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Detection of defective granulocyte function with flow cytometry in newborn infants

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Reactive oxygen species (ROS) production by phagocytic leukocytes is a critical factor in immunity against microorganisms. Human neonates are susceptible to overwhelming infections, for which abnormal granulocyte function may play an important role. In this study, we aimed to identify a convenient and quantitative method to measure ROS production by human granulocytes after cellular activation. We first compared the results of a flow cytometric assay with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF) probe or dihydrorhodamine-123 (DHR123) and an enhanced chemiluminescence test using granulocytes from a patient with chronic granulomatous disease and normal granulocytes stimulated with different concentrations of phorbol myristate-13-acetate. Peripheral blood granulocyte respiratory burst ability from 37 newborn babies with different gestational ages was then quantified using flow cytometric assay with H_2DCF probe. We found that flow cytometric assay of ROS production is sensitive and correlates with chemiluminescence measurements. The results showed that activated granulocytes from neonates with higher birth body weights and more advanced gestational ages tend to have higher levels of ROS production in respiratory burst (p=0.0042 and 0.063, respectively). This study demonstrates that flow cytometry is suitable for detecting the functional defects in granulocyte ROS production in human neonates of different gestational age.

Key words: Chemiluminescent measurements, flow cytometry, newborn infant, premature infant, respiratory burst

Developmental immaturity is believed to play an important role in the development of severe infection, an important contributor to the mortality and long-term morbidity in neonates [1,2]. While functional deficiencies of antibody, complements, and T lymphocytes are known to contribute to this susceptibility, abnormal granulocyte function appears to be a major host defense abnormality in neonates [3,4]. The ability of phagocytes to produce reactive oxygen species (ROS) is essential in order for neutrophils to kill infectious micro-organisms. Patients who have mutations in their genes encoding NADPH oxidase, which is responsible for producing ROS in granulocyte respiratory burst, have a severe immunodeficiency disease termed chronic granulomatous disease (CGD) and are susceptible to recurrent bacterial and fungal infections [5]. Likewise, weak granulocyte respiratory burst may predispose newborn infants to severe infections. An efficient method for measuring the strength of respiratory burst in a clinical setting is thus necessary for identifying these high-risk neonates. In the present study, we aimed to determine granulocyte respiratory burst ability in neonates with different gestational ages by using a convenient flow cytometric method.

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Materials and Methods

Patients

The study group included 42 admitted afebrile newborn infants, without apparent anomaly, at the Newborn Section of the Department of Pediatrics, National Cheng-Kung University Hospital, Tainan, Taiwan. Written consents were obtained from their primary caretakers. Five patients were excluded due to sepsis during their hospitalization. Among them, 4 were identified by positive bacterial culture results, and the other by a documented maternal fever. The 37 included neonates had gestational ages ranging from 25 to 42 weeks (mean ± standard deviation gestational age, 33.0 ± 4.5 weeks; birth weight, 2.0 ± 0.87 kg). Blood samples for respiratory burst assays were taken along with samples for routine hemogram examination. Total granulocyte

counts of the blood samples ranged from 3000 to 10,000/μL and did not appear to correlate with gestational age (not shown). Tests were performed within 48 h of birth.

Leukocyte preparation

Blood specimens (0.5 mL) from healthy volunteers or newborn infants were collected in tubes containing potassium salt of ethylenediamine tetra-acetic acid (K_3EDTA) and were tested immediately after blood sampling. We used Liu's stain to determine the percentage of granulocytes and Turk's solution to determine the leukocyte number per cubic millimeter. Fifteen mL of preheated lysis buffer (1 mM EDTA, 155 mM NH_4Cl, 20 mM $NaHCO_3$) was added to each sample. The mixture was incubated at 37°C for 3-5 min until the red blood cell lysis was complete. The mixture was centrifuged at 400 *g* for 5 min at 37°C to pellet leukocytes. Leukocyte preparations containing equal numbers of granulocytes were used for the subsequent experiments.

