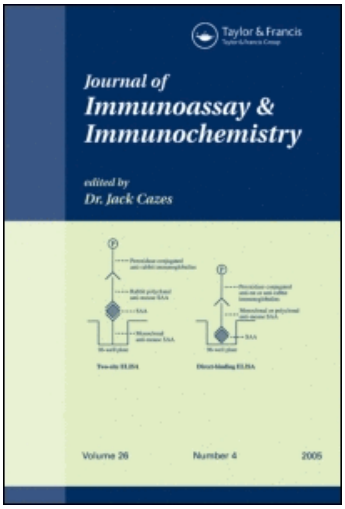

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Hyung Sim Choi[a]; Jun Woo Kim[a]; Young-Nam Cha[a]; Chaekyun Kim[a]
[a] Laboratory of Cell Signaling, Inha University College of Medicine, Incheon, Korea

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A Quantitative Nitroblue Tetrazolium Assay for Determining Intracellular Superoxide Anion Production in Phagocytic Cells

Hyung Sim Choi, Jun Woo Kim, Young-Nam Cha, and Chaekyun Kim
Laboratory of Cell Signaling, Inha University College of Medicine, Incheon, Korea

Abstract: Conventionally, a semi-quantitative microscopic nitroblue tetrazolium (NBT) assay is used to determine the production of superoxide anion (O_2^-) in various phagocytic cells. This microscopic assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by reduction of the membrane permeable, water-soluble, yellow-colored, nitroblue tetrazolium (Y-NBT) by O_2^-. However, this assay is semi-quantitative and is prone to observer bias. In the present study, we modified the NBT assay by dissolving the blue formazan particles using 2 M potassium hydroxide and dimethylsulfoxide and then measured its absorbance using a microplate reader at 620 nm. The absorbance of dissolved NBT increased in proportion to cell number ($r = 0.9907$), incubation time, and stimulus concentration. To test the usefulness of this modified assay, we compared the abilities of a number of types of phagocytic cells to produce O_2^-. The cells examined included murine macrophage cell lines (RAW 264.7 and J774), freshly prepared murine peritoneal macrophages and neutrophils, a human myeloid cell line (PLB-985), and freshly prepared human peripheral blood neutrophils. In addition, we demonstrate that nitric oxide produced by RAW 264.7 cells does not interfere with the modified colorimetric NBT assay. Taken together, our results indicate that the modified colorimetric NBT assay is simple, sensitive, and quantitative, and that it can be used to determine the amounts of intracellular O_2^- produced by phagocytic cells. Thus, this assay is sensitive enough to measure,

Address correspondence to Chaekyun Kim, Laboratory of Cell Signaling, Inha University College of Medicine, 7-206 3rd St, Shinheung-dong, Jung-Gu, Incheon 400-712, Korea. E-mail: chaekyun@inha.ac.kr

quantitatively, even the small amounts of O_2^- produced in monocytes and macrophages that are not detectable by the conventional microscopic NBT assay.

Keywords: Nitroblue tetrazolium (NBT), Superoxide anion, Nitric oxide, Phagocytes, RAW 264.7 cells, PLB-985 cells, Phorbol myristate acetate

INTRODUCTION

Professional phagocytes undergo NADPH oxidase catalyzed oxidative respiratory burst in response to various stimuli and, as a result, produce the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). These reactive oxygen species serve as inflammatory mediators and play important roles in host defense by killing invading pathogens,[1–3] and they also induce a variety of antioxidant enzymes in host cells. Many techniques are available for detection of O_2^- for basic research and clinical diagnostic purposes.[4] For example, extracellularly released O_2^- can be measured by spectrophotometric superoxide dismutase (SOD)-inhibitable ferricytochrome c reduction, peroxidase-dependent isoluminol chemiluminescence, and lucigenin-amplified chemiluminescence assays. Alternatively, to determine intracellular O_2^- production, one could use either microscopic NBT assay, fluorometric assays (like diaminobenzidine (DAB), 7-dichlorofluorescein (DCF), dihydrorhodamine (DHR), and homovanillic acid (HVA) oxidation assays), the peroxidase-dependent luminol-amplified chemiluminescence assay, or the oxidation of Fc OxyBurst. Among these assays, ferricytochrome c reduction and chemiluminescence assays that employ isoluminol or lucigenin enable the kinetic studies of O_2^- production to be easily followed; however, these two methods are relatively insensitive. Moreover, the conventional NBT assay and the fluorometric DAB oxidation assay are simply performed, but results are only semi-quantitative. In addition, the results obtained using these methods, alone or in combination, depend on cell types, the nature of stimulus applied, and on the locations and amounts of O_2^- produced.

