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Research Article

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Enhanced Phagocytic Capacity

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ABSTRACT The biologic basis for the elevated histochemical reduction of nitroblue tetrazolium dye (NBT) in neutrophils from patients with acute bacterial infection or polycythemia vera was studied. A precipitin reaction followed mixing NBT with heparin. NBT was reduced after phagocytosis of this complex (H-NBT) by polymorphonuclear leukocytes (PMNs). Ingestion required divalent cations and was facilitated by the presence of complement. H-NBT incubated with normal but not with C2-deficient human serum converted native C3 to its inactive form.

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These results suggest that increased phagocytic capacity of PMNs is the primary cause of increased histochemical NBT reduction. The PMNs of patients with acute bacterial infection or polycythemia vera may have alterations in their cell membranes which lead to an enhanced rate of phagocytosis.

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INTRODUCTION

An increased percentage of blood neutrophils reducing nitroblue tetrazolium dye (NBT) [1] to formazan has been utilized as an indicator of acute bacterial infection and other inflammatory states (1, 2). This increased reduction of NBT by neutrophils is most often seen in patients with acute bacterial infections; however, values may also be elevated in patients with polycythemia vera or neoplasia (3).

The reduction of NBT dye requires oxidative metabolism by the hexose monophosphate shunt, but the biologic basis for its increased histochemical reduction in certain diseases has not been defined. It has been suggested that NBT is impermeable to cell membranes, that it enters the cell during phagocytosis, after which it is reduced by diaphorase activity within the phagosome (4). Moreover, it has been demonstrated that the histochemical NBT reduction, at least to a certain extent, is dependent on the use of heparin as an anticoagulant (5). Recently, Segal and Levi determined that NBT interacts with heparin and fibrinogen to form a precipitate; electron microscopy revealed the presence of an amorphous material, probably the complexed NBT, within membrane bound vacuoles of polymorphonuclear leukocytes (PMNs) (6).

This investigation demonstrates that NBT reacts with heparin to form an insoluble complex which can be phagocytized, and that phagocytes collected from patients with elevated histochemical NBT reactions have

[1] *Abbreviations used in this paper:* ACD, acid citrate dextrose; CVF, cobra venom factor; HMS, hexose monophosphate shunt activity; H-NBT, heparin-nitroblue tetrazolium complex; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocytes.

enhanced phagocytic indices for several ingestable particles, including the complex of NBT and heparin. The total diaphorase activity of sonicated leukocytes obtained from patients with elevated histochemical reduction was not elevated. Enhanced phagocytosis is the likely cause of increased NBT reduction, ingestion occurring after the interaction of heparin, NBT, and complement.

METHODS

Isolation of leukocytes. Cells from patients with either acute bacterial infection or polycythemia vera were studied. All patients had histochemical NBT reductions of greater than 25% and PMNs from all patients also had marked toxic granulation.

Leukocytes were isolated by sedimentation from the heparinized blood of apparently healthy volunteer subjects and patients by the method previously described (7). Contaminating red blood cells were removed by hypotonic lysis and the leukocyte pellet was washed two times in phosphate-buffered saline (PBS) and adjusted to a final concentration of desired cells by the addition of PBS.

The number of phagocytes was determined in a standard white cell counting chamber observed under phase microscopy. Phagocytes are defined here as segmented neutrophils, band neutrophils, eosinophils, and monocytes. Lymphocytes accounted for less than 10% of the isolated cell suspension. When cells were sonicated for measuring diaphorase activity as described below, a Branson sonifier (Branson Instruments Co., Stamford, Conn.) with an output of 20 W was used for disruption. Each cell suspension was sonicated four times for 15 s each time with cooling in between. The whole sonicate was assayed immediately for activity.

Histochemical NBT reaction. Histochemical NBT reaction was performed as previously described (3). Briefly, 0.20 ml of heparinized venous blood (10 U of sodium heparin/ml; Upjohn Co., Kalamazoo, Mich.) was reacted with an equal volume of 0.1% NBT (Sigma Chemical Co., St. Louis, Mo.) for 25 min at 37°C. The mixture was then incubated for 15 min at room temperature; thin smears were made and stained with Pappenheimer's Stain. The reaction was graded on the basis of the number of neutrophils with intracellular deposits of formazan per 100 neutrophils counted, i.e., percent positive.

