Superoxide Dismutase in Polymorphonuclear Leukocytes of Term Newborn Infants and Very Low Birth Weight Infants

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ABSTRACT. We investigated the activity and the content of copper and zinc-containing superoxide dismutase (SOD) and superoxide anion (O_2^-) production in polymorphonuclear leukocytes (PMN) from healthy term newborn infants, very low birth wt infants, and healthy adults. SOD activity in PMN was measured with nitroblue tetrazolium reduction assay on PAGE, and the SOD content in PMN was determined with an ELISA using a monoclonal antibody against human copper and zinc-containing SOD. The activity and the content of SOD in the term neonatal PMN and VLBW-infants' PMN were significantly lower than those in the adults' PMN (term newborn infants, 6.6 ± 0.6 U/mg protein and 170.4 ± 16.3 ng/mg protein, $n = 10$; VLBW infants, 6.8 ± 0.9 and 173.0 ± 16.2 , $n = 6$; adults, 10.3 ± 0.6 and 241.9 ± 13.3 , $n = 10$; values were expressed as mean $\pm$ SEM). Both the phorbol myristate acetate- and the N-formyl-methionyl-leucyl-phenylalanine-induced O_2^- production rates of VLBW-infants' PMN were significantly higher than those of the term neonatal PMN. The phorbol myristate acetate-induced O_2^- production rate of the term neonatal PMN was significantly lower than that of the adults' PMN. The phorbol myristate acetate-induced H_2O_2 production rate of the term neonatal PMN was significantly lower than that of the adults' PMN. The conversion rate from O_2^- to H_2O_2 of the neonatal PMN was similar to that of the adults' PMN. These findings suggest that neonatal PMN have an equal ability to convert O_2^- to H_2O_2 to an adult's PMN, although the neonatal PMN had a lower SOD activity than the adults' PMN. In addition, the decreased oxygen metabolites production of the term neonatal PMN helps account for their defective bactericidal activity. (*Pediatr Res* 26:227-231, 1989)

Abbreviations

VLBW infants, very low birth wt infants
PMN, polymorphonuclear leukocyte
 O_2^- , superoxide anion
SOD, superoxide dismutase
Cu,Zn-SOD, copper and zinc-containing superoxide dismutase
PMA, phorbol myristate acetate
FMLP, N-formyl-methionyl-leucyl-phenylalanine
NBT, nitroblue tetrazolium

Newborn infants are susceptible to bacterial infection, and premature infants weighing less than 1500 g (VLBW infants) are even more susceptible than term infants. PMN play a primary role in the host defense system against bacterial infection. The respiratory burst, characterized by increased oxygen consumption, an increase in hexose monophosphate shunt activity, and an increased production of oxygen metabolites such as O_2^- , H_2O_2 , hydroxyl radical, and singlet oxygen, occurs upon the activation of PMN (1). These oxidant species are used as antimicrobial agents.

Defective bactericidal activity in PMN of normal newborn infants was described when the concentration of an opsonizing serum was reduced to 2% (2) or bacteria to the PMN ratio was large in the assay condition (3). And PMN of infants less than 12 h of age (4), preterm infants (5), and stressed newborn infants (6, 7) also have been reported to have defective bactericidal function.

O_2^- production is the key reaction in the initiation of the respiratory burst. The results of previous investigations of the O_2^- production rate of neonatal PMN have been varied (8-12), and there has been no report describing the O_2^- production of PMN obtained from VLBW infants.

PMN contain two distinct types of SOD, namely, a Cu,Zn-SOD localized in the cytosol, and a manganese-containing SOD in mitochondria (13). O_2^- , which has negligible antimicrobial activity itself (14), is dismutated spontaneously or enzymatically by Cu,Zn-SOD in the cytosol of PMN to generate H_2O_2 and oxygen (15). H_2O_2 has substantial bactericidal activity (16), and current evidence strongly suggests that hypochlorite, which is produced by the oxidation of Cl^- by H_2O_2 , and hydroxyl radical, the product of the interaction between O_2^- and H_2O_2 , are critically important antimicrobial oxidants generated in PMN (14, 17, 18). SOD in PMN also protects cells from self-inflicted oxidative injury by detoxifying O_2^- , but there has been very little information about the content or the activity of SOD in PMN of the neonates.

