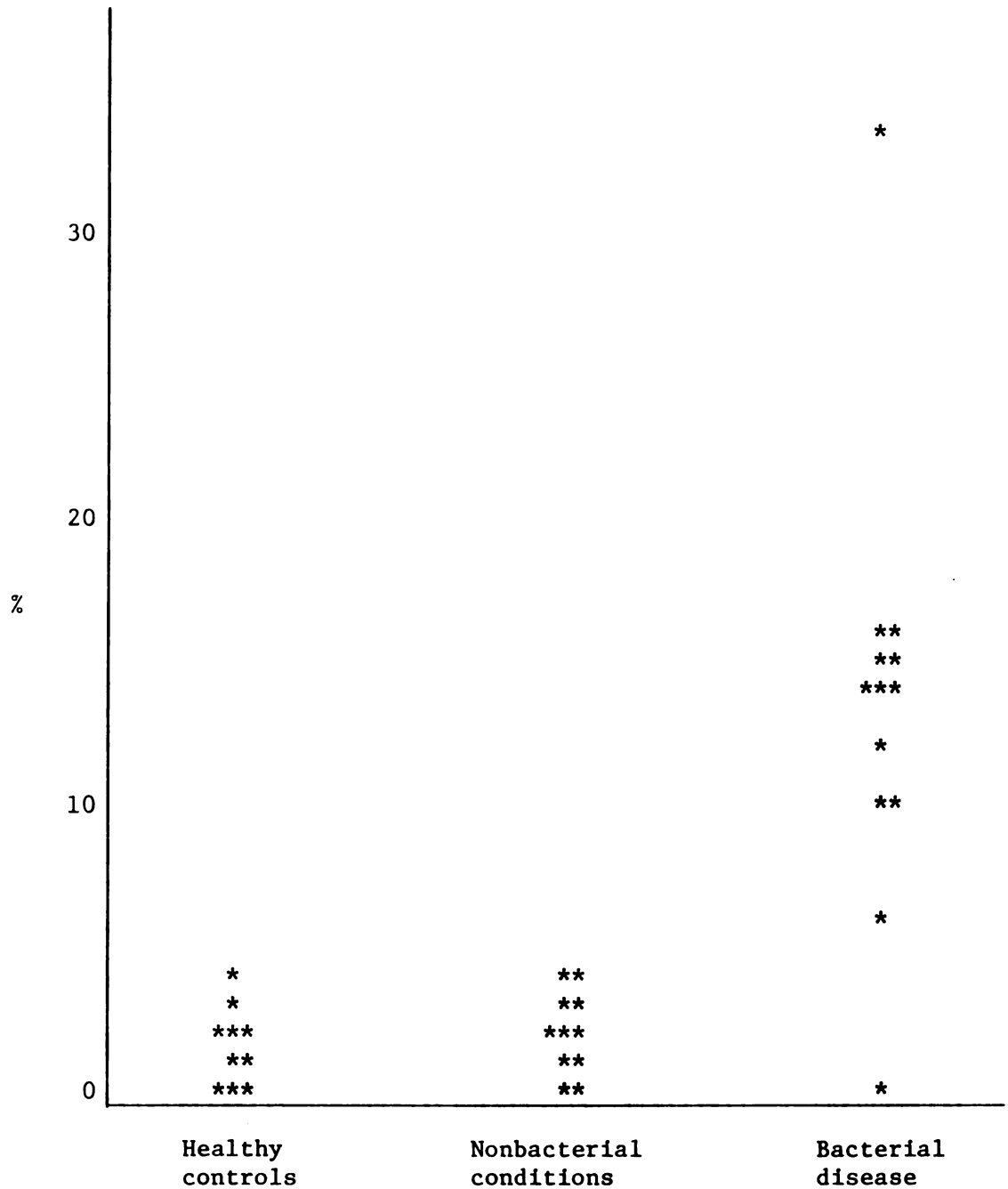

Figure 11. Distribution of NBT scores: cattle.