The abilities of phagocytic cells to produce O_2^- in response to a given stimulus vary depending upon cell types, and the animal species from which the cells originate. For example, the O_2^- producing capacity of various human phagocytes in response to a given dose of N-formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbol 12-myristate 13-acetate (PMA) varies, in the order, peripheral eosinophils $>$ neutrophils $>$ monocytes $\approx$ alveolar macrophages.[5] During phagocytosis, murine alveolar macrophages produced much larger amounts of O_2^- than peritoneal macrophages, while guinea pig peritoneal macrophages and neutrophils produced larger amounts of O_2^- than alveolar macrophages.[6] In general, monocyte and macrophage cell lines are known to produce only small amounts of O_2^-, at levels that are not easily measured. Thus, though the ferricytochrome c reduction assay is most commonly used to measure

extracellular O_2^- released by eosinophils or neutrophils, the assay is not sensitive enough to detect the O_2^- released by monocytes or macrophages. In particular, this assay is not useful for detecting O_2^- production by macrophage cell lines like RAW 264.7 or J774. Thus, variations in O_2^- producing capacity of cells compounded with the limited sensitivity of available detection methods, cause difficulty in understanding the roles of O_2^- produced in different cell types.

The conventional microscopic NBT assay is used to qualitatively detect the O_2^- produced in phagocytic cells. The method is commonly used in clinics to diagnose chronic granulomatous disease (CGD), an immunologic disease caused by a reduced oxidative burst[7–11] and, thus, a limited ability to kill phagocytosed bacteria.[12,13] In the conventional NBT assay, cells incubated with membrane permeable yellow-colored nitroblue tetrazolium (Y-NBT) are stimulated with various stimuli, bacterial lipopolysaccharide (LPS), PMA, or FMLP. Y-NBT absorbed by the cells is reduced to water-insoluble blue formazan particles (NBT) by intracellular O_2^-, and the number of cells containing the reduced blue formazan deposits are counted microscopically. However, the conventional microscopic NBT assay has numerous limitations. First, only small amounts of blue formazan are formed when a small amount of O_2^- is produced, which makes the microscopic evaluation difficult. Second, the visual results obtained are qualitative or semi-quantitative at best, and do not accurately reflect quantitative O_2^- production. Third, visual interpretation is subject to observer bias. And fourth, the method is difficult to standardize; for example, the reduction of Y-NBT to NBT is highly sensitive to the presence of electron donor species like O_2^-. To avoid these technical disadvantages, spectrophotometric methods have been developed which measure the absorbance of cells containing NBT deposits (densitometric NBT assay) or the absorbance of NBT dissolved in organic solvents (colorimetric NBT assay).[14–17]

In the present study, we modified the previously developed colorimetric NBT assays.[16,17] The formazan particles were dissolved with 2 M potassium hydroxide (KOH) and dimethylsulfoxide (DMSO) and the absorbance was determined using a microplate reader. Using this modified colorimetric NBT assay, we compared the O_2^- producing abilities of various phagocytic cells obtained from human peripheral blood and murine peritoneum under various conditions. The results suggest that the modified colorimetric NBT assay is sensitive, quantitative, and convenient, and is able to detect even the small amounts of O_2^- produced by weak O_2^- producers like RAW 264.7 cells.

