The Nitroblue Tetrazolium Reaction in Human Granulocytes Adherent to a Surface*

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INTRODUCTION

Normal human granulocytes are able to reduce the yellow dye, nitroblue tetrazolium (NBT), to insoluble blue formazan. This has been a useful reaction for the recognition of chronic granulomatous disease of childhood (CGD) and the carrier state, because granulocytes from these patients are deficient in their ability to reduce this dye(1).

It has also been suggested that granulocytes from patients with bacterial infections(2,3) and newborn infants(4,5) have increased capacity to reduce NBT, while cells from patients taking corticosteroid medications have decreased ability for NBT reduction(6). These observations are of interest because of the possible correlation of this intracellular biochemical reaction with the bactericidal capacity of the phagocyte.

The interpretation of such studies is hampered by the variation in results that are obtained with different methods. It has been shown that normal, resting granulocytes in suspension reduce only small amounts of NBT, and that phagocytosis of particles seems to be necessary for NBT to gain access to the living cell(7,8). With phagocytosis of latex particles, there is a significant increase in extractable formazan from the phagocytizing cells when compared to resting

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cells(9). In the course of developing a simple, cytochemical micromethod for recognizing CGD with granulocytes adherent to glass(10), we obtained results that appeared to conflict with these earlier published observations. For example, in our system, an average of 40% of normal granulocytes were able to reduce NBT without ingesting particles. These cells were distinctive, but bore no resemblance to those described by others(2,7,11). They are discussed in detail in this report. We suspected that technical differences might be responsible for the morphologic variability of formazan-containing cells. In the present report we have described the dynamic and morphologic characteristics of the formazan-containing leukocytes adherent to glass and compared these cells with those described by others.

METHODS

Isolation of Leukocytes

Human leukocytes from normal adult donors and from patients with CGD were isolated from a single drop of blood on a glass coverslip as described previously(10). In brief, a drop of whole blood was placed on a glass coverslip and incubated at 37° for 25 min in a moist chamber. The resulting clot was then washed away with normal saline, leaving a circular area replete with motile granulocytes and some monocytes adherent to the glass. These cells were utilized in all of the experiments unless otherwise specified.

Incubation of Leukocytes

Leukocytes were exposed to a variety of solutions and suspensions by inverting the glass coverslip over a drop of test media on a glass slide, blotting to remove excess fluid, and reincubating in a moist chamber at 37° (or other stated temperature) for variable lengths of time(10). For some experiments involving phagocytosis of particles, the glass slide itself was inverted during the incubation to insure maximal contact between cells and particles. The test media were always freshly prepared to a final volume of 1.4 ml by adding 0.85% saline to the other reagents listed below. Incubations were performed in 36% pooled human sera (0.5 ml) prepared from five healthy adults. P nitroblue tetrazolium (Nutritional Biochemical Corp., Cleveland) was added to some of the incubates from dilutions of a stock solution of 28 mg NBT dissolved in 10 ml of isotonic saline and then filtered through sintered glass. The final concentration of NBT when added to the incubates was either 0.05 or 0.12% except as stated. The osmolarity of the two test solutions differed by only 10 mOsm. Powdered zymosan (Nutritional Biochemical Corp., Cleveland) was boiled and washed according to the methods outlined by Hirsch(12). Sixty milligrams were suspended in 10 ml of physiological saline. For use, 0.3 ml of this stock suspension was added to 1.1 ml of other reagents. In most cases, this produced a numerical ratio of zymosan particles to leukocytes of at least 10. For some studies, sodium cyanide in a final concentra-
tion of $1 \times 10^{-3}$ M was added to the incubation medium. In some experiments the leukocytes were preexposed to one incubate and immediately reexposed to another by carefully removing the coverslip from the first slide and replacing it on another slide bearing the second incubate. At various stages of the incubation, viability was assessed by the cells' motility, observed directly on a warmed stage, and by their ability to exclude eosin(13) and to ingest particles.

**Microscopy**

(A) *Permanent preparations.* After incubation, the coverslip was carefully removed and quickly airdried (5 sec). The cells were fixed in absolute methanol and stained with safranin as described previously(10). The cells were viewed with plain light (for best discrimination of color) as well as phase microscopy (for morphology). Two hundred consecutive cells were examined in duplicate or quadruplicate for the presence and distribution of intracellular formazan and for the presence and numbers of particles.

(B) *Wet preparations.* Direct observation of the cells was possible at any stage of an experiment merely by removing the slide from the incubator and placing it on the warmed stage ($37^\circ$) of a phase microscope. In this manner one could watch and photograph the formation of formazan, the ingestion of particles, the degree of adherence of the cell to the glass, and, with the addition of eosin, evaluate the percentage of cells that were nonviable (stained pink).