Reagents

Catalase stock solution consisted of catalase (Sigma Chemical Company, St. Louis, MO, USA) dissolved in suspension buffer and stored in 10 μL aliquots at –80°C at a concentration of 1400 U/mL. 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF; Molecular Probes, Eugene, OR, USA) was prepared daily by dissolving 24.5 mg of H_2DCF in 5 mL of ethanol, and then making a 1:1000 dilution of this solution for a 1.0×10^4 nM (4.8×10^3 ng/mL) working solution. Dihydrorhodamine-123 (DHR123; Molecular Probes) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 57.7 mM, and stored in 10 μL aliquots at –80°C and then used after making a 1:200 dilution of stock solution. Phorbol myristate-13-acetate (PMA; Sigma) was dissolved in DMSO to give a 2 μg/μL stock solution and stored at –80°C. Working PMA was diluted to the desired concentration by suspension buffer.

Measurement of respiratory burst by flow cytometry

Leukocyte preparations containing 2×10^6 granulocytes were suspended in 300 μL of suspension buffer (0.1% w/v albumin, 1 mM EDTA in 1X Hanks' balanced salt solution) and preincubated with 4 μM H_2DCF or DHR123 for 15 min at 37°C, followed by activation of the oxidase with different concentrations of PMA for 20 min at 37°C. After the activation, catalase was added to the cell suspension to avoid diffusion of ROS between cells. The samples were then stored on ice and analyzed with a flow cytometer (Epics XL/MCL; Beckman Coulter, Miami, FL, USA) within 1 h. Granulocytes were gated based on forward scatter (size) and sideward scatter (granularity) and analyzed for their fluorescence intensity. 5000 granulocytes were recorded for each sample.

Measurement of respiratory burst by chemiluminescence test

In chemiluminescence (CL) experiments, respiratory burst activity was determined by enhanced CL with the Chemiluminescence Analyzer System (Tohoku Electronic Inc., Japan). Briefly, leukocyte preparations containing 8×10^5 granulocytes were suspended in suspension buffer, followed by activation with different concentrations of PMA for 10 min at 37°C. After activation, the CL count was measured in a completely dark chamber of the CL analyzing system for 200 sec. Lucigenin (2×10^{-5} M) or luminol (2×10^{-5} M) was then added to the sample and the enhanced CL was measured. CL counts were detected continually for a total of 600 sec and the mean CL counts of 10-sec intervals were recorded. Some samples were measured for 420 sec, when lucigenin or luminol was added at 100 sec.

Statistical analysis

Lineal regression was used to analyze the relationship between granulocyte ROS production ability and gestational age or birth body weight. Student's *t* test was used to analyze the ROS production ability of granulocytes from different patient groups. All the analyses were conducted using STATA version 8.0 (Stata Corporation, College Station, TX, USA).

Results

Flow cytometric analysis and CL measurement were performed on granulocytes from healthy adults. The cells were stimulated with different concentrations of PMA ranging from 80 to 400 ng/mL to generate increasing amounts of ROS. Higher CL counts were detected after stimulation with higher concentrations of PMA (Fig. 1A). Similarly, the mean fluorescence intensities of stimulated granulocytes also increased with increased PMA stimulation with H_2DCF (Fig. 1B) or DHR123 (Fig. 1C). Although both probes used in flow cytometry tended to generate higher levels of fluorescence in cells producing more ROS, marked