EXPERIMENTAL

Reagents

RPMI 1640 medium, Dulbecco's Modified Eagle's medium (DMEM), phosphate buffered saline (PBS), and Hanks' balanced salt solution (HBSS)

were purchased from GibcoBRL (Grand Island, NY); fetal bovine serum (FBS) was from HyClone (Logan, UT). The other chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO). Microscope cover glasses were from Fisher Scientific (Pittsburgh, PA), tissue culture plates (12-, 24-, and 96-well) from TPP (Trasadingen, Switzerland), and syringes and vacutainer tubes containing EDTA from Becton Dickinson (Franklin Lakes, NJ). All reagents were low endotoxin or endotoxin-free grades.

Isolation of Murine Peritoneal Cells

Mouse peritoneal cells were isolated from C57BL/6J mice (Jackson, Bar Harbor, ME). Mice were housed in microisolator cages under specific pathogen-free conditions and were fed autoclaved food and water *ad libitum*. Mice used in these experiments were 8–10 weeks of age. Macrophages and neutrophils were purified from peritoneal exudates as previously described.[18] Briefly, mice were injected, *i.p.,* with 1 mL of 3% thioglycolate and peritoneal cells were harvested 18 hr or 96 hr later for neutrophil or macrophage isolation, respectively. Cells were obtained by peritoneal lavage using 10 mL of cold HBSS at the indicated times following thioglycolate administration. Harvested cells were washed twice with cold PBS and cell numbers were assessed using Diff-Quick (Dade, Miami, FL). Cells isolated from peritoneal exudates were cultured in DMEM containing 100 U/mL of penicillin and 100 μg/mL of streptomycin for 1 hr at 37°C in 5% CO_2.

Preparation of Human Neutrophils

Human neutrophils were separated from the peripheral venous blood obtained from healthy volunteers, as approved by the Institutional Review Board at Inha University College of Medicine. Blood was taken into a Vacutainer tube containing 15% EDTA and layered onto 10 mL of Polymorphprep (Accurate Chemical and Scientific, Westbury, NY) in a 50 mL conical tube. After centrifugation at 400 × g for 30 min, cell pellets were harvested. Red blood cells were lysed by hypotonic lysis and cells were resuspended in DMEM containing 100 U/mL penicillin and 100 μg/mL streptomycin and cultured in 24-well culture plates for 1 hr at 37°C in 5% CO_2.

Cell Lines and Culture

The murine macrophage cell lines (RAW 264.7 and J774) were obtained from American Type Culture Collection (Manassas, VA). The human myeloid cell line (PLB-985) was a gift from M. Dinauer (Indiana University).

RAW 264.7 and J774 cells were grown in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2. PLB-985 cells were grown in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO_2. To induce granulocytic differentiation, PLB-985 cells (2×10^5 cells/mL at starting density) were exposed to 0.5% N,N-dimethylformamide (DMF) for 5 days.

Conventional Microscopic NBT Assay

Cells (5×10^5/well) were suspended in 0.4 mL DMEM and allowed to adhere to cover glasses placed in a 12-well flat bottomed tissue culture plate for 1 hr (prior to use, the cover glasses were disinfected with 1 N HCl for 10 min and washed with PBS). Cells adhering to cover glasses were treated with 100 μL of saturated Y-NBT solution containing 300 ng/mL of PMA for 20 min. As negative controls, some cells were incubated with specific inhibitors of NADPH-oxidase activity, namely, 5 μM diphenyleneiodonium (DPI, a heme-ferryl containing protein inhibitor) or 30 μg/mL SOD (a potent enzyme which catalyzes the dismutation of O_2^- to H_2O_2 and O_2). After incubation, cells were washed twice with warm PBS, air-dried, fixed with methanol, and counter-stained with 1% safranin O solution. The percentage of cells containing blue formazan particles (NBT-positive cells) was determined by evaluating 100 randomly selected cells under a microscope (Olympus CX-40, Olympus, Japan).