**RESULTS**

1. *Reduction of NBT by Nonphagocytizing Granulocytes*

   (A) *The formazan cell* (Figs. 1A and 6A). Many normal granulocytes, when exposed to 0.12% NBT without particles, were converted to large, distinctive, easily recognizable cells, hereafter designated formazan cells (see Figs. 1A and 6A). Although there was a wide variation in the amount of formazan seen within a given cell, such cells were always clearly recognizable by their distinct blue color which tended to be granular, diffusely spread within the cytoplasm, and more dense peripherally. Occasionally formazan was seen only at the tip of a pseudopod, or as a discrete collection in some part of the cytoplasm. In the fully developed cell, the presence of these precipitates was associated with cellular degeneration indicated by swelling, loss of nuclear reticular pattern, and coalescence of nuclear material. Although they superficially resembled monocytes, direct observation of individual leukocytes, before and after addition of NBT, confirmed that they were derived from granulocytes. Although almost all the cells were motile before the addition of NBT, many seemed to spread out thinly on the coverslip, indicating that a large part of the cell surface was flattened against the glass. These cells tended to become formazan cells whereas the others, that failed to reduce dye, seemed to be attached to the glass by a smaller area, with much of the cell free in the surrounding fluid medium as indicated by a more prominent refractile halo about the cell (Fig. 2).
Eosinophils and monocytes also contained formazan, although monocytes typically showed only scattered discrete precipitates without definite blue color. There was a direct correlation between the amount of formazan within a given cell and the extent of cellular degeneration. Granulocytes with only a small quantity of intracellular formazan retained their shape and characteristic nuclear structure.

Fig. 1. The effect of a 20-min exposure of granulocytes to 0.12% NBT without particles. (A) Normal adult showing several typical formazan cells. (B) CGD, showing absence of formazan cells (Safranin stain; phase microscopy).
Cells were designated as "formazan cells" only if there was a definite cytoplasmic collection of precipitates that had a distinctly blue color.

(B) The effect of the time of exposure to NBT on the production of formazan cells (Fig. 3). Granulocytes from a normal adult man were exposed to 0.12% NBT without particles for varying times. The percentage of formazan cells on each slide was then determined by counting 200 consecutive granulocytes and scoring each as positive or negative according to the above criteria. The upper curve in Fig. 3 shows the mean percentage of positive cells at each interval and indicates that formazan cells formed rapidly, reaching 20% of the total cells within 1 min and 40% by 20 min. After 40 min there were few additional positive cells. Many granulocytes did not become formazan cells at each interval although they were scattered among the formazan cells (Figs. 1A and 2), and thus were presumably subjected to the same experimental conditions.

Similar studies of granulocytes from two patients with chronic granulomatous disease showed a marked difference (see lower curve, Fig. 3). These granulocytes formed no typical formazan cells at any of the exposure times (10), and hence could be distinguished from normal cells at a glance (Fig. 1B).

![Fig. 2. Normal granulocytes exposed to 0.12% NBT for 20 min showing large formazan cells as well as refractile, motile, nonformazan cells (Wet preparation; phase microscopy).](image-url)
Similarly, CGD leukocytes were readily distinguished by this test when compared with granulocytes from other children. Nineteen children ranging in age from premature infancy to 13 years were examined (20-min exposure only). Although some of these children were healthy, most were hospitalized for diverse medical problems including recurrent infections. In each case, however, 30% or more of granulocytes became formazan cells.

From these trials, a 20-min exposure seemed to give the most reproducible results and was, therefore, selected for general use.

(C) Cell viability. To test the integrity of the cell membrane, normal granulocytes were exposed to eosin, added to the test media for a 20-min incubation without NBT. Only 2–6% of cells treated in this way admitted eosin, while 40% of cells became formazan cells in a simultaneous experiment with NBT. Related experiments showed that fully developed formazan cells admitted eosin, but that some partially developed formazan cells excluded eosin. Some cells admitted eosin but contained no formazan.

The adherent cells retained their capacity for phagocytosis; over 90% of cells readily ingested zymosan particles (see below). Granulocytes from patients with CGD remained motile and capable of phagocytosis, even after prolonged exposure to NBT.

These observations suggest that injury to cells is not responsible for the admission of NBT, but rather that cell degeneration takes place after the formation of sufficient intracellular formazan.