In our study, we have measured both the activity and the content of SOD in PMN of healthy term newborn infants and VLBW infants. In addition, we have evaluated the O_2^- production rate of PMN of term newborn infants and VLBW infants.

MATERIALS AND METHODS

Reagents. Cytochrome C (Type VI), PMA, FMLP, NBT, riboflavin, Cu,Zn-SOD (human erythrocytes), scopoletin, and horseradish peroxidase (type VI) were obtained from Sigma Chemical Co. (St. Louis, MO). Mouse antihuman Cu,Zn-SOD mAb and affinity-purified SOD were obtained from Ube Industries (Tokyo, Japan).

Study Population. For the determination of Cu,Zn-SOD in PMN, 10 healthy term newborn infants and six VLBW infants, all of whom were within 24 h after birth, and 10 healthy adults

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were studied. The mean birth wt of the healthy term newborn infants was 3186 g (range 2790–3576 g), and that of the VLBW infants was 1235 g (range 798–1486 g). The mean gestational age of the VLBW infants was 29 wk (range 25–32 wk). Diagnoses of the VLBW infants included four infants with respiratory distress syndrome, one with apnea, and one with hypoglycemia.

The O_2^- production rates were studied in PMN obtained from 12 healthy term newborn infants at 0, 3, and 6 d of age, 12 VLBW infants at 0, 6, and 30 d of age, and 19 healthy adults. The mean birth wt and the gestational age of the VLBW infants studied were 1084 g (range 789–1470 g) and 30 wk (range 25–35 wk), respectively. The population of the VLBW infants was as follows: five infants with respiratory distress syndrome, four infants with apnea, two infants with hypoglycemia, and the other with aspiration syndrome.

Isolation of PMN. One mL of heparinized blood was drawn from the vein and PMN were isolated by means of dextran sedimentation, centrifugation through Lymphoprep (Nycomed AS, Oslo, Norway) followed by hypotonic lysis of erythrocytes. Isolated cells, at more than 95% purity, were resuspended in PBS consisting of 10 mM phosphate (pH 7.4), 137 mM NaCl, and 2.7 mM KCl. The cell viability determined by trypan blue dye exclusion was always more than 95% throughout the experiments in this study.

Preparation of PMN Homogenate. Isolated cells suspended in PBS at pH 7.4 (1×10^7 PMN/mL) were sonicated for 45 s on ice with a sonifier cell disruptor (Kontes, Vineland, NJ) at maximum intensity and then centrifuged for 15 min at $1200 \times g$ at 4°C. The resulting supernatant was used for enzyme assays. The protein concentration in the samples was determined by the method of Lowry *et al.* (19) using BSA as a standard.

Measurement of Activity and Content of SOD. Photochemical NBT reduction assay on polyacrylamide gel electropherograms. The PMN homogenates (20 μ g protein) were electrophoresed on 10% polyacrylamide gel as described by Laemli (20) at a constant current at 20 mA, except SDS was omitted from the gel and the buffer. The activity staining procedure for SOD was performed as described by Salin and McCord (13). Although in the dark for 40 min, the gel was soaked in 50 mM potassium phosphate buffer at pH 7.8 containing 1 mM EDTA, 0.25 mM NBT, 20 mM tetramethylethylenediamine, and 30 μ M riboflavin.

After the soaking period, the gel was rinsed briefly with distilled water and exposed to an incandescent desk lamp for 15 min. During illumination the gel became uniformly purple except at the positions containing SOD. The gel was scanned at 560 nm with linear scanning densitometry using a Dual-wavelength TLC Scanner (CS-930, Shimadzu, Kyoto, Japan), and the areas under the troughs, which correspond with the bands of activity, were determined by weighing the tracing paper. Cu,Zn-SOD from human erythrocytes (Sigma) was used as a standard in each assay. SOD activity was expressed as U/mg protein.

ELISA using MAbs. Flat-bottomed 96-well microtiter plates were coated with affinity-purified mouse antihuman Cu,Zn-SOD (N-4). The homogenates of PMN diluted by distilled water to a concentration of about 200 μ g protein/mL were then added to the wells (100 μ L/well in duplicate), and incubated for 2 h at room temperature. Each plate also included 10 serial 2-fold dilutions of affinity-purified SOD standard (Ube Industries) ranging between 0 and 40 ng/well.