Modified Colorimetric NBT Assay

The proportionalities of the results were obtained using the modified colorimetric NBT assay, with respect to cell numbers, and the doses and durations of PMA stimulation. Different numbers of cells attached to a 24-well culture plate were incubated with 100 μL of Y-NBT solution containing PMA (300–600 ng/mL) for various times (20–60 min). As negative controls, some cells were incubated in Y-NBT solution containing PMA with 5 μM DPI or 30 μg/mL SOD. After incubation, cells were washed twice with warm PBS, then once with methanol, and air-dried; it is critical that extracellular Y-NBT be completely removed. The NBT deposited inside the cells were then dissolved, first by adding 120 μL of 2 M KOH to solubilize cell membranes and then by adding 140 μL of DMSO to dissolve blue formazan with gentle shaking for 10 min at room temperature. The dissolved NBT solution was then transferred to a 96-well plate and absorbance was read on a microplate reader at 620 nm (Power Wavex 340, Bio-Tek Instruments, Winooski, VT).

Nitrite Assay

To determine whether the NO produced in cells stimulated with PMA or LPS interferes with the results of the modified colorimetric NBT assay, the production of NO was measured in parallel with O_2^- production in RAW 264.7 cells. RAW 264.7 cells (2.5×10^5/well), seeded in a 96-well plate, were stimulated with either PMA (600 ng/mL) or LPS (1 μg/mL) for 30 min or 20 hr, respectively. As negative controls, 5 μM DPI or 0.5 mM aminoguanidine (AG) was added. To determine NO production, 100 μL of the culture medium was reacted with 100 μL of Griess reagent (1% sulfanilamide, 0.1% napthylenediamine dihydrochloride, and 5% o-phosphoric acid) for 10 min. Absorbance was measured at 550 nm using a Power Wavex 340 ELISA reader.

SOD-Inhibitable Cytochrome C Reduction Assay

Extracellular O_2^- production was measured based on SOD-inhibitable reduction of ferricytochrome c.[18] Briefly, cells were suspended in 250 μL PBSG (PBS with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 7.5 mM glucose) containing 75 μM ferricytochrome c and were activated by adding 200 ng/mL PMA. SOD (180 units/mL) was added to parallel samples to measure the SOD-inhibitable values. After incubation for 30 min at 37°C, the absorbance at 550 nm with a 490 nm reference filter was measured on a Power Wavex 340 ELISA reader.

Statistical Analysis

The two-tailed Student's t-test (paired) was performed using Microsoft Excel (Redmond, WA). Data are expressed as mean ± SD and a p value < 0.05 was considered significant.

RESULTS

Conventional Microscopic NBT Assay

Using the conventional microscopic NBT assay, we determined the percentage of NBT-positive cells in RAW 264.7 and PLB-985 cells after stimulation with PMA. 52% of PMA-stimulated RAW 264.7 cells were found to contain faint blue formazan particles, whereas all PLB-985 cells contained intense formazan deposits (Table 1). The addition of DPI blocked PMA-elicited blue formazan formation completely in both cell types. However, the addition of SOD, which barely penetrates the cell membrane, partially inhibited the intracellular production of blue formazan. These results are

Table 1. Percentage of NBT-positive RAW 264.7 cells and PLB-985 cells[a]

	RAW 264.7	PLB-985
No stimulus	0 ± 0	4 ± 7
PMA	52 ± 13	100 ± 0
PMA + SOD	42 ± 7	100 ± 0
PMA + DPI	0 ± 0	0 ± 0

[a]NADPH oxidase activity was evaluated in individual neutrophils using the NBT test. Shown are the percentage of NBT-positive cell (mean $\pm$ SD, n = 7 for RAW 264.7 and n = 3 for PLB-985).

consistent with those of others.[15,18] The microscopic photos shown in Fig. 1 indicate that only a small amount of O_2^- was produced (faint blue color) in PMA-stimulated RAW 264.7 cells, but that a substantial amount was produced in PLB-985 cells, thus demonstrating the disadvantages of the conventional microscopic NBT assay.