(D) The effect of different concentrations of NBT on the production of formazan cells (Fig. 4). Granulocytes of 13 normal adults were examined repeatedly by the method described. Cells from each donor were exposed to either 0.12% or 0.05% NBT, without particles, for 20 min. Figure 4 shows the results of 114 determinations with 0.12% and 107 determinations with 0.05% NBT expressed

Fig. 3. The effect of varying the time of exposure to NBT (0.12%). The number of determinations is shown in parentheses. The vertical lines indicate two standard errors of the mean.
NBT Reaction in Human Granulocytes

as mean values for individuals tested 2–74 times, almost always in paired experiments. There was wide variation in the percentage of positive cells formed by normal adults. On exposure to 0.12% NBT, an average of 42% of granulocytes became formazan cells. Paradoxically, an average of 67% of cells were positive on exposure to 0.05% NBT. In each case there was a greater percentage of formazan cells after exposure to 0.05% NBT than after the higher concentration. The difference between the means of the paired values was highly significant \( (P < 0.001) \). Concentrations of less than 0.05% or greater than 0.12% failed to further alter the percentage of formazan cells.

In order to estimate the variability of the test at the two concentrations, 74 paired tests were done on the same normal adult man over a 6-month period. The higher concentration of NBT (0.12%) produced 41% ± 1.6 (SEM) formazan cells; the lower concentration of NBT (0.05%) produced 64% ± 2.2 (SEM) formazan cells.

A possible explanation for this phenomenon came from observing live cells microscopically at 37°, as they were perfused with NBT. Many of the cells treated with the higher concentration appeared rapidly to become smaller in area and thicker (see Fig. 2); those cells did not become formazan cells. This process occurred more slowly in cells treated with the lower concentration of NBT, or untreated [see (E) below].

As with 0.12%, granulocytes from patients with chronic granulomatous disease produced no typical formazan cells after NBT 0.05%, even after 60 min incubation.

(E) The effect of preincubation without NBT on the production of formazan cells (Fig. 5). When we preincubated normal granulocytes with the usual reagents

![Fig. 4. The effect of two different concentrations of NBT (0.12% and 0.05%) on the production of formazan cells by 13 normal adults and two patients with CGD. The mean results of a series of paired experiments for each subject are represented by connecting lines. Cells were not given particles and were exposed to the test media for 20 min.](image)
(see methods) except for NBT, and then reincubated these same cells with 0.12% NBT for an additional 20 min, there was a significant decrease ($P < 0.001$) in the percentage of formazan cells formed when compared with simultaneous controls without preincubation (Fig. 5). With longer preincubation there was further diminution in the percentage of formazan cells. This phenomenon was not related to cell death because these same preincubated cells were capable of NBT reduction in vacuoles surrounding ingested zymosan (see below). However, preincubation did cause a large number of the cells to become less spread out and to assume the form of the refractile cells in Fig. 2. These cells were then able to exclude NBT unless given zymosan particles to ingest.

(F) The effect of other variables on the production of formazan cells. There was no significant effect on the percentage of formazan cells when the following experimental conditions were altered: (a) Surface:—Substitution of a plastic slide for glass; (b) Temperature:—Varying incubation temperatures from $37^\circ$ to $22^\circ$; (c) pH:—Varying pH from 8.0 to 7.4; (d) Serum concentration:—Variant concentrations of pooled human sera (or autologous serum) from 36 to 11%; (e) Protein source:—Substitution of bovine serum albumin (1 drop) for pooled sera. (Some protein was necessary for good surface adherence of the cells); (f) Sodium cyanide:—Preincubation of cells with sodium cyanide, $1 \times 10^{-3}$ M, for 20 min.

II. Reduction of NBT by Phagocytizing Granulocytes: The Effect of Zymosan Particles (Fig. 6B).

When normal granulocytes were exposed to our test system with added zymosan particles, very different results were observed. An average of 95% of such cells contained a homogeneous blue material that appeared to be limited to the space immediately adjacent to the ingested particle (Fig. 6B). This vacuolar precipitation of formazan had been described in detail by others(7).

In the preparation with zymosan, typical formazan cells were markedly diminished in number. Although diffuse, granular precipitates would be difficult to
distinguish in cells stuffed with large zymosan particles, the nuclei, often clearly seen, were generally intact. For example, in nine paired experiments, an average of 42% typical formazan cells were present after NBT alone, but only 19% typical formazan cells were present after NBT and zymosan.

To exclude the possibility that NBT was combining with the zymosan particles and thus being removed from solution(7), we centrifuged zymosan particles from test media, and incubated the supernatant with normal granulocytes. This supernatant still produced a normal percentage of diffusely blue formazan cells.

