INHIBITORY EFFECTS OF SUPEROXIDE DISMUTASES AND VARIOUS OTHER PROTEINS ON THE NITROBLUE TETRAZOLIUM REDUCTION BY PHAGOCYTIZING GUINEA PIG POLYMORPHONUCLEAR LEUKOCYTES

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Received July 16,1975

SUMMARY: The reduction of nitroblue tetrazolium by guinea pig peritoneal polymorphonuclear leukocytes was studied during the phagocytosis of latex particles. Superoxide dismutases from various sources inhibited the reduction of the dye. However, almost the same degree of inhibition was observed with a number of other proteins including albumins, γ-globulin, histone and trypsin inhibitor, as well as with the heat inactivated dismutases. The results suggest a non-specific inhibitory effect of superoxide dismutase proteins on the reduction of nitroblue tetrazolium rather than a specific enzymic inhibition of the superoxide-mediated reduction of the dye.

INTRODUCTION: Since the identification of erythrocuprein as superoxide dismutase (1), the inhibition observed with this enzyme has been interpreted frequently to indicate the participation of superoxide in a variety of reactions. Such a participation of superoxide has been implicated in the bactericidal action of polymorphonuclear leukocytes. Namely, an increased production of superoxide was observed during phagocytosis as shown by the dismutase-sensitive reduction of cytochrome c (2-4). An inhibitory effect of superoxide dismutase was observed on leukocytic bactericidal activity (5, 6). It was also found that superoxide was not produced by leukocytes obtained from patients with chronic granu-

lomatous disease (4, 7). Since the reduction of NBT[1/] is stimulated by phagocytosis in normal leukocytes (8) but is barely detectable in leukocytes from patients with chronic granulomatous disease (9), a possible involvement of superoxide in the reduction of this dye was examined. As will be described in this communication, various superoxide dismutases did inhibit the phagocytosis-dependent reduction of NBT. However, the results obtained raise serious doubts about the interpretation of the inhibitory effect of superoxide dismutase on the reduction of NBT by phagocytizing leukocytes.

MATERIALS AND METHODS: Shellfish glycogen was purchased from Nakarai Chemicals (Kyoto), heparin sodium salt from Novo Industry (Copenhagen), NBT from Sigma, and Bacto-latex (0.81 μ) from Difco Laboratories. Crystallized human serum albumin and human serum γ-globulin Fraction II were supplied by Nutritional Biochemicals Corporation; crystalline bovine serum albumin, crystallized egg albumin Grade VI, calf thymus histone Type VI (f_{2a}) and soybean trypsin inhibitor Type II-S were from Sigma. Bovine erythrocyte superoxide dismutase (specific activity, 3,300 units/mg protein) (1) was kindly provided by Dr. M. Fujiwara, *E. coli* superoxide dismutase (1,720 units/mg protein) (10) by Dr. N. Ogino, spinach superoxide dismutase (6,000 units/mg protein) (11) by Dr. K. Asada, Kyoto University Institute for Chemical Research, pseudomonad lysine monooxygenase (12) by Dr. T. Ohnishi, and pseudomonad protocatechuate 3,4-dioxygenase (13) by Dr. R. Yoshida.

Female guinea pigs weighing about 250 g were given intraperitoneally 25 ml of a 0.1% shellfish glycogen solution. After 5 hrs about 200 units heparin were injected intraperitoneally, and the

1/ Abbreviation used: NBT, nitroblue tetrazolium.

cells were washed out of the peritoneal cavity with 30 ml of 0.9% NaCl. The cell suspension was centrifuged at 120 X g for 5 min. The precipitated cells were suspended in 10 ml of distilled water and kept for 30 sec to rupture contaminating red blood cells. Then, the cell suspension was mixed with 10 ml of 1.8% NaCl. The mixture was centrifuged at 120 X g for 5 min, followed by two washes each with 20 ml of 0.9% NaCl. The cells were dispersed in 0.9% NaCl, and the final cell concentration was adjusted to 3 X 10^7 cells per ml. Usually 2 - 5 X 10^7 cells were obtained from one guinea pig, 80 - 85% being polymorphonuclear leukocytes as examined microscopically.

The NBT reduction was determined by the modified method of Baehner and Nathan (9). The reaction mixture (1.0 ml) contained 20 mM potassium phosphate buffer at pH 7.4, 0.2% NaCl, 10 mM glucose, 0.04% NBT, Bacto-Latex (1.7 X 10^9 particles) and the cells. The incubation was carried out at 37° for 15 min with shaking. The reaction mixture was acidified with 10 ml of 0.5 N HCl and then centrifuged. The precipitate was dissolved in 2 ml of pyridine, and the solution was kept in boiling water for 10 min. The absorbance at 515 nm was determined by a Union end-on type spectrophotometer model SM-401.

RESULTS: The rate of NBT reduction by guinea pig polymorphonuclear leukocytes increased as the amount of latex was raised, and 1.5 X 10^6 cells were saturated with 3.4 X 10^9 latex particles in the standard reaction mixture. In the presence of 1.7 X 10^9 latex particles, the rate of NBT reduction increased depending on the number of cells when the cell number was kept less than 2 X 10^6. Under these conditions the reduction of NBT proceeded almost linearly for 15 - 20 min. With 1.5 X 10^6 cells and 1.7 X 10^9

latex particles, the average increase in the absorbance at 515 nm was 0.4 - 1.0 in 15 min. The rate of NBT reduction was dependent on the concentrations of neutral salt and buffer in the reaction mixture. Thus, 20 mM potassium phosphate buffer at pH 7.4 containing 0.2% NaCl was found to be the optimal salt mixture and caused about three-fold stimulation of the NBT reduction. This salt-dependent activity was lost upon aging of cells; namely, 17 hours after the harvest of the cells the observed stimulation was only 0.5-fold, and after 44 hours there was no detectable stimulation. However, the cells still retained some activity unaffected by the salt.