Heparin-NBT reaction. It was observed that heparin mixed with NBT and plasma, whole blood, or PBS immediately formed a yellow precipitate (H-NBT). Morphologic studies of this reaction were performed on Wright's stained blood films by employing increasing concentrations of heparin. The reaction was also investigated after NBT was added to heparinized (10 U/ml) cell suspensions in PBS and the cells then were observed by wet mount by employing phase microscopy. Further study of the H-NBT reaction utilized immunodiffusion techniques employing Ouchterlony plates. These were carried out in 1.25% agarose in PBS at pH 7.35.

The ability of H-NBT precipitate to convert C3 to a faster migrating inactive form was studied by the two dimensional electrophoretic technique of Laurell (8). Conversion of C3 was determined after incubating an H-NBT (75 U of heparin and 1 mg NBT in 1 ml PBS) solution with equal volumes of fresh serum or serum from a subject lacking C2 at 37°C for 30 min. Zymosan-treated serum served as a positive control. Further studies of H-NBT and complement were performed by determining the histochemical NBT reaction in control Hyline rabbits and rabbits depleted to less than 10% of C3 by sequential doses of purified cobra venom factor (CVF) as described by Brown and Lachmann (9).

Studies of phagocytosis. An index of phagocytosis was determined by the following formula: phagocytic index = patient (phagocytic − resting value)/control (phagocytic − resting value).

Phagocytosis of H-NBT was compared with two standard particles by measuring oxygen utilization and hexose monophosphate shunt activity (HMS) in resting or phagocytizing cells isolated from both patients and controls.

The three particles (H-NBT, latex, zymosan) used for phagocytosis were all suspended in PBS. The standard suspension of latex particles (0.81 μm) was a 1:100 dilution of a stock dilution (Difco Laboratories, Detroit, Mich.). The suspension of zymosan was standardized to an OD of 1.0 at 525 nm in a spectrophotometer. The suspension of H-NBT was prepared in 15 ml of 0.1% NBT in PBS by adding 1.2 ml of 1,000 U/ml of sodium heparin.

Oxygen utilization was determined by standard techniques employing a Clarke oxygen electrode (7). In this assay the mixture contained 5×10^6 phagocytes, and a standardized quantity of the particle to be ingested in a final volume of 3.0 ml PBS. Serum was added in some experiments to give a final concentration of 10%. O_2 utilization was recorded during the first 5 min of phagocytosis.

HMS activity was measured by determining the evolution of $^{14}CO_2$ from oxidation of [1-^{14}C]glucose (7). The isotope was obtained from New England Nuclear of Boston, Mass. Specific activity of [1-^{14}C]glucose was 52.2 mCi/mmol. The reaction mixtures contained 5×10^6 phagocytes and a standardized quantity of particle. Serum was added in some experiments to give a final concentration of 10%. When serum was omitted, unlabeled glucose was added to give the same final glucose concentration. Reactions were initiated by the addition of cells, and the mixture was incubated at 37°C for 60 min in a metabolic shaker bath before measuring the $^{14}CO_2$ which evolved.

Ingestion of latex particles in control and patient phagocytes was also determined by visually counting cell-associated particles after phagocytosis. This third procedure for estimating phagocytosis employed 1×10^7 phagocytes and latex particles (0.81 μm) at a ratio of approximately 100 particles per phagocyte incubated for 15 min in a metabolic shaker at 37°C. 500 PMNs were then counted by phase microscopy and the number of particles associated with each cell was estimated. The counts were performed without knowledge as to whether the cells were obtained from controls or from patients with elevated histochemical NBT reactions. The phagocytic index was determined by recording the total number of all associated latex particles in 500 PMNs.