Cells preincubated without NBT, and then incubated with NBT and zymosan, were still able to produce vacuolar formazan in almost all cells. This finding is in contrast to preincubated cells subsequently given NBT without zymosan; as

![Image](image_url)

**Fig. 6.** Three different formazan-containing granulocytes. (A) The formazan cell near an unaffected granulocyte (Safranin stain; phase microscopy); (B) Vacuolar formazan (arrow) adjacent to zymosan particles (Safranin stain); (C) Discrete collection of formazan (arrow) (Wright's stain).
noted above, that procedure significantly reduced the percentage of diffusely blue formazan cells.

Thus, the ingestion of zymosan appeared to limit NBT reduction to endocytic vacuoles, thereby protecting the leukocytes from the degeneration associated with diffuse cytoplasmic precipitation of formazan. This phenomenon may be related to decreased surface area of cells ingesting zymosan.

In granulocytes from patients with CGD, we confirmed the findings of others(7) that no formazan is visible about the ingested zymosan particles.

III. A Third Type of Formazan-Containing Cell

After incubating heparinized whole blood with NBT and without particles, Park et al.(2) and others(3,5), have described a formazan-containing cell unlike our formazan cells, or the cells with vacuolar formazan illustrated by Nathan et al.(7). Park’s cells are normal-appearing neutrophils containing discrete chunks of black (Wright’s stain) material. We have found cells fitting this description (Fig. 6C) by repeating Park’s method(2) or by examining leukocytes in shaking suspensions, but never in leukocytes isolated on glass.

DISCUSSION

The variety of Formazan-containing Cells in Different Systems

Almost all normal granulocytes have the capacity to reduce NBT if the dye can gain access to reactive sites within the cell. Since the number and appearance of cells producing formazan vary in different experimental systems, it seems likely that these differences are due to factors which influence permeability of the cell to NBT.

Nathan and his coworkers have concluded that phagocytosis of particles is necessary for NBT to enter the living cell(7,8). Using zymosan particles, they showed that the site of NBT reduction appeared to be within the endocytic vacuole, and postulated that the soluble NBT was swept into the cell at the time of particle ingestion(7). Within minutes, nearly 100% of the normal phagocytizing granulocytes or monocytes contained at least one blue-stained zymosan particle. We have confirmed these observations in cells adherent to glass (Fig. 6B). These cells are distinctive, as they are filled with large particles, many of which are outlined by a blue rim of formazan of variable thickness and density. Although granulocytes from patients with CGD also ingest zymosan readily, there is no visible formazan in the surrounding vacuole.

On the other hand, our studies provide evidence that under certain conditions, NBT can penetrate granulocytes in the absence of particles and be reduced to granular precipitates of formazan spread diffusely throughout the cytoplasm (Figs. 1A, 2, 6A). No exogenous substrate is necessary. These, which we have called “formazan cells,” have not been described by others. An average of 42% of normal living granulocytes sticking to a glass coverslip are transformed in this way. This reaction occurs primarily in cells that have a large part of their surface area adherent to the glass. This suggests that NBT may gain access to the cell as
it tries to "phagocytize" the glass surface; or that the cell membrane becomes more permeable to NBT as the cell thins and flattens itself against the coverslip. The diffuse precipitation of formazan apparently is often fatal to the adherent cell, causing nuclear degeneration and a relative inability to ingest particles(10). It is of interest that Humbert et al., in studying leukocyte adhesiveness on passage through glass beads, found that an average of 44% of normal granulocytes adhered to the glass after a 15-min exposure(14). This figure is remarkably close to the 42% of granulocytes that became formazan cells in our system, and provides additional evidence that the formation of these distinctive cells may be related to the relative "stickiness" of the cells to glass.

**The effect of preincubation without NBT**

Normal granulocytes preincubated without NBT, lose much of their ability to produce diffusely blue formazan cells when subsequently reincubated with NBT (Fig. 5). During the preincubation period there is a definite decrease in the average area of surface attachment which correlates with the decreased production of formazan cells. However, these same preincubated cells retain their ability for phagocytosis and for vacuolar deposits of formazan about ingested particles.

**The Paradoxical Effect of NBT Concentration**

The significant, inverse correlation between the concentration of NBT and the percentage of formazan cells produced may be explained by two observations. It has been shown by others that rat liver succinate-neotetrazolium reductase can be inhibited by increasing the concentration of neotetrazolium(15). Alternatively, direct observation of normal granulocytes at the time of exposure to NBT suggested that the higher concentration tends to decrease surface area of granulocytes with resultant cells that are less permeable.

