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Sensitive and Quantitative Nitroblue-Tetrazolium Test for Detecting Chronic Granulomatous Disease

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New method for nitroblue-tetrazolium (NBT) test was developed by the following modifications.

(1) After 10 N KOH treatment, bathochromic shift in the maximal absorption took place from 515 nm to 710 nm and the optical density of the new peak was 5 times higher than that of non-treated sample. In this assay, dimethylformamide extraction was substituted for pyridine.

(2) Because of the smallness of the incubation mixture (100 μl), the background of the test was greatly reduced.

(3) Increase in the concentration of latex particles elevated the NBT reducing activity. In consideration with the background absorption, $2.9—5.7 \times 10^8$ particles/assay was set as the optimal condition. The particles themselves, however, did not interrupt the reading of optical density in this system. In this modified method, 3 ml of blood was enough to estimate the NBT reducing activity in the duplicate tests (6 samples). This assay system is, therefore, applicable for weak children, from whom the collection of the large volume of the blood is not desirable.

Introduction

Phagocytic and bactericidal activity of leukocytes is well known as an important defense mechanism against bacterial infection. It has been reported that the redox activity of leukocytes is involved in the bactericidal activity[2] and that reducing activity of nitroblue-tetrazolium (NBT) relates to the redox activity.[3] It is also known that leukocytes of patients with chronic granulomatous disease (CGD) which can not kill bacteria, are defective in NBT reducing activity.[4] Thus, to detect such kind of patients, NBT-test has been used clinically in both biochemical and histochemical ways. In the case of weak patients such as baby patients, however, it is sometimes very undesirable to collect the blood so much as 15 ml for the measurement in the ordinary NBT-test. It is required nevertheless to examine quantitatively the ability of NBT-reduction in leukocytes from such kind of patients.

The development of the sensitive NBT-test is not only useful in the clinical test but also in biochemical elucidation of the mechanism of the bactericidal function in polymorphonuclear (PMN) leukocytes. For these purposes, development of a sensitive NBT-test by the modification was attempted in the present study.

Materials and Methods

Preparation of Human Peripheral Polymorphonuclear (PMN) Leukocytes——Human peripheral blood, 2—3 ml and 20—25 ml, for the sensitive assay and ordinary assay, respectively, was collected with heparinized syringe, transfered into test tubes, and stood at room temperature for 1 hr to let red cells sediment.

1) Location: *a*) *b*) *1-2-3, Kasumi, Hiroshima-shi, Hiroshima.*
2) *a*) S.J. Klebanoff, *J. Exp. Med.*, **126**, 1063 (1967); *b*) R.C. Allen, S.J. Yevich, R.W. Orth, and R.H. Steele, *Biochem. Biophys. Rec. Commum.*, **60**, 909 (1974); *c*) N.I. Krinsky, *Science*, **186**, 363 (1974).
3) J.R. Humbert, G.P. Gross, A.E. Vatter, and W.E. Hathaway, *J. Lab. Clin. Med.*, **82**, 20 (1973).
4) R.L. Baehner and D.G. Nathan, *N. Engl. J. Med.*, **278**, 971 (1968).

The upper layer containing PMN leukocytes was transfered into another tube and mixed with 2 vol. of 0.87% (pH 7.4) ammonium chloride to disrupt contaminated red blood cells. The solution was mixed thoroughly, and centrifuged (100 $g \times 5$ min) immediately to collect leukocytes. The supernatant was aspirated off, and the cells were suspended in Krebs-Henseleit bicarbonate buffer (pH 7.4) (K-H buffer). The cells were washed twice and were resuspended in a small volume of the buffer. The number of PMN in the suspension was counted and K-H buffer was added to make the number of PMN leukocytes adequate for the assay.