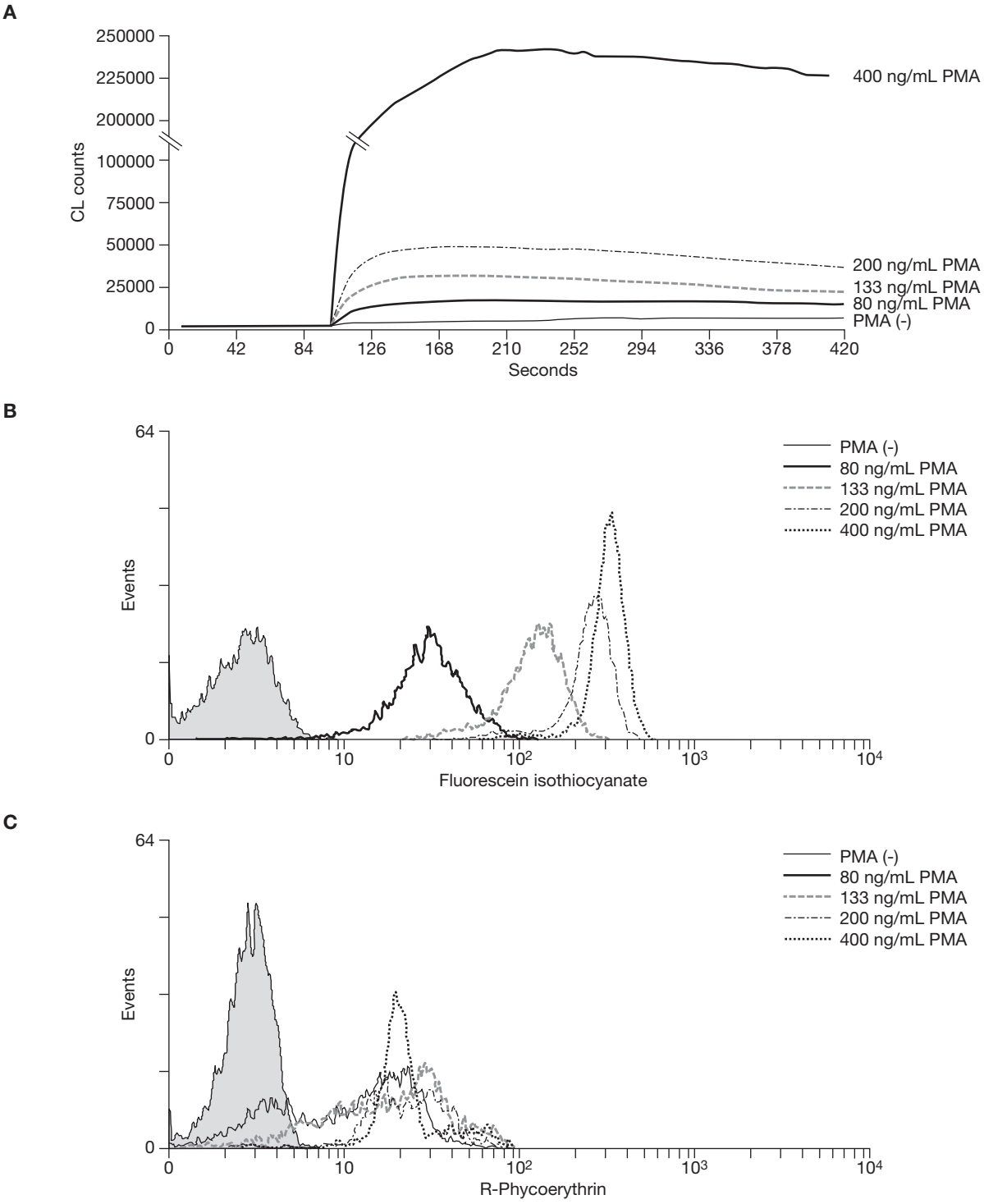

Fig. 1. Dose-dependent reactive oxygen species (ROS) production by activated granulocytes measured with flow cytometry and chemiluminescence (CL) detection. Respiratory burst response of granulocytes without stimulation or stimulated with phorbol myristate-13-acetate (PMA) [80 ng/mL, 133 ng/mL, 200 ng/mL, and 400 ng/mL] was measured by CL detection (A) or flow cytometry with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF) [B] or flow cytometry with dihydrorhodamine-123 (DHR123) (C). The mean fluorescence intensities tested by H_2DCF for granulocytes treated with 0 ng/mL, 80 ng/mL, 133 ng/mL, 200 ng/mL, and 400 ng/mL PMA were 2.33, 29.63, 113.17, 234.62 and 286.57. The mean fluorescence intensities tested by DHR123 for granulocytes treated with 0 ng/mL, 80 ng/mL, 133 ng/mL, 200 ng/mL, and 400 ng/mL PMA were 2.81, 11.81, 17.6, 25.87 and 31.43. Similar results were observed in 3 repeats of the experiment.