Determination of diaphorase activities. Isolated cells were suspended in PBS to a final concentration of 5×10^7 cells/ml and disrupted by sonication as described. The sonicate was assayed immediately in a recording spectrophotometer. Each cuvette contained in a total volume of 3.0 ml: 50 μmols sodium phosphate buffer, pH 7..0; 0.15 μmol NBT dye; 0.23 μmol NADH or 5.4 μmol NADPH both obtained from Sigma Chemical Co. These conditions were experimentally determined to give optimal activity; a much higher concentration of NADPH was required

for maximal activity than of NADH. The reaction was initiated by the addition of 0.10 ml sonicate, and the formation of reduced NBT dye was determined by the increase in OD at 560 nm, followed for at least 5 min in the spectrophotometer. Values were expressed as change in OD per minute per milligram protein. Protein concentrations of the leukocyte extracts were determined by the biuret procedure of Gornall, Bardawill, and David (10) with bovine serum albumin as a standard.

RESULTS

NBT reactions. Table I gives results of histochemical NBT reduction in control neutrophils and neutrophils taken from patients with either acute infection or polycythemia vera. Results are compared by using 10 U of heparin per 1.0 ml of venous blood, 0.02 M EDTA, or 1:5 vol/vol acid citrate dextrose (ACD) solution as the anticoagulants. The histochemical reaction was elevated only in heparinized blood.

Fig. 1 shows the effects of varying the concentration of heparin on the histochemical NBT reduction of control neutrophils. The number of cells reducing the dye increases to a peak between 50 and 100 U/ml of heparin. High concentrations of heparin inhibited the reaction. Peak reductions occurred at a concentration of heparin which was near the equivalence zone which will be discussed below.

Wright's stained blood films with increasing concentrations of heparin showed increasing quantities of an amorphous pink precipitate outside of cells, which could be reduced to formazan by adding a reducing agent such as ascorbic acid. In the studies in which NBT was added to heparinized (10 U/ml) cell suspensions in PBS and the cells observed under phase microscopy by wet mount, the precipitate of H-NBT formed at the cell surface and reduction to formazan occurred after phagocytosis of the complex. Fig. 2 demonstrates the precipitated unreduced H-NBT complex in close association with the PMNs. Reduction of NBT appeared to always occur intracellularly rather than at the surface of the PMN where the complex of H-NBT first formed. In 200 individual PMNs in which reduced NBT was associated with the cell, reduction of NBT was never observed beyond the periphery of the cell, that is, formazan precipitates were always present

TABLE I

Effect of Anticoagulants on Histochemical NBT Reactions

	Patients (n = 7)	Controls (n = 10)
Heparin (10 U/ml)	31±8*	5.9±2.0
EDTA (0.02 M)	2.0±0.5	1.0±0.2
ACD	2.6±1.1	1.0±1.1

* Percent positive ±SD.

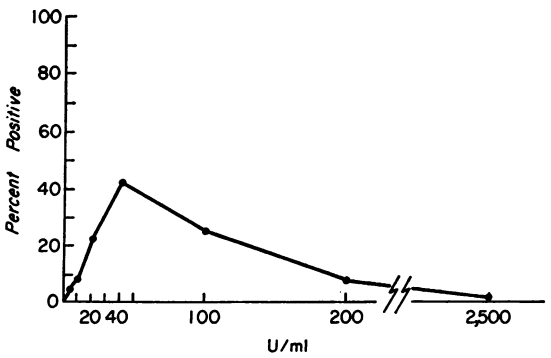

FIGURE 1 The effect of varying concentrations of heparin on the percent of resting control PMNs reducing NBT.

within the circular edge of the cell. This substantiated the concept that intracellular rather than surface reduction of H-NBT complex was occurring. These results were further substantiated when the supravital dye neutral red was added to the wet mounts. Both neutral red dye and reduced NBT were observed in close proximity within the cell (data not shown). Since neutral red dye is a lysosome "indicator" (11), these results suggested that reduction of NBT was occurring within the phagocytic vacuole.