Preparation of PMN Leukocytes from Guinea Pig——PMN leukocytes were prepared as method of Sbarra and Karnovsky.[5] To induce the peritoneal leukocytes, 12% casein (Difco Lab.) in 0.9% NaCl solution (25 ml/kg body weight) was injected intraperitoneally in guinea pigs (Hartley strain). After 18 hr, the peritoneal exudate was removed and was filtered through 4 sheets of gauze. In case red blood cells were found in the cell preparation, the cell suspension was treated with 2 vol of 0.87% ammonium chloride. The precipitate was washed with K-H buffer twice, and the number of PMN leukocytes was counted. More than 95% of the cells were identified as PMN leukocytes under a light microscope.

The original latex particle suspension (0.81 μ, Difco Lab.) was centrifuged (1000 $g \times 50$ min), and was resuspended in K-H buffer to make the concentration of 2.4×10^{11} particles/ml. The number of the latex particles was counted on the picture taken under a microscope.

Ordinary NBT-Test——The ordinary NBT-test developed by Baehner and Nathan[4] was examined as the control procedure. The following solution was mixed in test tubes to prepare the incubation solution; K-H buffer 0.35 ml, 0.85% sodium chloride containing 0.1% NBT (Sigma Chem. Co. Grade III) 0.4 ml, and 0.01 M KCN 0.1 ml. To one of the tubes 0.05 ml of latex particle was added (phagocytic series), to another 0.05 ml of K-H buffer was added instead of latex particles (resting series). Then 0.1 ml of leukocytes suspension was added onto 0.9 ml of incubation solutions. The reaction was allowed to proceed for 15 min, and was stopped by the addition of 10 ml of 0.5 N hydrochloric acid. The mixture was centrifuged (1000 $g \times 15$ min) and the precipitate was extracted twice with 2 ml of pyridine. Optical density of extracts was read at 515 nm against the no incubation blank. NBT reducing activity was calculated from the difference between phagocytic and resting samples.

Sensitive NBT-test——The following procedure was carried out unless otherwise mentioned. Potassium cyanide solution (0.01 M) 2 ml, K-H buffer 4 ml, 10 times concentrated phosphate buffered saline (0.1 M phosphate, 8% NaCl, 0.2% KCl) 0.4 ml, and H_2O 2.6 ml were mixed. NBT dye 8 mg was dissolved in the mixture and pH of the solution was adjusted to 7.4, which was used for an incubation solution. The incubation solution 50 μl, was divided into 2 series in conical centrifuge tubes with a cap. Latex suspension (1.0×10^{11} particles/ml, 5 μl) was added to one series of the tubes (phagocytic series), and the same amount of K-H buffer was added onto another series of the tubes instead of latex particles (resting series). Those tubes were preincubated at 37° for 5 min, and 50 μl of the cell suspension was added to start the reaction. The reaction was allowed to proceed for 15 min, except for time course experiment, and was stopped by the addition of 2 ml of 0.5 N hydrochloric acid. The mixture was kept in ice for more than 30 min and then centrifuged (1000 $g \times 10$ min). The precipitate was washed twice with 2 ml of 0.5 N hydrochloric acid.

Dimethylformamide (DMF) 1 ml was added to the precipitate, and the mixture was stirred and stood for 10 min at 100° in a water bath to extract the NBT-formazan. To increase the coloration, 1 ml of 10 N KOH was added onto the DMF extract and mixed thoroughly. The mixture was centrifuged (1000 $g \times 15$ min) at room temperature to separate the upper DMF layer from the lower aqueous layer. The latex was packed between the DMF and aqueous layers, so that the latex particles did not disturb the subsequent reading of optical density. Pyridine was not suitable as the solvent for the extraction after the 10 N KOH treatment, because pyridine itself was also developed slight red color, which brought about the deviation in the results of NBT-test. Optical density of DMF layer was read at 710 nm against no incubation blank. NBT reducing activity was calculated from the difference between phagocytic and resting samples.