differences were noted in groups using H_2DCF and DHR123. While cellular fluorescence was more homogeneous in cells probed with H_2DCF (Fig. 1B), the fluorescence was more widespread in terms of fluorescence intensity in the cell population probed with DHR123 (Fig. 1C). Moreover, the fluorescence intensity increased progressively in cells probed with H_2DCF, while the fluorescence intensity in DHR123-probed cells appeared to reach a plateau in cells producing higher amounts of ROS. The differences between using H_2DCF and DHR123 to measure the amount of ROS production were tested using leukocytes from different subjects (Fig. 2). While a dose-dependent increase in H_2DCF-generated fluorescence was evident in granulocytes stimulated with increasing concentrations of PMA (Fig. 2A), little increase in DHR123-generated fluorescence was noted in cells stimulated with higher (200 ng/mL and 400 ng/mL) concentrations of PMA (Fig. 2B). We hence used H_2DCF in the following experiments to measure the strength of PMA-stimulated granulocyte respiratory burst.

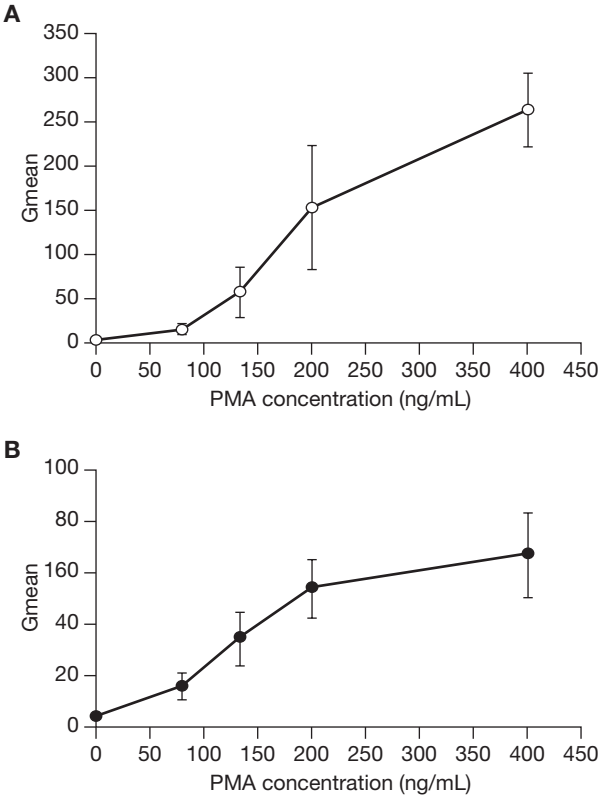

Fig. 2. Dose-dependent reactive oxygen species production by activated granulocytes measured with flow cytometry with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF) and dihydrorhodamine-123 (DHR123). Respiratory burst response of granulocytes without stimulation or stimulated with phorbol myristate-13-acetate (PMA) [80 ng/mL, 133 ng/mL, 200 ng/mL, and 400 ng/mL] was detected by flow cytometric analysis with H_2DCF (A) and DHR123 (B). The Gmean value of granulocytes after stimulation by different concentrations of PMA are shown as mean and standard error of the mean of 4 experiments using cells from subjects.

We then compared the chemiluminescent and flow cytometric methods in measuring ROS production by granulocytes from a CGD patient after stimulation with PMA (400 ng/mL). No ROS production could be detected in CGD granulocytes with the CL method (Fig. 3A). However, slightly increased cellular fluorescence was detected in leukocytes from the CGD patient after stimulation with PMA (Fig. 3B). As this CGD patient has been characterized as having an X91-gene mutation [6], minimal amount of intracellular ROS may be produced after stimulation.