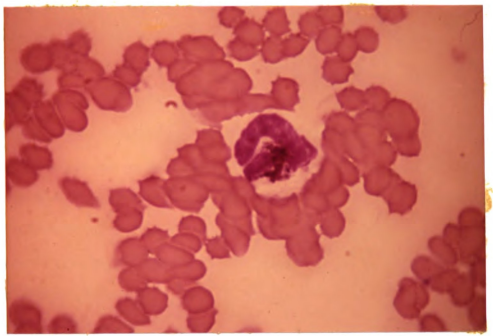

Figure 12. Bovine neutrophil with a formazan deposit. Wright's stain; x 1200.

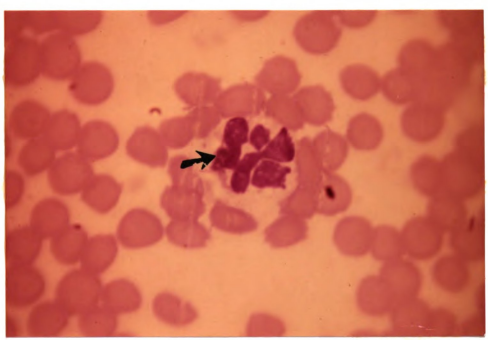

Figure 13. Less distinct and smaller formazan deposit (arrow) in a bovine neutrophil. Wright's stain; x 1200.

to the veterinary clinic. Postmortem cultures were positive for *Pasteurella* sp. and *Streptococcus* sp. Scores were also low in a few cattle with bacterial mastitis. This could have been due to localization of the infections.

Fibrinogen levels were increased in others. The increases did not correspond to increased reduction of NBT, increased leukocyte counts or positive bacterial cultures. This would seem to indicate that the increases in fibrinogen were associated with inflammatory changes regardless of whether or not they were due to pathogenic bacteria.

Leukocyte counts in nonbacterial conditions ranged from 4400 to 8900 WBC/cmm and from 3600 to 23,700 WBC/cmm in bacterial infections.

DISCUSSION AND CONCLUSIONS

Normal Values

Mean scores obtained in control blood samples of dogs, cats and horses were approximately 3% and in cattle approximately half that value. Hallett and Wilson (1973) reported 9.6% as a normal mean value in the dog. However, these investigators used the method reported by Park *et al.* (1968). An entirely different method using EDTA as an anticoagulant was used in this study. Freeman and King (1972a) had reported lower NBT values when using EDTA rather than heparin. Park *et al.* (1968), Matula and Paterson (1968) and Gordon *et al.* (1973), using the same technique in normal human beings, reported mean scores of 7, 3, and 6%, respectively. Thus there is a considerable amount of variation which is probably due to slight differences in testing methods or in individual interpretations.

Problems with Methods

The method originally attempted in this study was that of Park *et al.* (1968). However, difficulty was encountered in interpreting the slides obtained due to extensive leukocyte clumping, rupture of cell membranes and indistinct nuclear staining. The use of EDTA partially rectified these problems, and the subsequent use of plastic tubes in which to incubate the mixture

appeared to lessen cell disruption. Also, the change to the standard solutions suggested by Hicks and Bennett (1972) alleviated many technical problems. The inert synthetic sucrose polymer, Ficoll, would presumably have been useful in stabilizing the cell membrane and in counteracting the reduction of the NBT scores due to the use of EDTA (Stuart and Simpson, 1970). However, Ficoll was not available at the time this study was undertaken.

Overlap: False-Negatives and False-Positives

The incidence of false-positive and false-negative scores reported in animals was comparable to the situation in human patients. Many of the animals may have been referral cases or subjected to treatment by the owners. Therefore, the exact admission status of many animals regarding length of illness and prior antibiotic or steroid administration, for example, remained undetermined. Also, although the majority of the animals with positive NBT scores were cultured, most of the animals with negative scores were not. Therefore, the incidence of overlapping scores between the groups of bacterial disease versus nonbacterial conditions may have been far greater than that reported. Further studies under controlled conditions are necessary to determine the exact incidence.