Modified Colorimetric NBT Assay

Some phagocytic cells produce insufficient O_2^- to be detected extracellularly, which causes some difficulties when attempting to detect O_2^- production using the microscopic NBT or ferricytochrome c reduction assays. To overcome these difficulties, a colorimetric NBT assay, based on dissolving blue formazan particles with organic solvents, has been developed, and was used to measure the intracellular production of O_2^- in human macrophages

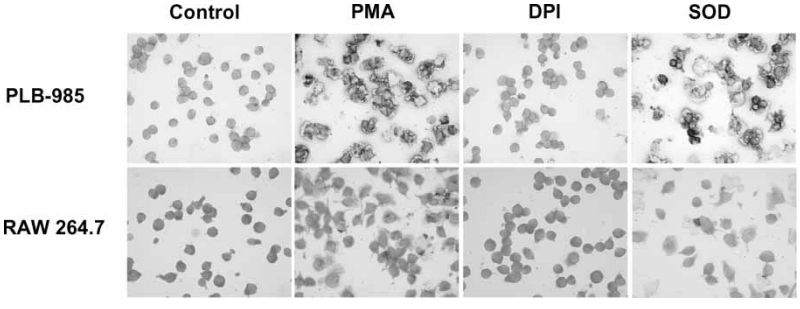

Figure 1. Detection of superoxide anion production using microscopic NBT assay. RAW 264.7 cells and PLB-985 cells attached to chamber slides were stimulated with 600 ng/mL PMA in the presence of Y-NBT for 20 min at 37°C. Attached cells were washed with PBS and stained with safranin O. One representative photo from three independent experiments is shown.

treated with γ-interferon,[16] and in bovine polymorphonuclear leukocytes ingesting group B *Streptococci*.[17]

To determine the sensitivity and quantitative ability of our modified colorimetric NBT assay, we examined the O_2^- produced in PMA-stimulated RAW 264.7 cells, a weak O_2^- producing cell line. The results shown in Fig. 2 demonstrate that the O_2^- produced in RAW 264.7 cells in response to PMA-stimulation increased in proportion to cell numbers (Fig. 2a), the duration of stimulation (Fig. 2c), and with the concentration of PMA (Fig. 2d). Moreover, the correlation between the number of cells and the absorbance of dissolved NBT was linear ($r = 0.9907$) (Fig. 2b), and variations between triplicate samples were small. We standardized the assay by using neutrophils (1×10^5 cells) and macrophages (2.5×10^5 cells) stimulated with PMA (600 ng/mL) for 30 min and 45 min, respectively. In establishing these assay, we considered the relationship between optimal absorbance and