These were then washed three times with PBS at pH 7.4 containing 0.1% Tween 20, and the wells were filled with 100 μ L of peroxidase-conjugated, affinity-purified mouse F(ab')₂ antihuman Cu,Zn-SOD-(N-6). After 2 h incubation at room temperature, the plates were again washed three times with PBS-Tween 20 solution, and subsequently 100 μ L of 0.44 mg/mL *o*-phenylenediamine dihydrochloride in citrate buffer at pH 5.0 with 0.007% H_2O_2 was added to each well. After a 15-min incubation in the dark at room temperature, the reaction was stopped by the addition of 50 μ L of 2 N-hydrochloric acid.

Optical density of each well was measured at 492 nm using a

Titertek Uniskan (LabSystems and Flow Laboratories, Finland). Values were determined by reference to a standard line. The content of SOD was expressed as ng/mg protein.

Measurement of O_2^- Production. Spectrophotometric measurement of O_2^- -dependent cytochrome C reduction was performed in a special double-beam spectrophotometer with different circuits of 550 and 540 nm equipped with a thermoregulator set at 37°C (model SA-1, Arrows, Osaka, Japan) as described previously (21). The sample cuvette contained 20 μ M ferricytochrome C, 1×10^5 cells of PMN, and 5 mM glucose in 1 mL of PBS at pH 7.4. PMN were stimulated by either PMA (0.1 μ g/mL) or FMLP (4 nM). The rate of cytochrome C reduction was measured by the change of absorbance at 550–540 nm.

When PMA was used as a stimulant, the linear change of the absorbance was observed between 2 and 4 min after the addition of the agent and the absorbance continued to increase over 10 min at the lower rate. When FMLP was used as a stimulant, the absorbance reached the plateau level 3 min after the addition of the agent. Thus, the O_2^- production rates were calculated from the linear slope of cytochrome C reduction for PMA and from the amount of cytochrome C reduced in 3 min for FMLP using an absorbance coefficient of cytochrome C at OD_{550–540} of 19.1. The O_2^- production rate was expressed as nmol/min/ 10^6 PMN for PMA-stimulated cells and nmol/3 min/ 10^6 PMN for FMLP-stimulated cells.

Measurement of H_2O_2 Release. H_2O_2 production by PMN was determined by measuring the decreased fluorescence of scopoletin (22). The sample cuvette contained 2 μ M scopoletin, 20 nM horseradish peroxidase, 5×10^5 cells of PMN, and 5 mM glucose in 2 mL of Krebs-Ringer bicarbonate buffer at pH 7.4.

Fluorescence was measured using the fluorescence spectrophotometer (650-10S, Hitachi, Tokyo, Japan) equipped with the thermoregulator set at 37°C and a magnetic stirring device. Peak excitation and emission wave lengths were determined to be 355 and 460 nm, respectively. H_2O_2 standard solutions (0–2 μ M) were prepared just before the measurement using a molar extinction coefficient of 81 at 230 nm. The intensity of the emission fluorescence was recorded continuously on the Hitachi recorder type 561, and the rate of H_2O_2 release after the addition of PMA (0.1 μ g/mL) was calculated from the linear portion of reduced scopoletin.

Statistical Analysis. Statistical analysis was performed using Student's *t* test except that the Wilcoxon rank sum test was used in the experiments of O_2^- production rate of PMN. Possible relationships between SOD activity and SOD content in individual samples were investigated with linear regression analysis.

RESULTS

Activity and content of SOD in PMN. SOD dismutates O_2^- to generate H_2O_2 , not only to produce potent antimicrobial agents such as hydroxyl radical and hypochlorite, but also to protect the PMN membrane from O_2^- toxicity. Strauss *et al.* (23) reported that the neonatal PMN had a similar content of SOD to the adults' PMN, but they used a xanthine oxidase-cytochrome C assay which is highly affected by SOD-insensitive cytochrome C reduction (24). Therefore we used polyacrylamide gel-NBT reduction assay and ELISA using a MAb against human Cu,Zn-SOD to eliminate the influence of other factors in the measurement of SOD.