Fig. 3 depicts an Ouchterlony plate in which the center well contains 0.1% NBT dye and the peripheral wells of varying size contain 100 U/ml of heparin. The equivalence zone varied somewhat in different studies, perhaps due to the heterogeneity of heparin solutions. However, it usually ranged between 75 and 100 U of heparin/ml reacting with 1 mg/ml of NBT.

Studies of phagocytic indices. The phagocytic indices are depicted in Fig. 4 and represent the mean values obtained from both oxygen utilization and HMS activity since the results of the two methods were virtually identical. No differences were observed between patients with acute bacterial infection or patients with polycythemia vera; therefore, these data were pooled. The phagocytic indices of all three types of particles were significantly increased in all patients with elevated histochemical NBT reduction. This increase was observed in the presence of fresh AB serum, heat-inactivated AB serum, and in the case of H-NBT with no serum in the assay. With each experimental condition the differences between patients and controls were statistically significant ($P < 0.01$). There was no difference among the increases in phagocytic indices of the three different particles in patients with elevated histochemical NBT reactions when fresh AB serum was utilized. However, with heat-inactivated AB serum, zymosan had a lower index than H-NBT or latex, suggesting that its uptake was more dependent on the presence of heat-labile opsonin, probably complement.

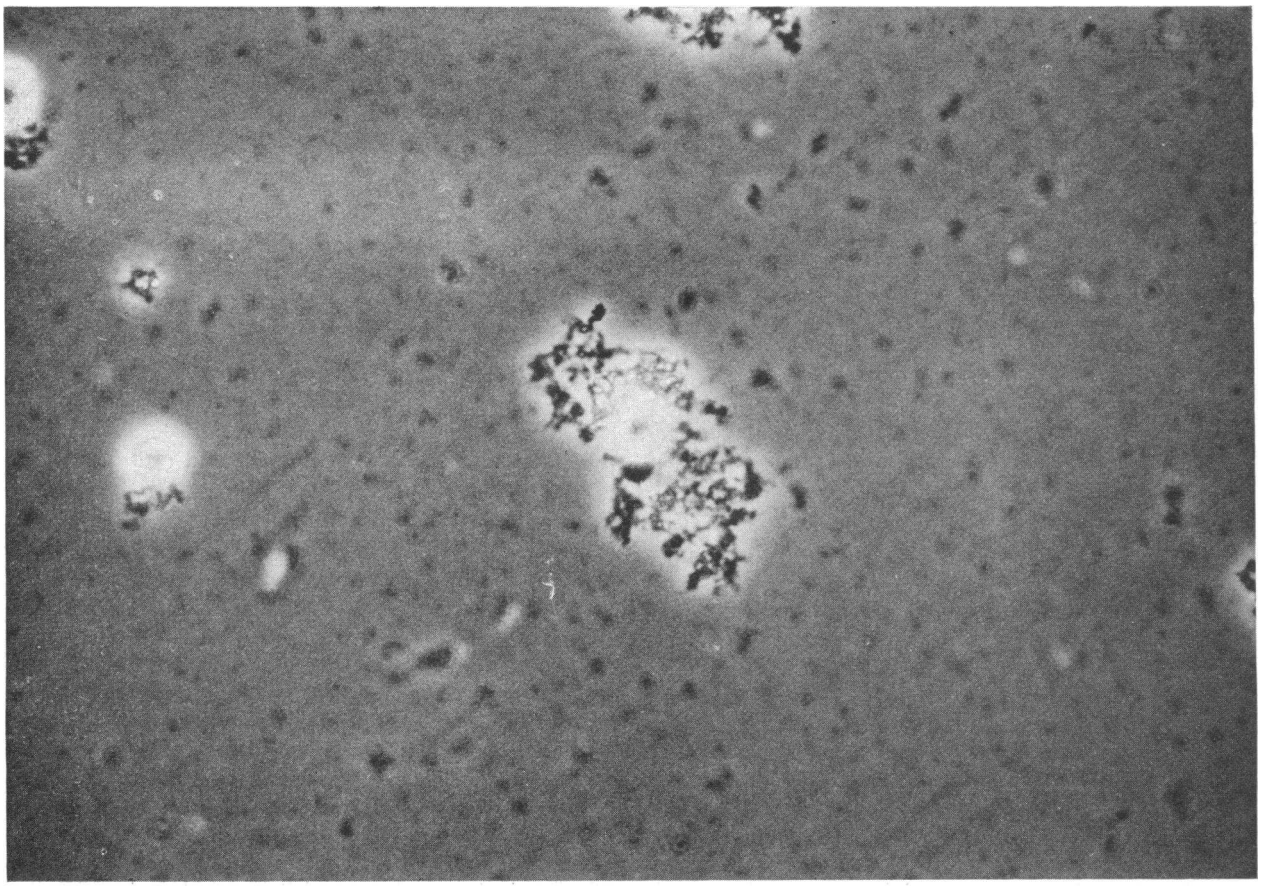

FIGURE 2 The association of heparin (10 U/ml) and NBT (0.1 mg/ml) around the periphery of a PMN before the ingestion. The NBT has not been reduced. (Phase contrast × 1,200)

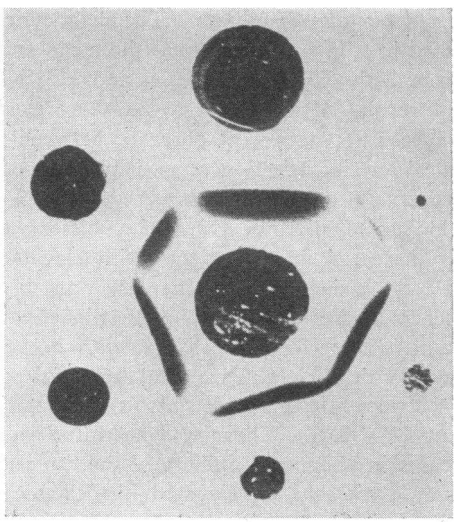

FIGURE 3 Precipitin reaction of heparin and NBT. Center well contains 0.1% NBT and the peripheral wells of varying size contain heparin (100 U/ml). After precipitation the plate was soaked in ascorbic acid solution which reduced NBT at the line of precipitation.