Results

Effect of the Alkaline Treatment on the Coloration of NBT-formazan

Absorption spectrum and the optical density of NBT-formazan (NBT-dye reduced form) were compared before and after the treatment with 10N KOH. As shown in Fig. 1, the absorption of optimal NBT-formazan extracted by DMF was 515 nm which was the same with that extracted by pyridine. By the addition of the equal volume of 10N KOH onto the DMF extract the peak was shifted bathochromically from 515 nm to 710 nm. NBT-formazan was prepared after the incubation of NBT solution with the cells (5×10^6/ml) for 60 min and was extracted with pyridine. An aliquot of 0.1 ml pyridine extract diluted with 0.9 ml of pyridine and DMF gave almost same optical density, 0.242 and 0.245 at 515 nm, respectively.

5) A.J. Sbarra and M.L. Karnovsky, *J. Biol. Chem.*, **234**, 1355 (1959).

On the other hand, the sample diluted by DMF gave OD 1.18 at 710 nm after 10N KOH treatment. In other words, OD at 710 nm after 10N KOH treatment was about 4.8 times higher than that at 515 nm. Presence of 0.1 ml of pyridine in DMF solution did not interfere the optical density at both 710 nm and 515 nm. To get higher sensitivity, reduction of the background coloration was attempted, because blank was around 0.37—0.50 (in optical density at 710 nm) after the treatment with 10N KOH, which may depend on the small amount of contaminating NBT itself. This problem was solved by the reduction of the incubation mixture from 1 ml to 100 μl and complete washing of the precipitate with 0.5N HCl. As the washing solution, 0.5N HCl was superior to ethanol, methanol and trichloroacetic acid. The absorption of the no incubation blank was reduced to around 0.04—0.07 at 710 nm by this modification.

The color developed after the treatment with 10N KOH was markedly stable up to 4 hr as shown in Fig. 2.

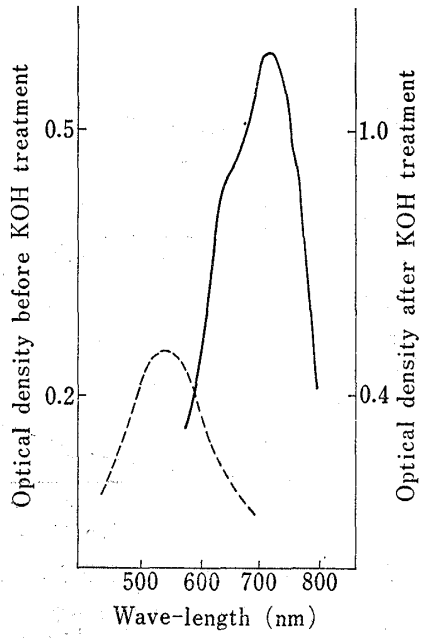

Fig. 1. Absorption Spectrum of NBT-formazan in DMF before (broken line) and after 10N KOH Treatment (solid line)

Effect of the Concentration of Latex Particles

Apparent NBT reducing activity of PMN tended to increase with the number of latex particles up to 8.6×10^8 particles/assay in the presence of 2.6×10^5 cells/assay as shown by open circle in Fig. 3. In higher concentration of latex particles, however, the coloration of the blank increased slightly (closed circle). Optimal reducing activity judged from difference between the apparent activity and blank, as indicated by open triangle, was observed around $2.9—5.7\times10^8$ latex particle/assay.