In the NBT reduction assay on PAGE, the photogenerated O_2^- reduced NBT to form insoluble purple formazan except for an achromatic band at the position of SOD activity. Figure 1A represents the typical densitometric scans of the stained gel. When the areas under the troughs, which correspond with the bands of human erythrocyte Cu,Zn-SOD activity, were plotted against the amount of SOD applied, there was a direct linear correlation between the two in the range of 0–0.5 μ g of SOD (2500 U/mg) (Fig. 1B) as previously described by Misra and Fridovich (25). We measured Cu,Zn-SOD activity of PMN ho-

mogenates by this method using 0.05, 0.1, and 0.15 μg of human erythrocyte Cu,Zn-SOD to produce a standard line in each assay. The results are shown in Table 1. SOD activity of the VLBW infants or the term-neonatal PMN was significantly lower than that of the adults' PMN ($p < 0.005$).

Although the NBT reduction assay on PAGE was useful to measure Cu,Zn-SOD activity in PMN, we also measured the content of Cu,Zn-SOD using a MAb against human Cu,Zn-SOD to verify the NBT reduction assay. Again, the content of SOD in PMN of the term newborn infants or the VLBW infants was significantly lower than that in the adults' PMN ($p < 0.005$) (Table 1).

Figure 2 represents the correlation between the SOD measured with ELISA and the SOD activity determined with the NBT reduction assay on PAGE. There was a significant direct correlation between the values by two methods ($r = 0.84$, $p < 0.005$, linear regression analysis).

O_2^- production rate of PMN. The O_2^- production rates of PMN of the VLBW infants, the healthy term newborn infants, and the adults are shown in Table 2. PMA-induced O_2^- production rates of PMN obtained from the VLBW infants at 0, 6, or 30 d of age were similar to that of the adults' PMN. The PMA- or FMLP-induced O_2^- production rate of PMN obtained from the VLBW infants at 0 d of age was significantly higher than that

Fig. 2. The relationship of SOD activity measured with NBT reduction assay on PAGE and SOD content measured with ELISA in PMN from VLBW infants (Δ), healthy term newborn infants ($\circ$), and adults ($\bullet$).

Table 2. O_2^- production rates of PMN obtained from VLBW infants, healthy term newborn infants, and adults in response to PMA or FMLP*

	Age (d)	n	Stimulus	
			PMA (nmol/min/ 10^6 PMN)	FMLP (nmol/3 min/ 10^6 PMN)
VLBW infants	0	12	$13.0 \pm 1.1^\dagger$	$24.8 \pm 2.8^{\dagger\ddagger}$
	6	12	11.9 ± 1.1	$20.4 \pm 2.8^\ddagger$
	30	9	15.2 ± 2.1	14.5 ± 2.2
Term newborn	0	12	$7.2 \pm 0.9^\ddagger$	10.4 ± 1.5
	3	10	$8.8 \pm 0.7^\ddagger$	15.9 ± 1.6
	6	9	$10.9 \pm 1.1^\S$	16.4 ± 1.8
Adults		19	13.2 ± 0.8	12.7 ± 1.0

* PMN were suspended in PBS at pH 7.4 (1×10^5 PMN/mL) containing 20 μM cytochrome C and 5 mM glucose. And PMN were stimulated by either 0.1 $\mu\text{g/mL}$ PMA or 4 nM FMLP. Spectrophotometric measurement of O_2^- dependent cytochrome C reduction was performed as described in "Materials and Methods." Values represent mean $\pm$ SEM from the number of the subjects as indicated.

$^\dagger p < 0.005$ when compared to term newborn infants' values at 0 d of age.

$^\ddagger p < 0.005$, $^\S p < 0.05$ when compared to adults' values.

Fig. 1. A, densitometric scans of PAGE stained for SOD activity. The gel containing (1) 0.05, (2) 0.1, or (3) 0.15 μg of Cu,Zn-SOD from human erythrocytes was scanned at 560 nm. The area under the trough represents the quantity of SOD activity. This represents typical results from 10 similar independent experiments. The scale in the figure represents 0.1 ΔOD at 560 nm. B, estimation of SOD activity. The Cu,Zn-SOD-containing areas under the troughs produced by densitometric scanning of the gel were plotted against the quantity of SOD applied to the gel. The relative area under the trough was measured by weighing the tracing paper.