In order to examine the ROS production of activated granulocytes from newborn infants with flow cytometry, the leukocytes were stimulated with PMA at a suboptimal concentration of 200 ng/mL and then analyzed by flow cytometry. The results are shown as stimulation index (SI) values, calculated as follows:

$$\text{SI} = \frac{\text{Mean channel fluorescence intensity of PMA-stimulated granulocytes}}{\text{Mean channel fluorescence intensity of unstimulated granulocytes}}$$

There was a significant trend showing that SI tended to increase with the increase of birth body weight (R^2=0.2165; p=0.0042). The SI values for infants with birth body weight ≤2000 g were significantly lower than those of infants with higher birth body weight (p=0.01) [Fig. 4A]. Our results thus show that respiratory burst ability of PMA-stimulated granulocytes increases with the increase of birth body weight. We also analyzed the relationship between gestational age and strength of respiratory burst in these neonates. We found a similar trend of increase in respiratory burst activity with the advancement of gestational age (R^2=0.0978, p=0.063). Moreover, the SI values in infants with gestational age ≤34 weeks were significantly lower than those in infants with more advanced gestational ages (p=0.02) [Fig. 4B].

Discussion

Human neonates are susceptible to severe bacterial and fungal infections [7-12]. While a number of abnormalities have been described in the host defense system of newborn infants [2,8], defective granulocyte