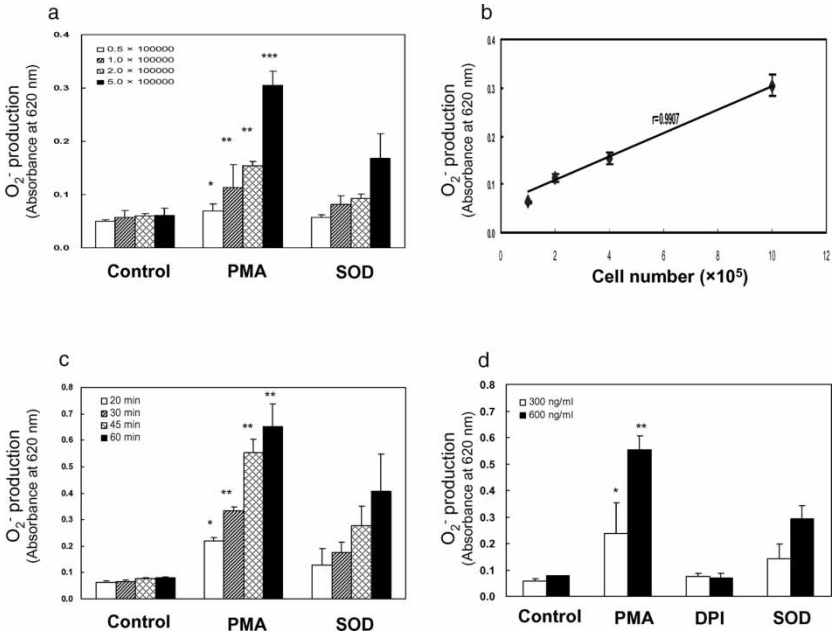

Figure 2. Measurement of intracellular superoxide anion production using the modified colorimetric NBT assay. (a) Different numbers of RAW 264.7 cells were incubated with Y-NBT containing 600 ng/mL PMA for 45 min ($n = 5$). (b) and (c) Absorbance of dissolved NBT was increased in proportion to the cell number ($r = 0.9907$) (b), and to the duration of stimulation (c) ($n = 3$). (d) Absorbance of dissolved NBT was increased in proportion to PMA concentration ($n = 4$). Data are expressed as mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (control vs PMA, paired *t* test).

the time required to reach an absorbance plateau, and the elimination of non-specific increases in NBT production.[15]

Comparison of the O_2^- Producing Abilities of Different Phagocytic Cells

The simplified and standardized colorimetric NBT assay was used to compare the abilities of various phagocytic cells to produce intracellular O_2^- (Fig. 3). The PMA-stimulated cells tested included the murine macrophage cell lines (RAW 264.7 and J774), freshly isolated murine peritoneal macrophages (m-mp), and polymorphonuclear neutrophils (m-pmn), freshly prepared human peripheral blood neutrophils (h-pmn), and the human myeloid neutrophil cell line (PLB-985). Intracellular production of O_2^- in all tested cells increased significantly in response to PMA stimulation, except in J774 cells. However, the ferricytochrome c reduction assay was unable to detect O_2^- production in J774 and RAW 264.7 cells (data not shown).

Lack of Interference by Nitric Oxide

Activated macrophages are known to produce both O_2^- and nitric oxide (NO). As Y-NBT can also be reduced to blue formazan by electron donation from NO radical, the colorimetric NBT assay has been used to measure the NO

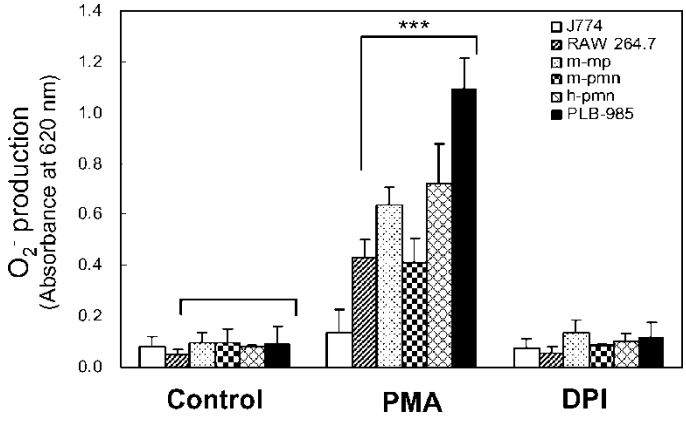

Figure 3. Comparison of intracellular superoxide anion production in various phagocytic cells. Attached RAW 264.7 cells (2.5×10^5) were stimulated with 600 ng/mL PMA in the presence of Y-NBT for 45 min and superoxide anion production was measured using modified colorimetric NBT assay (n = 3). Data are expressed mean ± SD. ***$p < 0.001$ (control vs PMA, paired *t* test).

produced by iNOS in infected renal tissues.[12] Thus, to determine whether the NO produced in PMA- or LPS-stimulated RAW 264.7 cells interferes with O_2^- as determined by the modified colorimetric NBT assay, experiments were conducted in parallel to determine O_2^- and NO levels in RAW 264.7 cells. We found that, although PMA-stimulation did not produce NO in RAW 264.7 cells (data not shown), LPS-stimulation caused strong iNOS induction and high levels of NO production beginning at 6 hr stimulation and lasted more than 30 hr.[19,20] Using the modified colorimetric NBT assay, PMA-stimulation was found to evoke strong O_2^- production at 30 min, but not NO production (Fig. 4a). LPS-stimulation did not provoke detectable production of O_2^- or NO at 30 min. And, although the Griess reaction conducted at 20 hr after incubating with PMA or LPS detected no NO production in PMA-stimulated cells, strong NO production was evident in LPS-stimulated macrophages (Fig. 4b). Aminoguanidine, an inhibitor of constitutive and inducible NOS activity, inhibited LPS-induced NO production (at 20 hr) whereas DPI inhibited PMA-elicited O_2^- production (at 30 min and 20 hr). These data suggest that the modified colorimetric NBT assay is useful for measuring the intracellular production of O_2^- and is not subject to interference from NO produced concomitantly in LPS-stimulated macrophages.

