Ultracytochemistry for Detection of O_2^- in Polymorphonuclear Leukocytes Using the Tetrazolium Method: Comparison of Various Tetrazolium Salts

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The reduction of nitroblue tetrazolium (NBT) produces diformazan which shows localization of superoxide anion (O_2^-) in polymorphonuclear leukocytes (PMNs). The detection of O_2^- in PMN is performed exclusively by using a microscopic NBT test, while an ultracytochemical method in NBT has seldom been employed. The present study compared various tetrazolium salts, including nitroblue tetrazolium (NBT), tetranitroblue tetrazolium (TNBT) and 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl) tetrazolium chloride (BSPT), by detecting O_2^- using a histochemical and an ultracytochemical method. Peripheral rabbit leukocytes which ingested opsonized zymosan were prepared for detection of O_2^- . The reacted leukocytes in each tetrazolium salt contained a reaction medium which was examined by

light and electron microscopy. Each reaction of the tetrazolium salts took place in the phagosomal membrane. The color of formazan in the TNBT was black rather than dark purplish-blue, and darker than that of NBT. On the other hand, the reaction color of BSPT was purplish-red. The reaction formazans of NBT and TNBT were coarse and electron-dense by ultracytochemistry. Some of these diformazans were not associated with the membrane, while the formazan of BSPT demonstrated fine deposits and was electron-dense. These reaction products corresponded well with the membrane. These results suggest that BSPT could be employed for detection of O_2^- not only in the histochemical method but also in ultracytochemistry and can be used as a new method.

Key words: Polymorphonuclear leukocytes, Superoxide anion, NBT, TNBT, BSPT

I. Introduction

Numerous attempts have been made to demonstrate that the superoxide in polymorphonuclear leukocytes (PMNs) phagocytose foreign substances such as bacteria, opsonized zymosan and lipopolysaccharide.

Superoxide anion, O_2^- , has been easily detected by the histochemical method using nitroblue tetrazolium (NBT). NBT reduction has been widely used in diagnostic tests since Nathan [3, 21] developed the technique to discriminate patients with chronic granulomatous disease. Many investigators have improved the histochemical method with NBT. However, there are few reports in ultracytochemistry using NBT. Since the diagnosis needs

to discriminate the symptoms quickly and easily, a light microscopy method may mainly be used. Only Segal [26] and Briggs *et al.* [5] employed this method in ultracytochemistry to observe the localization of O_2^- which PMNs produce during phagocytosis. Peskin *et al.* [23], used tetranitroblue tetrazolium (TNBT) and demonstrated the ability to detect the reactive oxygen species in the stimulated macrophage with opsonized candida albicans by the ultracytochemical method.

The NBT method is the only method which can directly detect O_2^- in histochemistry. However, there are some differences in the characteristics of the reaction products of NBT reduction between investigators [5, 13, 26]. Moreover, the formazans of both NBT and TNBT produce an amorphous material. On the other hand, 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl) tetrazolium chloride, BSPT, another kind of tetrazolium salt, has been mainly used for the detection of succinate dehydrogenase [15], the reduction of which demonstrates an osmiophilic formazan and is electron-dense.

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In the present study, we compared three kinds of tetrazolium salts, NBT, TNBT and BSPT, and demonstrated O_2^- using electron microscopy, and devised a new method.

II. Materials and Methods

Polymorphonuclear leukocytes (PMNs) were prepared from rabbit peripheral blood using a 6% dextran sedimentation technique. Granulocytes were separated with blood cells by suspension in a 0.2% saline solution followed 30 seconds later by an additional equal volume of 1.6% saline and then subjected to centrifugation at $120 \times g$. This hypotonic lysis of erythrocytes was repeated twice. The leukocytes were then suspended in Hanks' balanced salt solution (Hanks' BSS), without phenol red, pH 7.5. The concentration of each tetrazolium salt was as follows: NBT: 0.005 mg/ml–0.125 mg/ml, TNBT: 0.025 mg/ml, BSPT: 0.0125–0.125 mg/ml. Both NBT and TNBT were dissolved in Hanks' BSS, and BSPT was dissolved in 0.1 ml dimethylformamide and then was diluted in Hanks' BSS.

Opsonized zymosan was prepared as follows: Ten mg zymosan A was incubated in 1 ml fresh rabbit serum for 30 min at 37°C and then was centrifuged and washed in Hanks' BSS.

The complete incubation medium consisted of opsonized zymosan, 10 mM azide, PMNs and Hanks' BSS with an additional 1.0 mg/ml of glucose. Each of the tetrazolium salts was then incubated for 15 min at 37°C.