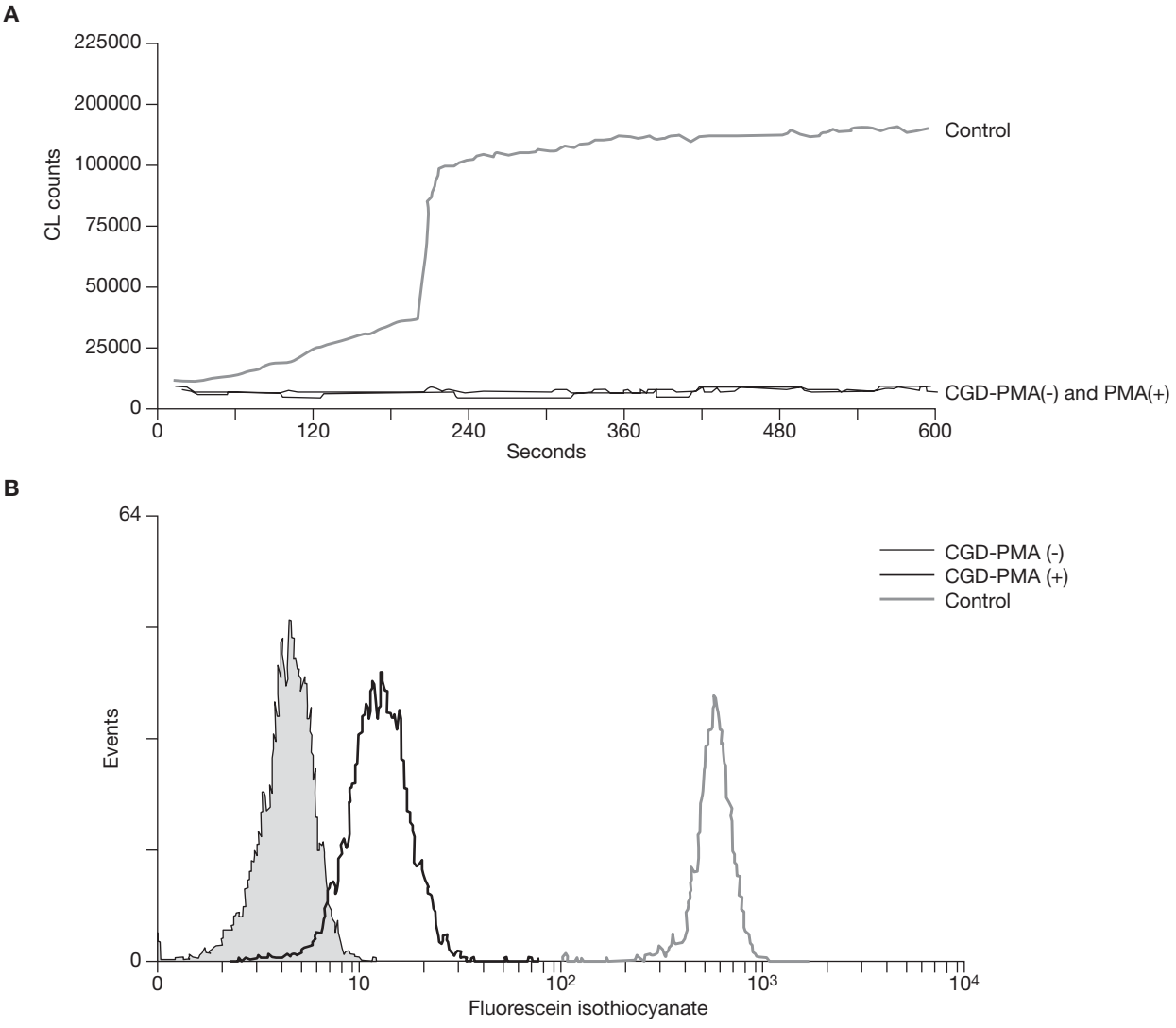

Fig. 3. Reactive oxygen species production by activated granulocytes from an X91-chronic granulomatous disease (CGD) patient. Respiratory burst response of X91-CGD granulocytes without stimulation or stimulated with phorbol myristate-13-acetate (PMA) [400 ng/mL] was measured by chemiluminescence (CL) detection (A) or flow cytometry (B). Granulocytes from a normal volunteer were used as controls. The mean fluorescence intensities of unstimulated and stimulated CGD granulocytes were 4.34 and 12.27, respectively. The mean fluorescence intensity for the normal control was 552.97.

function has been known to be among the most apparent deficiencies. Wright et al [13] first observed that the granulocytes of stressed neonates with a variety of infectious and non-infectious illnesses had decreased respiratory burst ability and bactericidal activity when compared to healthy infants [4]. A number of investigators have since evaluated granulocyte oxidative metabolism in newborn infants without active infection. Among those studies, a few groups also included preterm infants [9,14-16]. Driscoll et al evaluated granulocyte ROS production with a CL method in preterm infants and reported that ROS production capability during the first week of life was equal to that of healthy adults [17]. Another study by Bektas et al also showed similar results [18]. On the contrary, Usmani et al noted that within the first 10 days of life, granulocytes from stable preterm neonates demonstrated significantly decreased capability of ROS production when compared to granulocytes from healthy full-term neonates and adults [3]. Peden et al reported that in neutrophils from preterm neonates, PMA stimulation did not yield differences between severely and moderately preterm neonates. However, neutrophils from both preterm groups had significantly less capability of ROS production after stimulation than did cells from either adult or term neonatal groups [19] .