Regardless of the mechanism, concentration of NBT in the test media appears to be an important factor when one is trying to compare results of different in-

| Author                  | Intracellular formazan distribution | Phagocytosis necessary | Associated with cellular degeneration | Final concentration NBT | Found in CGD |
|-------------------------|------------------------------------|------------------------|--------------------------------------|-------------------------|--------------|
| Gifford and Malawista(10) (also Figs. 1A, 2, 6A) | Diffuse, granular                  | No                     | Yes                                  | 0.12%–0.025%           | No           |
| Nathan et al.(7) (also Fig. 6B)                          | Vacular, homogeneous               | Yes                    | No                                   | 0.08%                  | No           |
| Park et al.(2) (also Fig. 6C)                             | Clumps                             | No                     | No                                   | 0.05%                  | No           |
vestigators (Table 1), and should be kept constant for studies of granulocytes from a series of patients.

**Diffuse Formazan Cells Vacuolar or Localized Formazan Deposits**

The deposits of formazan about ingested zymosan particles appear to be morphologically different from those in the nonphagocytizing formazan cells. The latter are diffuse and granular (Fig. 6A), while those about the ingested particles are localized and homogeneous (Fig. 6B). When adherent granulocytes ingest zymosan particles in the presence of NBT, the cell seems to be protected from the diffuse formazan precipitation and degeneration seen when such particles are not employed, perhaps because of sequestration of the reactants and their potentially lethal products in endocytic vacuoles, or because the process of phagocytosis has caused the cell to be less adherent to the glass surface. The granular appearance of the formazan deposits in the diffuse reaction might be due to its association with cytoplasmic granules. Neither cell occurs when granulocytes from patients with CGD are tested.

Park and his associates(2) as well as others(3,5), have produced a formazan-containing cell by smearing and staining incubated, heparinized whole blood to which NBT without particles has been added. They then determined the percentage of neutrophils containing “large black deposits” (Wright's stain) of formazan. Normal controls showed only about 8.5% of such cells. These cells were increased in patients with certain bacterial infections(2,3) and in newborn infants(4), but were absent in cells from patients with CGD(4). These formazan-containing cells do not resemble those seen when particles are used with isolated granulocytes(7), or the diffusely blue formazan cells described here. The formazan in Park's cells appears as discrete chunks of black material in a localized area of cytoplasm, without cellular degeneration (Fig. 6C). Some deposits have the appearance of material that has been ingested rather than formed within the cell. We have never seen these deposits in leukocytes isolated on glass. The increased percentage of these cells in certain types of bacterial infection may be due to an increased phagocytic capacity of blood leukocytes during infection. The percentage of our formazan cells is also usually increased in untreated bacterial infection(16).

**CONCLUSIONS**

Our observations suggest that the variability of NBT reduction by normal human granulocytes is a function of the test conditions. Permeability of the granulocyte to NBT may be the critical variant and can be altered by changes in NBT concentration, particle ingestion, and adherence to a surface. Reports which describe an increase or decrease in NBT reduction associated with certain clinical disorders(2–6) need not be interpreted to represent a stimulation or depression of the enzyme system(s) necessary for NBT reduction (or bactericidal capacity) as in CGD.
SUMMARY

We have compared nitroblue tetrazolium (NBT) reduction by granulocytes adherent to glass with that described by others in whole blood or by isolated granulocytes in suspension. After standard treatment, live granulocytes were rapidly transformed into large, distinctive, degenerated cells diffusely laden with blue formazan precipitate ("formazan cell"). The ability to become formazan cells was directly related to the degree of spreading of the granulocytes on the glass surface. These changes were inversely related to the concentration of NBT and to the length of preincubation before exposure to NBT. The reaction was insensitive to cyanide, did not require the ingestion of particles, and was absent in granulocytes from patients with chronic granulomatous disease of childhood (CGD).

When normal granulocytes were presented zymosan particles under these conditions, almost all cells contained formazan limited to vacuoles surrounding the ingested particle; whereas there were many fewer "formazan cells." If heparinized whole blood was incubated with NBT without particles, however, a small percentage of granulocytes contained the discrete collections of formazan described by others.

The diverse morphologic results of these various techniques may be related to the way in which NBT enters the cell. In our system, the more adherent cells are able to admit and reduce NBT diffusely throughout the cytoplasm. In contrast, when large particles are phagocytized, NBT is swept in with the particle, and is reduced primarily in the phagocytic vacuole. Our observations suggest that alterations in the production of formazan by human granulocytes in many clinical states may be due in part to changes in cell permeability to NBT rather than to changes in enzymatic activity as is clearly the case in CGD.

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