DISCUSSION

Phagocytes produce different levels of O_2^- in response to various stimuli. Intracellular O_2^- is used to kill the phagocytosed pathogens and also to enhance the expression of various enzymes in phagocytes to protect them

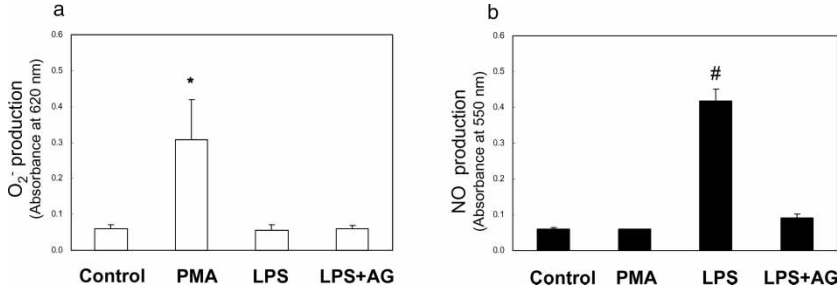

Figure 4. Effect of nitric oxide on modified colorimetric NBT assay. (a) Production of intracellular O_2^- in RAW 264.7 cells was determined at 30 min and 20 hr after stimulations with PMA (600 ng/mL) or LPS (1 μg/mL). (b) Production of NO was determined by the Griess reaction at 30 min or 20 hr after stimulation with PMA or LPS, respectively. Data are expressed mean ± SD from three independent experiments. *p or #p < 0.01 (control vs PMA- or LPS-treated, respectively, by paired *t* test).

from toxic metabolites. Alternatively, extracellular O_2^- can function as a physiological signal mediator to enhance the expression of various antioxidant enzymes in surrounding cells. Much interest has been expressed in the standardized quantification of the amount of O_2^- produced by phagocytes. The O_2^- radical donates its unpaired electron readily to yellow, water-soluble nitroblue tetrazolium (Y-NBT) and converts it to the blue-colored, water-insoluble blue NBT. Utilizing this property of Y-NBT and its ability to cross the plasma membrane, Y-NBT is used to detect the production of O_2^- in activated phagocytes. Based on this principle, the conventional microscopic NBT assay was developed to detect the intracellular production of O_2^-, and this assay is widely used to diagnose CGD, an immuno-deficiency disease caused by reduced O_2^- production and an inability to kill phagocytosed bacteria.[7] However, the conventional microscopic NBT assay has several disadvantages and provides only semi-quantitative information on the amount of O_2^- produced by phagocytes. As shown in Fig. 1, PMA-stimulated RAW 264.7 cells produce only small amounts of NBT. Thus, using the conventional microscopic NBT assay, it is difficult to discriminate between faintly positive cells and negative cells and, thus, the technique is prone to observer bias. To avoid these difficulties, densitometric and colorimetric NBT assays have been developed.[15,17,21] These assays utilize spectrophotometric absorbance to quantify the amount of NBT in adherent cells (densitometric NBT assay) or to quantify the amount of NBT dissolved in organic solvent (colorimetric NBT assay). We modified the colorimetric NBT assay by dissolving cells with KOH and DMSO and determining its absorbance on a microplate reader at 620 nm.