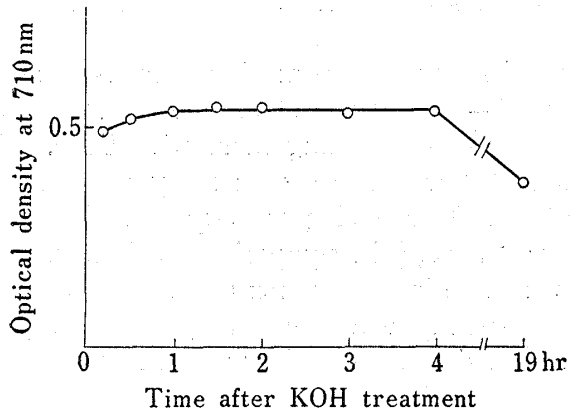

Fig. 2. Stability of the Color of NBT-formazan developed after 10N KOH Treatment

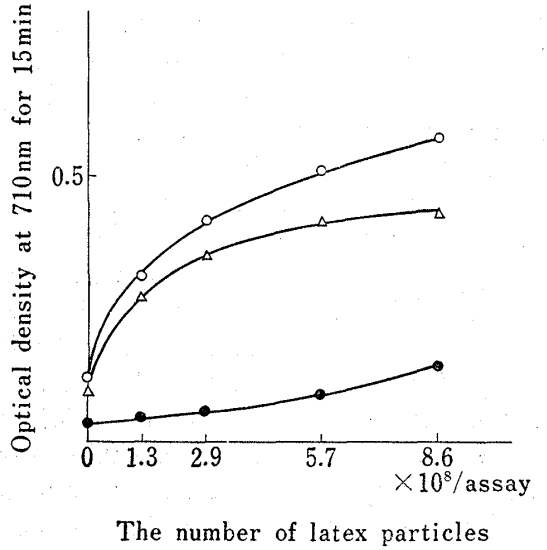

Fig. 3. Relationships between NBT reducing Activity and the Number of Latex Particles

Apparent NBT reducing activity (○—○), no incubation blank (●—●), difference between apparent activity and the blank (△—△), incubation was carried out for 15 min at 37°.

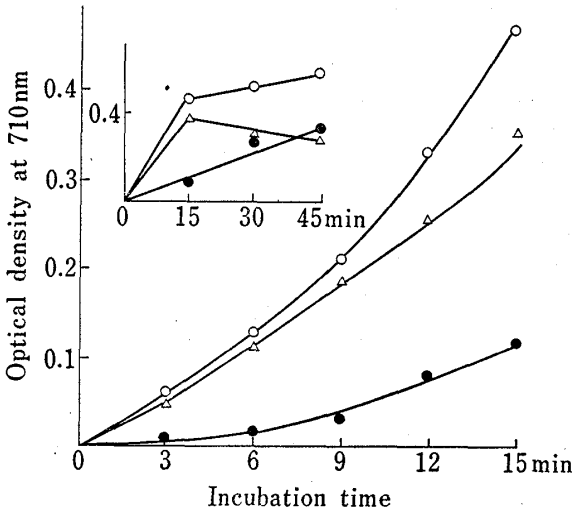

Fig. 4. Time Course of NBT reducing Activity

phagocytosis (O—O), resting (●—●), and △ (OD) (△—△; difference between phagocytosis and resting)

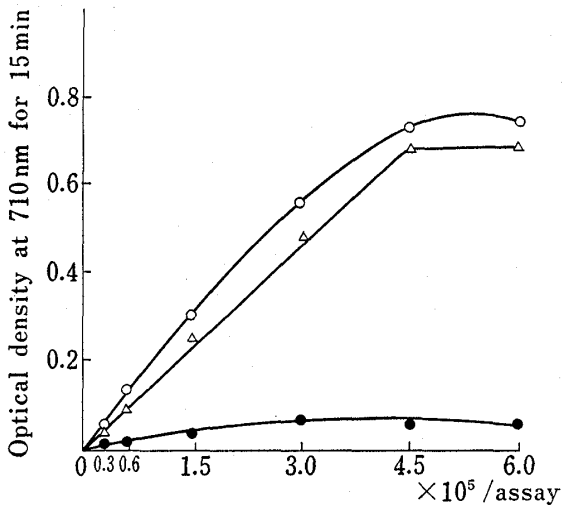

Fig. 5. Relationships between NBT reducing Activity and the Number of PMN Leukocytes

phagocytosis (O—O), resting (●—●), and △ (OD) (△—△; difference between phagocytosis and resting). Fifteen minute incubation was carried out at 37°.