The effect of various superoxide dismutases on the NBT reduction was examined under the conditions described above. As shown in Table I, when the amount of superoxide dismutase was increased, the rate of NBT reduction decreased. The inhibition was not complete, and the maximal inhibition observed was 60 - 70%. However, heat inactivated superoxide dismutases were almost as inhibitory as the native enzymes. To examine whether the inhibition was a non-specific effect of the superoxide dismutase protein itself, various other proteins were tested. All the proteins tested were inhibitory. These proteins include albumins of various origins, γ-globulin, histone and trypsin inhibitor. They showed a 50% inhibition with amounts of the order of a few μg per ml. Bovine serum albumin did not inhibit the aerobic reduction of NBT by xanthine oxidase. Moreover, two crystalline oxygenases obtained in this laboratory were also inhibitory although they were less effective than the albumins. Since several proteins obtained from mammalian serum inhibited the NBT reduction, serum of either guinea pig or human blood was tested. The addition of 1 μl of serum showed an almost complete inhibition of NBT reduction. As pres-

Table I. Inhibitory effects of various proteins on the phagocytosis-dependent reduction of NBT

The NBT reduction was determined in the standard reaction mixture (1 ml) containing 1.5 X 10^6 cells and the protein as indicated. Since erythrocyte superoxide dismutase kept in boiling water for 30 min still retained about 2% of the original activities, the dismutases were inactivated by autoclaving for about 20 min.

Added protein			NBT reduction	Added protein		NBT reduction
	μg	(units)	%		μg	%
None			100	Bovine serum albumin	5	46
None (Latex omitted)			10		10	22
					20	9
Erythrocyte					50	5
superoxide dismutase	1	(3)	90			
	5	(16)	79	Human serum albumin	5	70
	10	(33)	68		10	22
	50	(165)	46		20	6
	100	(333)	39		50	4
	150	(500)	33			
(Autoclaved)	5		118	Egg albumin	5	47
	10		90		10	30
	50		46		20	26
					50	19
Spinach				Human serum γ-globulin	5	68
superoxide dismutase	3	(17)	61		10	43
	11	(66)	32		20	37
	28	(166)	30		50	38
	56	(333)	25			
(Autoclaved)	3		93	Histone f_{2a}	10	53
	11		62			
	28		53	Soybean trypsin		
	56		18	inhibitor	5	58
					10	51
E. coli					20	21
superoxide dismutase	6	(10)	68		50	16
	12	(20)	54			
	35	(60)	51	Lysine monooxygenase	10	85
	58	(100)	55		50	25
	116	(200)	40			
(Autoclaved)	6		103	Protocatechuate		
	12		93	3,4-dioxygenase	10	72
	35		77		50	12
	58		64			
	116		72			

ented in Fig. 1, when the rate of NBT reduction was plotted against the amount of protein, the inhibition curves of both guinea pig and human serum were similar to that obtained with bovine serum albumin.

DISCUSSION: The experimental results described in this communication suggest that any result concerned with the possible participation of superoxide should be carefully examined if it is based solely on the inhibitory effect of superoxide dismutase. However, our results do not necessarily rule out an involvement of superoxide anion in the reduction of NBT since the native superoxide dismutases may inhibit the NBT reduction through their enzymic activities whereas the boiled enzymes may exert their non-specific inhibitory effects. Xanthine oxidase (14) and leukocyte sonicate (15) have been reported to transfer electrons to NBT either by a direct anaerobic reaction or by an indirect aerobic reaction via a univalent reduction of molecular oxygen. In spite of the practical use of the NBT test in clinical laboratories, the precise mechanism of NBT reduction by phagocytizing leukocytes is still

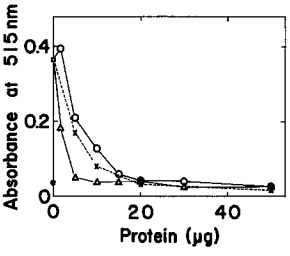

Fig. 1. Inhibitory effects of human and guinea pig sera on the phagocytosis-dependent reduction of NBT. The rate of NBT reduction under the standard conditions was determined in the presence of 1.5 X 10^6 cells and serum as indicated. The amount of serum was expressed in terms of the amount of serum albumin assuming the albumin content to be 5 g/100 ml of blood. △———△, human serum; o———o, guinea pig serum; X-----X, bovine serum albumin; ■, assayed in the absence of latex particles.

unknown (16). The mechanism of how albumins and other proteins inhibit the NBT reduction remains unclear. The ingestion of latex particles by the cells may be hindered although superoxide dismutase was reported not to inhibit the ingestion (6). These proteins may interact with either an enzyme or a reductant responsible for the reduction of the dye and thus inhibit the reductive reaction. Moreover, the proteins may protect leukocyte cells from the damage caused by the simultaneous presence of NBT and latex particles, which results in the enhanced reduction of the dye (17). Although the NBT reduction performed by the method of Baehner and Nathan (9) is an important diagnostic test, variable results are frequently encountered by clinicians who use this method. This may be partly attributable to inhibition of the NBT reduction by contaminating serum in the leukocyte preparation.

ACKNOWLEDGEMENT: This work has been supported in part by the Scientific Research Fund of the Ministry of Education and by a grant for the research of immunologic disorders of the Ministry of Welfare of Japan. The authors wish to thank Dr. C. M. Edson for his critical reading of this manuscript.

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