Table II compares the number of cell-associated latex particles in control cells with cells from patients with elevated histochemical NBT reactions. Patients with elevated histochemical NBT reaction had a significant increase in the mean phagocytic index.

Conditions affecting phagocytosis of H-NBT complex. Fig. 5 demonstrates the conditions which affected the ingestion of H-NBT complex in control phagocytes with O_2 utilization and HMS activity as the indices of phagocytosis. Mean values, combining both assays, are expressed as percent of control. Fresh

TABLE II
Phagocytosis of Latex Particles

	Normal (n = 10)	Patients (n = 11)
Phagocytic index*	1,568±326	2,164±246
	($P < 0.01$)	

* Particles in 500 phagocytes were counted, and the total ±SD expressed.

pooled AB serum was used in the control assay. Serum obtained from five patients with elevated histochemical NBT scores did not alter the phagocytic index of H-NBT. A decrease in the phagocytic index, however, was observed in heated AB serum, serum obtained from a patient with systemic lupus erythematosis which contained 70 mg/100 ml of C3, or serum depleted of complement (less than 20% of control) by treatment with purified CVF for 30 min at 37°C. Addition of 0.02 M EDTA was completely inhibitory. Surprisingly, the phagocytic index of H-NBT was greater when no serum was present in the phagocytic assay. Similar results have been observed in studies of phagocytosis of antigen-antibody complexes (12).

Interaction of H-NBT and complement. Fig. 6 demonstrates by diagramming results from two dimensional electrophoresis that incubation of H-NBT with fresh human serum converted native C3. With both zymosan and H-NBT, more than 40% of C3 was converted. Conversion did not occur in serum obtained from a human with isolated C2 deficiency, suggesting that activation of complement occurred by the classic pathway. These results have been substantiated by observing that H-NBT can precipitate C1q (unpublished observations).