Table 1. SOD activities and SOD contents in PMN obtained from VLBW infants, healthy term newborn infants, and adults*

	n	SOD activity (U/mg protein)	SOD content (ng/mg protein)
VLBW infants	6	$6.8 \pm 0.9^\dagger$	$173.0 \pm 16.2^\dagger$
Term newborn infants	10	$6.6 \pm 0.6^\dagger$	$170.4 \pm 16.3^\dagger$
Adults	10	10.3 ± 0.6	241.9 ± 13.3

* The PMN homogenate was assayed for the activity with NBT reduction assay and the content of Cu,Zn-SOD with ELISA as described in "Materials and Methods." Values represent mean $\pm$ SEM from the number of the subjects as indicated.

$^\dagger p < 0.005$ when compared to adults' values.

of PMN obtained from the term newborn infants at 0 d of age ($p < 0.005$). The PMA-induced O_2^- production rate of the term neonatal PMN at 0 d of age was significantly lower than that of the adults' PMN ($p < 0.005$), and increased with age, but it was still lower than that of the adults' PMN at 3 and 6 d of age ($p < 0.005$ and $p < 0.05$, respectively).

The FMLP-induced O_2^- production rate of PMN obtained from the VLBW infants at 0 d of age was significantly higher than that of the adults' PMN ($p < 0.005$), and decreased with their age to be equal to the adults' PMN value at 6 d of age. The FMLP-induced O_2^- production rate of the term neonatal PMN

Table 3. O_2^- production, H_2O_2 release, and the conversion rate from O_2^- to H_2O_2 of PMN obtained from healthy term newborn infants, and adults*

	<i>n</i>	O_2^- production (nmol/min/ 10^6 PMN)	H_2O_2 release (nmol/min/ 10^6 PMN)	Conversion rate from O_2^- to H_2O_2
Term newborn infants	6	$6.8 \pm 1.3^\dagger$	$1.0 \pm 0.1^\ddagger$	0.17 ± 0.03
Adults	6	12.4 ± 1.4	1.3 ± 0.1	0.11 ± 0.02

* PMN were suspended in Krebs-Ringer bicarbonate buffer at pH 7.4 (2.5×10^5 PMN/mL) containing 2 μ M scopoletin, 20 nM horseradish peroxidase, and 5 mM glucose, and were stimulated by 0.1 μ g/mL PMA. The amount of H_2O_2 released was determined by measuring the decrease of fluorescence of scopoletin. The peak excitation and emission wave lengths of scopoletin were 355 and 460 nm, respectively. Values represent mean $\pm$ SEM from the number of the subjects as indicated.

$^\dagger p < 0.01$, $^\ddagger p < 0.05$ when compared to adults' values.

increased with their age, but there was no significant difference as compared with the adults' values.

H_2O_2 release and the conversion rate from O_2^- to H_2O_2 of PMN. H_2O_2 release and the conversion rate from O_2^- to H_2O_2 of PMN obtained from the term newborn infants at 0 d of age and the adults are shown in Table 3. Again, the PMA-induced O_2^- production rate of PMN obtained from the term newborn infants at 0 d of age was significantly lower than that of the adults' PMN ($p < 0.01$). Also the amount of H_2O_2 produced by the neonatal PMN was significantly lower than that produced by the adults' PMN ($p < 0.05$). Although the conversion rate from O_2^- to H_2O_2 of the neonatal PMN was slightly higher than that of the adults' PMN, there was no statistically significant difference ($p > 0.1$).

DISCUSSION

Strauss *et al.* (23) demonstrated that SOD activity in PMN measured by the xanthine oxidase-cytochrome C system was similar in newborn infants, their mothers, and adult controls. However, their assay was not appropriate to measure SOD activity in crude PMN homogenates, because the mixture included other substances capable of directly reducing cytochrome C (24).

We could not obtain reliable results with the same method. To solve this problem, we used an NBT reduction assay on PAGE, and the results indicated a high correlation with the SOD content measured by ELISA. Accordingly, both methods are useful for measuring Cu,Zn-SOD in a very small quantity of crude extracts. In our study, both the activity and the content of Cu,Zn-SOD of PMN of the term newborn infants and the VLBW infants were lower than the adults' PMN (Table 1). Decreased activity and content of Cu,Zn-SOD in the neonatal PMN might be attributed to the fact that newborn infants have a lower serum copper concentration than adults (26).