The suspended reacted PMNs with tetrazolium salts were smeared on a glass slide. Thereafter, a routine light microscopic procedure was performed.

Electron microscopy

The reacted cells were briefly washed in a phosphate buffer at pH 7.5 three times, and then were fixed in 2.5% glutaraldehyde in phosphate buffer for 1 hr. Thereafter the cells were postfixed with 1% OsO_4 and dehydrated in a graded ethanol series and propylene oxide, and

then embedded in Epon 812. One μm semithin section was prepared for light microscopy.

Controls

The exclusion of opsonized zymosan from the incubation medium containing PMNs was employed. NAD(P)H oxidase inhibitor, diphenyleneiodonium chloride (DPI, 10 μM) [22] was added to the reaction medium. P-benzoquinone (1 mM) [27] was also examined for the inhibition test.

These control tests were performed using the NBT and BSPT methods.

To prevent the diffusion of NBT diformazan, we employed 2% polyvinyl pyrrolidone (PVP) to overcome a deficiency of the NBT method.

III. Results

Light microscopy

When the PMN phagocytosed the opsonized zymosan, the NBT reduction appeared blue around the phagosomal membrane. The concentration of NBT was 0.025 mg/ml. The semithin section embedded in Epon resin completely kept the diformazan (Fig. 1). The precipitate of the formazan of NBT was scattered in the cytoplasm of the zymosan. In contrast, as compared to the NBT method, the TNBT method demonstrated a darker blue at the concentration of 0.0125 mg/ml (Fig. 2). Although the reacted color with the NBT or TNBT methods was the same between the light microscopic procedures and the semithin sections, in the case of BSPT, it was different. The reacted color of BSPT in semithin sections was brown. However, the formazan of BSPT in the light microscopic procedure showed purplish-red around the phagocytic vacuole at the concentration of 0.025 mg/ml (Fig. 3). When the concentration of BSPT was reduced to 0.0125 mg/ml, the reaction products were apparently diminished even though the precipitates were observed (Fig. 4).

Fig. 1. Light microscopic photograph of PMN ingested opsonized zymosan. Reduced NBT produced diformazan which appeared purplish-blue on the phagosomal membrane in the ultrasemithin section (arrows). The color of diformazan was the same as seen in the light microscopic material.

Fig. 2. The reaction product of TNBT showed black rather than dark-blue by light microscopy (arrows). The reacted PMN were suspended, and then thin smears were made on glass slides and observed under light microscopy. The extra-cellular zymosan was not stained.

Fig. 3. In the case of BSPT, the color of the formazan appeared purplish-red around the phagosomal membrane (arrows). The procedure was done in the same manner as TNBT.

Fig. 4. When the concentration of BSPT was 0.0125 mg/ml, the reaction products diminished dramatically (arrows).

Fig. 5. Electron microscopy revealed the formazan of NBT which accumulated in the phagosomal membrane (arrows). Some formazan precipitated into granules.

Fig. 6. The formazan of NBT exhibited dot-like reaction products. The deposits had an adequate electron-density, but diffusion of formazan was occasionally observed.

Fig. 7. The reaction products of TNBT (0.025 mg/ml) were associated with the phagosomal membranes. However the "blobs" of reaction products were observed in the ingested zymosan (arrows).

Fig. 8. The formazan of BSPT (0.025 mg/ml) on the phagosomal membrane and on the cell to cell contact demonstrated fine deposits and electron-density (arrows).

Electron microscopy

When the PMN engulfed zymosan was stained with the NBT method, the diformazan precipitated on the phagosomal membrane as a dotted, amorphous material which was electron-dense by electron microscopy. These reaction products were localized mainly within the

phagosomal membrane (Fig. 5). Fig. 6 also exhibited the reaction products in the NBT method. Both Figs. 5 and 6 demonstrated the reaction products which were electron-dense, but a diffusion of formazan was sporadically observed. In the case of the TNBT method, the reaction products in which PMN engulfed the zymosan were coarse

Figs. 1-8

and electron-dense (Fig. 7). In contrast to the NBT and TNBT, the formazan of BSPT was fine and possessed an adequate electron-density at the concentration of 0.025 mg/ml (Fig. 8). When the concentration of BSPT was increased to 0.125 mg/ml, most of the PMNs developed a disrupted membrane, and were necrotic due to the cytotoxicity of BSPT. When the concentration of BSPT was decreased to 0.05 mg/ml, fine reaction products were observed, but some blobs of the reaction product appeared in the phagosomal membrane (Fig. 9).