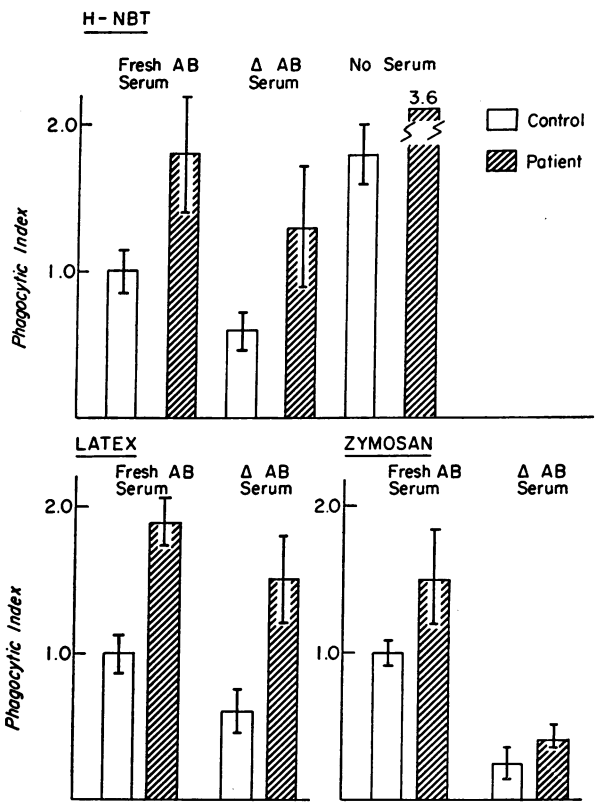

FIGURE 4 Phagocytic indices of control PMNs ($n=8$) and PMNs isolated from patients with acute bacterial infection ($n=4$) or polycythemia vera ($n=4$). The three particles are H-NBT, latex, and zymosan.

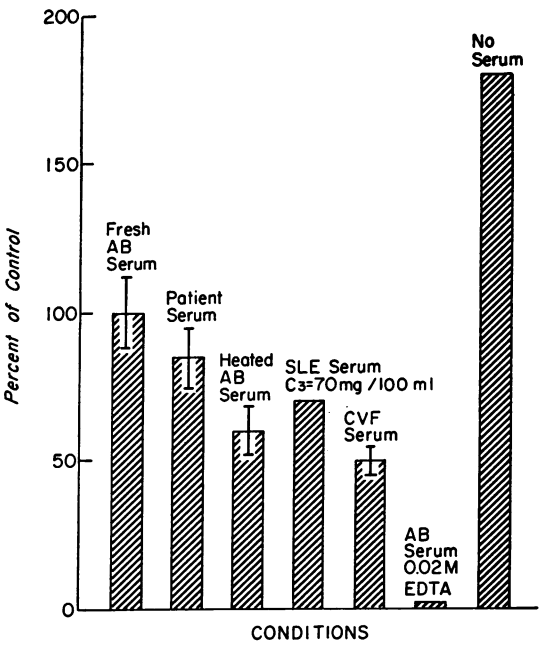

FIGURE 5 Conditions which alter the phagocytic index of H-NBT (see Methods). Fresh AB serum served as the control. $n=5$ for patient serum. SLE, systemic lupus erythematosis.

Table III demonstrates that the percent NBT histochemical reduction is significantly reduced in the rabbit

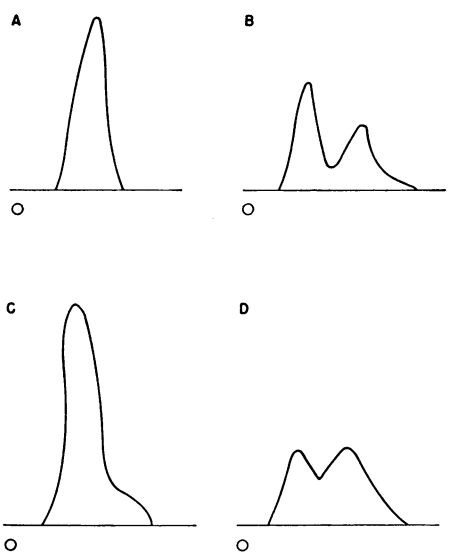

FIGURE 6 Diagrammatic representation of two dimensional electrophoresis for C3. The anode for the first dimension is to the right and for the second dimension to the top. C3 is largely converted to the faster inactive form in B (incubation of serum with H-NBT) and in D (incubation of serum with zymosan). A is an untreated control serum and C is isolated C2-deficient serum treated with H-NBT.