Effect of the Incubation Time and Concentration of PMN Cells

The relationships between NBT reducing activity and incubation time were examined in the presence of PMN leukocytes 2.7×10^5/assay and latex 3.9×10^8 particles/assay. Diffrence in the optical density between phagocytic and resting samples, linearly increased at least up to 15 min, and declined thereafter (Fig. 4). For the quantitative NBT-test, the effect of cell numbers on the NBT reducing activity must be examined. As shown in Fig. 5, the optical density at 710 nm of phagocytic group increased gradually in parallel with the number of PMN leukocytes. The optical density of the resting group also increased slightly. The difference in the optical density between phagocytic and resting group, was well correlated with the cell number up to 4.5×10^5/assay under this condition. All of these experiments were carried out with the use of the leukocytes from guinea pig peritoneal exudate, essentially the same results, however, were observed in leukocytes from human peripheral blood.

Application to Clinical Test

According to the newly developed sensitive assay system, clinical specimens were examined. For the preparation of PMN leukocytes, 3—5 ml of the blood was collected from healthy subject as well as from various types of patients including those with CGD. The number of PMN cells and latex particles were 1.34×10^5 and 3.9×10^8 per assay, respectively, and incubation was carried out for 15 min at 37°. NBT reducing activity of PMN from 2 CGD patients was negligible (absorbance 0.009 on average) in comparison with that of 15 controls (0.292 ± 0.102) (Table I). PMN from various kinds of patients such as systemic lupus erythematodes, chronic granulomatous nephrosis, atypical pneumonia, abdominal pain, and nick crump gave similar activity to that of normal control. The present assay system, therefore, seems to be applicable to the detection of CGD patients.

Discussion

NBT-test was originally devised by Baehner and Nathan[4] for the detection of CGD patients who were defective in bactericidal function. This assay system has been very useful for this purpose. However, a volume as much as 15—20 ml of blood is required for this assay.

Table I. Applicability of This Method to Clinical Test

		Sample	OD 710 Activity	OD 710 Average	OD 710 ±SD
Control	Normal control	F 28	0.425		
		M 23	0.298		
		M 31	0.293		
		F 25	0.277		
		M 27	0.512		
		M 22	0.218		
		M 22	0.415		
		M 25	0.200	0.292	±0.102
		M 25	0.311		
		M 28	0.199		
		F 25	0.404		
		M 31	0.182		
		F 20	0.228		
		M 23	0.133		
		M 37	0.288		
	Patients	M 6 atypical pneumonia	0.347		
		M 6 abdominal pain (unknown)	0.265		
		M 4 nick crump	0.308		
		F 37 SLE mother	0.298		
		M 5 SLE	0.248		
		M 8 CGN	0.160		
CGD		M 5 months	0.025	0.009	
		M 9	−0.007		

SLE: systemic lupus erythematodes, CGN: chronic granulonatous nephrosis, CGD: chronic granulomatous disease
M: male, F: female

Histochemical NBT staining developed by Park, *et al.*,[6] and Gifford and Malawista[7] is applicable to those weak patients, but this assay is inferior in quantification.

It has been assumed so far that redox reaction system has close relationship with the bactericidal mechanism[8] from the result of studies on myeloperoxidase-halide system,[2] NADPH oxidase,[9] O_2 consumption,[10] and HMP shunt,[11] in PMN leukocytes. NBT reducing activity may also be involved in the bactericidal mechanism in PMN leukocytes. So, more sensitive and quantitative NBT assay system has been desired for both clinical and investigative purpose.