The permeability of the reaction products in BSPT was confirmed by serial semithin and ultrathin sections. Serial sections showed the continuities of formazan of BSPT which O_2^- caused in eosinophils, and kept the localization of the reaction products (Fig. 10a, b).

Control and inhibitor

Ten μ M of DPI, an inhibitor of NADH oxidase [22], completely inhibited the reaction products (Fig. 11). The other inhibitor, p-benzoquinone also inhibited the reaction products (Fig. 12). Unstimulated PMN were free of reaction products in each case of tetrazolium (data not shown).

Characteristics of BSPT and improvements for the NBT method

The problem with tetrazolium salts is that they have a tendency for the formazan to easily diffuse. To confirm this, the reacted production with BSPT, which was fixed in 2.5% glutaraldehyde, followed by immersion in phosphate buffer, pH 7.5, was kept in the refrigerator for 3 weeks. The result showed that there was no fading or diffusion in the phagosomal membrane with BSPT. The color of the reaction products remained purplish-red (data not shown).

The NBT method is very popular and widely used. This study indicates a problem in that the reaction products were coarse. We, therefore, tried to prevent this problem. When 2% PVP was added to the NBT reaction medium, fine reaction products appeared, in spite of the reduced density (Fig. 13).

IV. Discussion

In the present study, each of the tetrazolium salts was sufficiently electron-dense to observe the localization of the reaction products by electron microscopy even after

the embedding procedure was performed. The method of BSPT confirmed that the reaction deposits were fine and had permeability into the tissues. Briggs *et al.* [5] emphasized that the reaction products of NBT have a low density by electron microscopy. On the other hand, Segal *et al.* [26] demonstrated that neutrophils exposed to endotoxin had the electron-dense diformazan of NBT. Furthermore, Peskin *et al.* [23] described that TNBT had adequate electron-dense reaction products in stimulated macrophages. These differences are considered to be due to the preparation of the PMN and the consistency of the incubation medium.

Our results were also in agreement with Peskin's results [23], the reaction products of TNBT had an adequate electron-density, but the blob that Briggs [5] indicated was also observed. Furthermore, the localization of reaction products was limited to the phagosomal membrane, but didn't show the reaction deposits on the whole surface of PMN. It should be pointed out that when PMNs adhered to the coverslip, the PMNs were stimulated and the NBT reduction increased [16, 19]. However, our present study demonstrated that the PMNs that ingested the opsonized zymosan were seldom stained around the whole of their surfaces with tetrazolium salts, but were stained only locally at the phagosomal membrane. Many investigators demonstrated that the formazan deposits of reduced tetrazolium were mainly in the phagocytic vacuoles. Even if the incubation time of the reaction is prolonged, the reaction products localize on the phagosomal membrane, but not the plasma membrane. However, when NAD(P)H was added to the incubation medium, the reaction rapidly progressed, followed by an increment of NBT reduction. Some PMNs exhibited diformazan on the plasma membrane (data not shown). Briggs *et al.* [4] demonstrated that when exogenous NADH was added to the cerium reaction medium, the cytochemical reaction products increased and located on the plasma membrane. Manes [18] also demonstrated that exogenous NAD(P)H caused the appearance of reaction products on the plasma membrane of rabbit blastocyst using the cerium method. Cohen *et al.* [6] suggested that membrane-enriched particles (podosomes) have a NAD(P)H dependent O_2^- production. This podosome is located in the plasma membrane. Nakamura [20] showed that exogenous NADPH raised O_2^- consumption in the phagocytosed PMN when the permeability was improved. Tanaka [28]

Fig. 9. When the concentration of BSPT was 0.05 mg/ml, the fine reaction product was found within the phagocytic vacuole, but the "blobs" appeared in the phagosomal membrane (arrows).

Fig. 10. a, b: Serial ultrathin sections were made. The eosinophil engulfed zymosan showed reaction products within the phagosomal membranes. These two photographs demonstrate the permeability of BSPT. Note that the reaction products of extra cellular debris occasionally were stained.

Fig. 11. Effect of DPI (10 μ M), an inhibitor of NADH oxidase. No reaction deposits of BSPT were found (arrows).

Fig. 12. Effect of p-benzoquinone. Reaction deposits were absent in the phagosomal membrane in PMN (arrows).

Fig. 13. Improvement of the NBT method. PVP added to reaction medium prevented the diffusion of reaction product except for a reduction in electron-density.