Observations

An interesting observation which confirmed prior observations was the prompt decrease in the number of positive neutrophils following effective antibiotic therapy. In some cases, the NBT

score remained high during antibiotic therapy which was apparently not efficacious, only to be followed by a prompt decrease following the change to a new, more effective drug.

In all 4 species studied, the leukocyte counts varied from frank leukopenia to leukocytosis in both the NBT-positive and NBT-negative groups. Examination of the tables reveals that the NBT test more often accurately predicted the presence of bacterial infection than the total white blood cell count (WBC). Therefore, the NBT test showed a greater reliability than WBC count in diagnosing potential bacterial infections.

Conclusions

The results of this study indicate that the NBT test is an effective diagnostic aid in differentiating bacterial infections of horses, cattle, cats and dogs from nonbacterial conditions. In all of these species, there were striking differences between the average NBT scores of animals with bacterial infections and those of normal animals and animals with nonbacterial diseases. The test was most reliable in dogs and horses. Some false results occurred in all 4 species. In addition to enabling tentative diagnosis of bacterial infection before cultural confirmation is possible, the NBT test can provide, in animals as in man, an index of the efficacy of antibacterial therapy. Consequently, the NBT test should be a good prognostic aid and should prove particularly useful in monitoring high risk animals such as post-surgical patients for early indications of bacterial infections.

SUMMARY

In human patients, the occurrence of bacterial infection is reflected in an increase in the percentage of nitroblue tetrazolium (NBT)-positive neutrophils above the levels observed in healthy controls and in individuals with nonbacterial conditions.

In this study, a modified technique was employed to perform the NBT test on blood samples of 4 species of domestic animals. The study utilized blood samples from 139 dogs, 39 cats, 62 horses, and 34 cattle.

Average scores obtained in healthy control animals were as follows: dog - 3.0%, cat - 3.5%, horse - 3.2%, and cow - 1.5%. Bacterial infections with involvement of the systemic circulation resulted in increases in NBT reduction in all 4 species similar to those reported in human patients. Most of the positive scores ranged from 15 to 35%. A few values over 70% were obtained in dogs and horses. The highest scores observed in cats and cattle were approximately 35%, but fewer individuals of these 2 species were sampled.

Clumping of cells and dense deposits of formazan which obliterated cell nuclei and ruptured cytoplasmic membranes were often seen in samples with high percentages of positive cells.

Although the incidence of false-negative and false-positive scores was low, false scores did occur in all 4 species. In many cases, explanation of these results was extremely difficult. Moreover, since in some cases treatment histories were incomplete and/or cultures could not be performed, it is possible that there were some additional false results which were unrecognized. In view of the rather high incidence of false results in human patients, it is likely that several of the conditions resulting in false scores in human beings are duplicated in animal species and could be detected by controlled studies.

The results obtained in this study indicated that the NBT test is useful in differentiating bacterial infection from non-bacterial conditions in all 4 of the species sampled. The NBT test would appear to be especially useful: 1) in monitoring high-risk patients for early bacterial infection, and 2) in serial tests to determine the efficacy of antibacterial therapy in animals with severe infections.

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VITA

VITA

Deborah Lees Banas was born on February 26, 1949, in Quantico, Virginia. After attending school in Virginia, Mississippi and Arizona and graduating as valedictorian from Woodrow Wilson High School in Portsmouth, Virginia, she entered Michigan State University as a mathematics major in the fall of 1967. The following fall, she changed to a pre-veterinary major and entered veterinary school in the fall of 1969. After five terms of veterinary school, she received the B.S. degree and was awarded the John A. Hannah award for being the female graduate with the highest grade-point in the university. In June 1972 she received her D.V.M. and the Michigan Veterinary Medical Association award for having the highest grade-point average in the graduating class. Beginning in the summer of 1972, Deborah entered graduate school seeking a Master's Degree in Veterinary Pathology.

Dr. Banas is currently working at Hazleton Laboratories in Vienna, Virginia as a Staff Pathologist. She met her husband, Gary A. Banas, while attending veterinary school, and they were married September 4, 1971. Gary also completed his veterinary degree and is a small animal practitioner in Springfield, Virginia.

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