There have been several reports studying decreased functions of PMN caused by autooxidative reactions. Decreased PMN functions such as chemotaxis, phagocytosis, and bactericidal activity can be induced by exposing PMN to oxidants (27). Skosey *et al.* (28) reported that products of oxidative metabolism adversely influenced lysosomal enzyme release from PMN and SOD retrieved enzyme release in those cells. PMN preexposed to cytotoxins showed decreased spontaneous and chemotactic migratory responses, whereas these changes were not observed in chronic granulomatous disease patients (29).

We have shown that the conversion rate from O_2^- to H_2O_2 of the neonatal PMN was similar to that of the adults' PMN (Table 3). This finding suggests that neonatal PMN have an equal ability to convert O_2^- to H_2O_2 as adults' PMN, although the neonatal PMN had a lower SOD activity than the adults' PMN (Table 1). There are two possible explanations for these results: 1) O_2^- is dismutated more spontaneously than enzymatically in PMN, or, 2) the concentrations of SOD in the microenvironment in PMN are sufficient to convert O_2^- in both term newborn infants and adults, although the total content is different between the two. In either case, the low content of Cu,Zn-SOD in PMN of the

term newborn infants or the VLBW infants may not account for the autooxidative damage. However, the term neonatal PMN generated a lower amount of H_2O_2 than the adults' PMN as expected from a decreased production rate of O_2^- , and this may have caused a decreased hydroxyl radical production (12) which resulted in defective bactericidal activity in the neonatal PMN.

There have been several reports studying the O_2^- production rate of neonatal PMN (8–12), but the results have been varied. Some studies have demonstrated equal O_2^- production rates of PMN from healthy term newborn infants when compared to adults' PMN (8–10). However, it was also reported that the O_2^- production rate of PMN from infants delivered vaginally, or by cesarean section with labor, was lower than that of adults' PMN (11). And the O_2^- production rate was higher in PMN of neonates than that in adults' PMN when opsonized zymosan was used as a stimulant (12).

In this report, we have shown that the PMA-induced O_2^- production rate of PMN from the healthy term newborn infants was significantly lower than that of the adults' PMN (Table 2). However, the FMLP-induced O_2^- production rate of PMN from the healthy term newborn infants was similar to that of the adults' PMN (Table 2). The difference between the O_2^- production rate elicited by PMA or FMLP may be due to the different modes of action of these two stimuli. PMA directly stimulates protein kinase C in PMN, whereas FMLP binds the surface receptors on the PMN cell membranes to elicit phosphatidylinositol metabolism and subsequent responses (30–32). But the exact reason for the different results with these two stimulants is unclear.

The PMA- or FMLP-induced O_2^- production rate of PMN from the VLBW infants at 0 d of age was higher than that of the term newborn infants' PMN ($p < 0.005$) (Table 2). PMN of stressed neonates have been reported to produce higher amounts of O_2^- than those of adults or healthy term newborn infants (10). We did see a statistical difference in the O_2^- production rate between PMN of the VLBW infants with respiratory distress syndrome and those of the other VLBW infants ($p > 0.2$). The O_2^- production rate of PMN of the hypoglycemic VLBW infants did not differ from the values of the other VLBW infants ($p > 0.1$). None of the VLBW infants was given any medication other than glucose infusion at the sampling time at 0 d of age. Therefore, the difference in the O_2^- production rate between the VLBW infants' PMN and the term infants' PMN may not be attributed to specific complication such as respiratory distress and hypoglycemia.

The exact reason that the VLBW infants' PMN had a higher O_2^- production rate than did the term infants' PMN is unclear. However, O_2^- may be one of the major causes of retinopathy of prematurity and bronchopulmonary dysplasia, which are known as the side effects of the use of oxygen as a therapeutic agent (33, 34). The fact that PMN of the VLBW infants had the capacity to produce higher amounts of O_2^- than that of healthy term newborn infants may indicate that PMN contribute to the occurrence of bronchopulmonary dysplasia or retinopathy of prematurity of the VLBW infants. The exact role of PMN in triggering these diseases remains to be clarified.

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