Figs. 9-13

suggested that NADPH oxidase is located on the outside surface of the plasma membrane, and that the added NAD(P)H was rapidly oxidized. In contrast to this report, Yamaguchi [29] suggested that the NADPH binding site is located in the inside of the plasma membrane. Liang *et al.* [17] suggested that NAD(P)H is translocated to the phagosome prior to oxidation and that NAD(P)H, including its sources and/or carriers, accumulate near the phagosome. As NAD(P)H is not permeable to membranes, our findings suggest that when exogenous NAD(P)H is added to the reaction medium, PMN interacts with NAD(P)H as an electron donor [1, 2], resulting in the appearance of reaction products on the plasma membrane. In fact, some PMNs without stimulated zymosan showed blue formazan in the presence of NAD(P)H.

Concentration of tetrazolium salts

All of the tetrazolium salts, NBT, TNBT and BSPT, precipitate reaction deposits, depending on their concentration. When the concentration of NBT was increased, non-specific reaction deposits appeared as well as TNBT or BSPT. However, when the concentration of NBT was decreased to 0.0125 mg/ml, the amount of reacted cells was dramatically reduced and weakened reaction products appeared. With the concentration of NBT at 0.05 mg/ml, there was no staining in the background, e.g., opsonized zymosan, lymphocyte, platelet, etc.. Resting PMN showed no reactions. In addition, the number of positively reacted cells increased more than at the concentration 0.025 mg/ml of NBT. Babior [1] and Digregorio [8, 9] suggested that the rate of O_2^- production increases as the NBT concentration increases. Many investigators used a NBT concentration of more than 0.25 mg/ml. Hirai *et al.* [11] employed a NBT concentration of 0.025 mg/ml to avoid artificial production. Gifford *et al.* [10] found that a lower concentration of NBT produced more positive cells than did a higher concentration of it. Thus, it is difficult to determine the appropriate concentration of NBT.

Either way, our findings suggest that the suitable concentration of NBT is between 0.025 mg/ml and 0.05 mg/ml.

Since the color of the formazan of TNBT was a dark-blue, which appeared almost black at a concentration of 0.025 mg/ml, it may be suitable to use a concentration of 0.025 mg/ml in TNBT. On the other hand, the present concentration of BSPT used was 0.025 mg/ml–0.05 mg/ml. This concentration of BSPT was almost one or two-tenths, and was used in the detection of succinate dehydrogenase (SDH) [15]. These investigators employed a paraformaldehyde fixation and preserved the membrane structure. However, we used living cells. Our present study demonstrated that a higher concentration of BSPT caused the cytotoxicity. However, more recently, Rothe *et al.* [25] reported NADH-diaphorase (NADH-d) using BSPT at a concentration of 0.2–0.4 mM instead of the

NBT method. Hope *et al.* [12] initially adapted BSPT for NADH-d detection in electron microscopy. Following this report, many investigators have gradually employed the BSPT method for NADH-d due to the electron-dense reaction product. On the other hand, however, other authors [24] support the suitability of NBT for NADH-d detection. They show adequate electron-dense reaction products from NBT in NADH-d. In fact, we observed electron-dense diformazan of NBT in PMN. Therefore, it seems that the nature of formazan in tetrazolium salts shows many characteristics. Because the results obtained from the same tetrazolium salt differed between investigators, it is suggested that the techniques employed and the use of tetrazolium were different.

Although NBT contains this fault, this method was the most available tetrazolium salt, which contains TNBT and BSPT, in the laboratory. Thus, we tried to overcome this deficiency of the NBT method. To avoid diffusion artifacts in enzyme histochemistry, several procedures have been developed. One of them, polyvinyl alcohol (PVA) was used [14]. However, the concentration of NBT will be raised with PVA. We, therefore, could not adopt it. PVP was used to prevent the diffusion of lactate dehydrogenase. Dahl *et al.* [7] employed 7.5% PVP, which seemed to have a high viscosity, so we reduced the concentration of PVP to 2%. Our findings showed that PVP prevented the diffusion of diformazan except in weaker reaction products. As Dahl mentioned [7], the reaction product reduced the electron-density, although the reason for this was obscure. We will investigate further to overcome this deficiency and devise a new method.

In conclusion, each reaction of the tetrazolium salts was produced in the phagosomal membrane. The color of the reaction products in all the tetrazolium salts remained in semithin sections. The reaction formazans of NBT and TNBT were coarse and electron-dense. On the other hand, the reaction products of BSPT were fine and electron-dense. Our present results suggest that the BSPT method is convenient for the detection of O_2^- in ultracytochemistry. We will further improve upon this method, including the NBT and the TNBT methods, and make a useful tool for the detection of superoxide anion.

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VI. References

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