According to Altmann,[12] it was shown that glycine–NaOH buffer (pH 11.5) treatment of the DMF extract from histochemical specimens made the bathochromic shift of the maximal absorption from 515 nm to 710 nm. On basis of this finding, development of a quantitative and sensitive NBT assay system was attempted. First of all, 10N KOH was adopted instead of the glycine–NaOH buffer in these experiments, because the NBT-formazan after 10N KOH treatment was more stable (Fig. 2). There are further advantages of this treatment that

6) B.H. Park, S.M. Fikrig, and W. Smith, *Lancet*, **2**, 532 (1968).
7) R.H. Gifford and S.E. Malawista, *J. Lab. Clin. Med.*, **75**, 511 (1970).
8) R.B. Johnston, B.B. Keele, H.P. Misra, J.E. Lehmeyer, L.S. Webb, R.L. Baehner, and K.V. Rajagopalan, *J. Clin. Invest.*, **55**, 1357 (1975).
9) P. Patriarca, R. Cramer, P. Dri, L. Fant, R.E. Basford, and F. Rossi, *Biochim. Biophys. Acta*, **53**, 830 (1973).
10) R.S. Weening, D. Roos, and J.A. Loos, *J. Lab. Clin. Med.*, **83**, 570 (1974).
11) B. Holmes, A.R. Page, and R.A. Good, *J. Clin. Invest.*, **46**, 1422 (1967).
12) F.P. Altmann, *Histochemie*, **17**, 319 (1969).

alkaline solution neither dissolves in DMF solution nor dilutes DMF solution. Furthermore removal of latex particles could readily be achieved as the particles were packed in the interphase between DMF and 10N KOH in this condition.

Bathochromic shift of NBT (not reduced form) attaching nonspecifically to the cellular protein and latex particles brought about another problem of high background absorption.

This problem was almost solved by reduction of volume of the incubation mixture and washing it with 0.5N HCl 3 times. The concentration of latex particles was also a matter to be examined. According to the ordinary assay method, high concentration of latex particles increased the turbidity which interrupted the reading of the optical density. Optimal concentration of latex particles for the assay was obtained around $2.9—5.7\times10^8$/assay (Fig. 3), concentration of which was almost 4 times higher than that in the ordinary method. This result is well coincident with that of the studies on O_2 consumption after phagocytosis of various numbers of latex particles.[10]

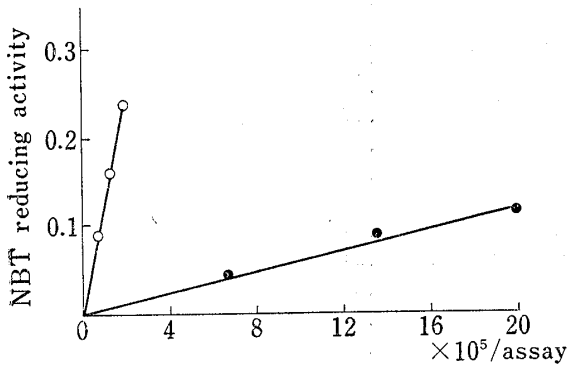

Fig. 6. Sensitivity of the newly Developed Assay in Comparison with That of the Ordinary Assay

In the modified assay (○—○), 0.67, 1.33, and 2.0×10^5 PMN cells, and 5.7×10^8 latex particles per assay were incubated. After 10N KOH treatment against NBT-formazan extracted in DMF, optical density at 710 nm was read. In regular assay (●—●), 6.7, 13.3, 20.0×10^5 PMN cells (which is about ten times larger number of cells) and 1.3×10^9 latex particles per assay were incubated. NBT-formazan extracted by pyridine was read at 515 nm. Incubation was carried out in both cases at 37° for 15 min. NBT reducing activity was calculated from the difference between phagocytosis and resting.

Sensitivity of newly developed NBT-test can be calculated in comparison with that of ordinary test. There were linear relationships between NBT reducing activity and the number of PMN leukocytes up to 2.0×10^5 and 20×10^5 in modified and ordinary assay respectively. According to the result from Fig. 6, 2.0×10^5 PMN/assay gave 0.231 in modified assay and 20×10^5 PMN/assay, 0.110 in ordinary assay, indicating that the sensitivity of this test seems to be almost 20 times higher than that of the ordinary NBT-test. This sensitive assay may be apparently applicable to the analysis of CGD patients especially of the baby patients. This method may also be useful for the biochemical elucidation of the bactericidal mechanism in leukocytes.

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