TABLE III

Effect of Complement Depletion by CVF on Histochemical NBT Reaction in Rabbit Neutrophils (n = 5)

	Percent NBT reduction	Phagocytic Index,* zymosan	Phagocytic Index, alexinated zymosan
Pre-CVF	18	720	760
Post-CVF	2	54	810

* Phagocytic index = number of phagocytized zymosan particles in 100 neutrophils. Alexinated zymosan are preopsonized with complement. See Methods.

blood depleted to less than 10% of C3. The results are compared with the phagocytic index of cell-associated particles utilizing washed zymosan or zymosan preopsonized by incubating it first with fresh human serum for 30 min at 37°C and washing three times in PBS.

NBT-diaphorase activities. Table IV compares the diaphorase activities of NADH and NADPH in sonicated leukocytes obtained from controls, patients with bacterial infection, and patients with polycythemia vera. No differences in either total NADH or NADPH diaphorase activity were observed.

DISCUSSION

The difficulties in differentiating acute bacterial infections from other febrile disorders have led to a search for a test with sufficient sensitivity and specificity to aid the clinician in making a diagnosis. The NBT histochemical test and the presence of toxic granulation and vacuolization of PMNs which may occur during acute bacterial infection have both been utilized for this purpose (1, 11). Unfortunately, neither is totally specific nor altogether sensitive. However, they may be valuable aids if interpreted with caution, and perhaps a better definition of their specific nature will lead to the development of more useful diagnostic tests.

The pathogenesis of alterations causing toxic granulation and vacuolization of PMNs is unknown, but data have suggested that there are changes in the cell membrane (11). These PMNs become more permeable to Romanovsky stains after which the azurophil granules stain deeply as "toxic granulation." The Döhle bodies frequently seen in such cells represent an aggregate of rough endoplasmic reticulum, and the vacuolization of these neutrophils probably results from formation of secondary lysosomes which occur as phagolysosomes or autophagic vacuoles (11). The presence of toxic granulation correlates with an elevated histochemical NBT reaction (7). These earlier observations interpreted with the data of this investigation suggest that toxic PMNs more actively phagocytize NBT and that both conditions may reflect alterations at the cell surface.

TABLE IV

Diaphorase Activities in Sonicates of Leukocytes

	Histochemical	NADH diaphorase	NADPH diaphorase
Control (n = 7)	4.6±2.7*	0.0183±0.004‡	0.0142±0.004
Bacterial infection (n = 5)	52.2±11.2	0.0196±0.006	0.0144±0.007
Polycythemia vera (n = 2)	57.5±17.5	0.0177±0.011	0.0178±0.012

* Mean percent reduction ±SD.

‡ Mean OD per minute per milligram protein at 560 nm ±SD.

Theoretically, the increase in histochemical NBT reduction observed during infections and other acute inflammatory states and in polycythemia vera could be caused by enhanced reduction of the dye at the cell surface, increased uptake of the dye with subsequent intracellular reduction, or an increase in diaphorase activity of the cell. Our interpretation of this investigation is that increased phagocytic capacity of such cells with subsequent intracellular reduction of the NBT dye and heparin complex is responsible for the elevated histochemical reduction of NBT dye. The NBT dye complexes with heparin by an unknown mechanism to give a classic precipitin reaction which can fix complement and convert C3. Complement as an opsonin facilitates uptake by the PMN. Similar reactions between heparin and other cationic substances have been reported (13).