Using the modified colorimetric NBT assay, the absorbance of KOH/DMSO-solubilized NBT was found to increase in proportion to cell number ($r = 0.9907$) (Fig. 2b), incubation time (Fig. 2c), and the dose of PMA employed to stimulate RAW 264.7 cells (Fig. 2d). After considering optimal absorbance and incubation time needed to reach an absorbance plateau, we standardized the method using 2.5×10^5 cells per well and stimulated with PMA (600 ng/mL) for 30–45 min. Optimal reaction conditions for other phagocytic cells may be determined by adjusting cell number, incubation time, and stimulus concentration.

The conventional microscopic NBT assay is highly sensitive. As PMA is a strong O_2^- producing stimulant, and the neutrophils are efficient O_2^- producers, the high sensitivity of the microscopic NBT assay can cause difficulties. For example, previous studies by Dinauer group demonstrated that some PMA-stimulated neutrophils produce only 20–30% of the O_2^- produced by normal neutrophils, become NBT-positive cells that are as intense as normal neutrophils.[11,22] Thus, when the ability of PMA-stimulated wild-type and *rac2*$^{-/-}$ neutrophils were compared for their ability to produce O_2^- using the conventional microscopic NBT assay, the relative number of NBT-positive cells was found to be similar in both genotypes.[18,23] When the ferricytochrome c reduction assay was employed, *rac2*$^{-/-}$ neutrophils

were found to produce only 25% of the O_2^- produced by wild-type cells.[18,24] By employing the modified colorimetric NBT assay, and using reduced numbers of cells, difficulties caused by the hypersensitivity of the conventional microscopic NBT assay can be avoided. The modified colorimetric NBT assay proved sensitive but versatile, and could be applied to measure the intracellular production of O_2^- in a quantitative manner using only 5×10^4 cells. Though the modified colorimetric NBT assay is not complete for measuring total O_2^- production, nevertheless, it can be used in combination with other methods to determine the ability of phagocytic cells to produce O_2^-.

In addition, the modified colorimetric NBT assay was able to discriminate O_2^- production from NO production, regardless of incubation time or stimulus (Fig. 4). Results obtained at 30 min showed that PMA caused a marked increase in intracellular O_2^- production, but that this O_2^- did not enhance NO production, as determined with the Griess reaction at 20 hr, perhaps due to a lack of iNOS upregulation. Conversely, whereas LPS-stimulation did not increase O_2^- production significantly at 30 min or at 20 hr, it was found to enhance NO production caused by the LPS-derived iNOS induction at 20 hr.

In conclusion, the modified colorimetric NBT assay is more sensitive and quantitative than the conventional microscopic NBT assay or previously developed colorimetric NBT assays for measuring intracellular O_2^- production in various phagocytic cells. Furthermore, the assay can measure intracellular production of O_2^- quantitatively and is free of NO interference and from non-specific increases in NBT absorbance. Although future studies will need to compare this modified colorimetric NBT assay with other sensitive assays, such as chemiluminescence or fluorometric assays, and to test the effectiveness using the cell lines that have low levels of NADPH oxidase activity, the method is sensitive enough to measure O_2^- production in macrophage cell lines, such as RAW 264.7, that produce very small amounts of O_2^- which were barely detectable using the conventional microscopic NBT assay or the ferricytochrome c reduction assay. Furthermore, the modified assay can be easily modified to eliminate the hypersensitivity of conventional NBT assays with respect to cells that produce massive amounts of intracellular O_2^- when stimulated.

ABBREVIATIONS

NBT, nitroblue tetrazolium; Y-NBT, yellow-colored nitroblue tetrazolium; NO, nitric oxide; O_2^-, superoxide anion; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; DAB, diaminobenzidine; DCF, 2,7-dichlorofluorescein; DHR, dihydrorhodamine; HVA, homovanillic acid; FMLP, N-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetate; CGD, chronic granulomatous disease; DMEM, Dulbecco's

Modified Eagle's Medium; PBS, phosphate buffered saline; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; DMF, N,N-demethylformamide; KOH, potassium hydroxide; DMSO, dimethyl sulfoxide; DPI, diphenyleneiodonium chloride; LPS, lipopolysaccharide.

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