Increased phagocytic indices are not only present in acute bacterial infection, since many of the patients had polycythemia vera with elevated histochemical NBT reductions and no detectable bacterial infection. This corroborates the increased phagocytic activity previously reported in patients with polycythemia vera (14). Increased phagocytosis of oil red O and NBT has been reported in patients with bacterial infection; although, the increase was attributed to serum factors in that study (15). The genesis of the increased phagocytic capacity is unknown. It was not related to factors present in the patient's serum. Moreover, since there was increased phagocytic indices of H-NBT, latex, or zymosan in fresh serum, heat-inactivated serum, or even in the absence of serum in the case of H-NBT, it is unlikely that there is an alteration in a specific cell receptor such as the receptor for C3 or the Fc fragment of immunoglobulin. A more likely explanation would be that there are alterations in the cell membrane which are associated with increased phagocytic rates, regardless of the method of attachment.

Explanations other than an enhanced phagocytic capacity per se of PMNs resulting in increased NBT

reduction must be examined. First, the H-NBT complex might more avidly become attached to the cell surface after which it could induce activation of oxidative metabolism of the cell and be reduced itself at the cell surface without being taken into phagocytic vacuole. Activation of oxidative metabolism can be induced without ingestion of particulate matter and particles may be ingested in the absence of appropriate oxidative metabolic machinery (16, 17). However, our microscopic studies of individual cells suggest that cell surface interaction is an unlikely possibility. For example, we did not detect reduction of NBT dye in a position extrinsic to the cell circumference. Segal and Levi have demonstrated that amorphorous particulate matter which probably represents an NBT and heparin complex can be demonstrated within a PMN by electron microscopy (6). An interaction simply at the cell surface seems even less likely since both zymosan and latex particles give similar reactions to the H-NBT complex and these particles are rarely observed to simply adhere to the cell surface by phase microscopy.

Another problem in interpretation concerns the nature of the histochemical NBT test. In this test cell particle contact which could result in phagocytosis would not be expected to occur easily, since there is no agitation of the cell utilized in this method, whereas in other in vitro methods of phagocytosis tumbling or shaking of the phagocytes is employed. However, as described under Results and demonstrated in Fig. 2, NBT appears to complex with heparin at or near to the cell surface, thus, providing contact with the cell without agitation. Our observations by phase-contrast microscopy demonstrated uptake of the precipitated complex into a phagosome after which the NBT dye was reduced to formazan.

Another consideration for the increased NBT reduction concerns the possibility of an elevation of cellular NBT-diaphorase activity. It is generally accepted that an NADH or NADPH diaphorase activity within the phagosome is responsible for the reduction of NBT dye (18). Our observations employing whole cell sonicates which demonstrated no increase in the total NADH or NADPH diaphorase activity does not prove that a single diaphorase might not be elevated in neutrophils of patients with infections or polycythemia vera which would then be responsible for the increased NBT reduction. Holmes and Good have demonstrated by disk gel electrophoresis four district NADH diaphorases and three NADH diaphorases in cell sonicates (18). Thus, a single one of these could conceivably be involved in the reduction of NBT within the phagocytic vacuole. Indeed a slight increase in NBT diaphorase activity has been reported in leukocytes obtained from patients with bacterial infection, but the difference between patients and controls was quite small (15). It remains remotely possible that a single diaphorase might be increased in patients with increased histochemical reduction and that this diaphorase isoenzyme is delivered specifically into the phagosome and is responsible for the histochemical NBT reduction within the cell. If this diaphorase is also rate limiting for the oxidative metabolism of the cell, then its increase could also account for the increased oxidative metabolism noted when other phagocytic particles such as zymosan and latex particles were employed in this study. However, the marked increments in histochemical NBT reduction present within the neutrophils of our patients associated with a normal total diaphorase activity for both NADH and NADPH in cell sonicates makes this explanation very unlikely. Irrefutable proof, however, will require further investigations of the specific diaphorases responsible for NBT reduction within neutrophil phagosomes.

Thus, an increased rate of phagocytosis not specific for a surface receptor is the most plausible explanation for our data. Further delineation of the specific nature of this alteration of the cell surface may lead to a more complete understanding of neutrophil physiology and to the development of more specific diagnostic tests for infection.

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