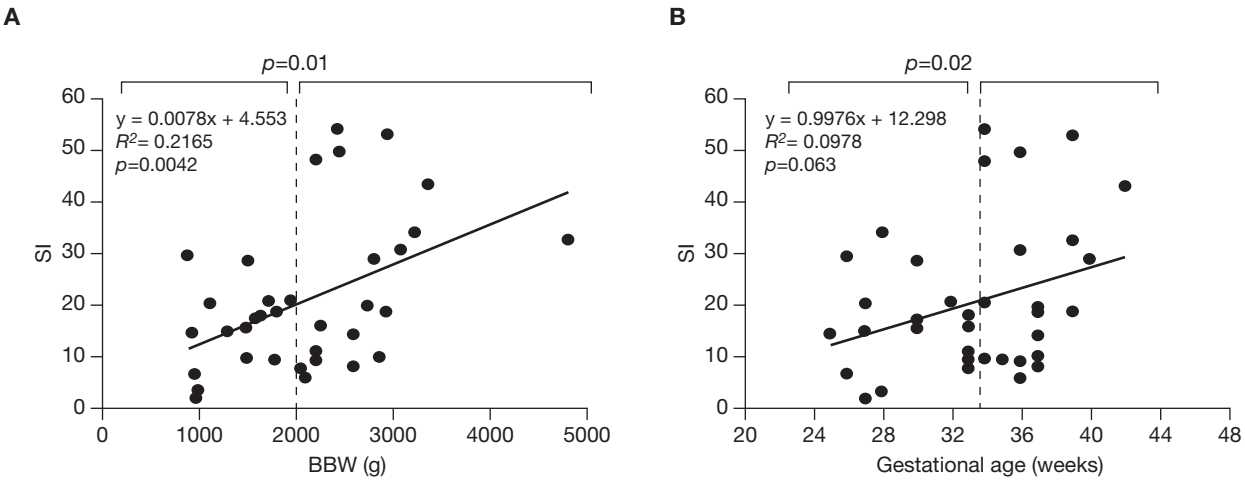

Fig. 4. Flow cytometric measurements of reactive oxygen species production by granulocytes from neonates with different birth body weight and gestation age. Respiratory burst stimulation index (SI) of granulocytes from each neonate was determined by the ratio of mean fluorescence intensities with or without stimulation of phorbol myristate-13-acetate (400 ng/mL). The relationship between SI and birth body weight (BBW) [A] and gestational age (B) are shown. The correlation between SI and BBW or gestation age was analyzed by linear regression. Student's *t* test was used to evaluate differences between groups.

In this investigation, we aimed to clarify whether prematurity affects the amount of ROS production in granulocyte respiratory burst and, meanwhile, establish a clinically feasible way to measure this critical immune function. Several different methods are currently used to investigate the production of ROS, such as the cytochrome C reduction assay, CL test, the nitroblue tetrazolium (NBT) test, or assays based on fluorogenic dyes [20-22].

Earlier tests using NBT reduction have been suggested as relatively non-quantitative for assessing the ability of granulocyte ROS production. In human neonates, the amount of blood available for testing is relatively limited. An ideal assay thus should require only a small blood volume and be technically simple. Among these methods which are used to evaluate the respiratory burst ability of granulocytes, flow cytometry tests best satisfy these requirements. Flow cytometry can be used to detect ROS production at a single-cell level using small amounts of blood and is widely available in clinical settings. Thus, we compared the results of flow cytometry and CL detection, which is one of the most sensitive methods for ROS detection. We used the same numbers of leukocytes stimulated with different concentrations of PMA, which triggers NADPH oxidase-mediated production of superoxide through activation of protein kinase C [23,24]. In the CL test used in this study, lucigenin reacts with superoxide and produces CL [25]. In the flow cytometric analysis, H_2DCF or DHR123 probes were used. Non-fluorescent H_2DCF is trapped intracellularly and oxidized to highly fluorescent DCF in the presence of ROS. H_2DCF was originally reported to be sensitive only to H_2O_2 [26,27], but it has recently been reported to be also sensitive to other ROS [28]. DHR123 is freely permeable through the plasma membrane, localized in the mitochondria, and after oxidation by H_2O_2 and O_2^- to rhodamine-123, emits a bright fluorescent signal upon excitation by blue light (488 nm) [22]. Based on the dose-dependent ROS production in response to PMA stimulation in this investigation, the relative fluorescence intensity generated by H_2DCF correlated with the superoxide anion generation measured with CL in activated granulocytes. Although the 2 probes were both effective flow cytometric probes for assessing the oxidative burst in human granulocytes for the diagnosis of CGD, our results showed that the value of DHR123 as a probe to measure the amount of ROS production is limited by the fact that the maximal amount of fluorescence was generated in cells stimulated with suboptimal concentrations of PMA. Flow cytometric analysis with H_2DCF thus appears to be an appropriate test for measuring the strength of respiratory burst in granulocytes in small amounts of human whole blood. Thus, we performed flow cytometric analysis with H_2DCF.

We also used CL and H_2DCF flow cytometric methods to measure the respiratory burst of granulocytes from a CGD patient who has an inherited gene defect in the leukocyte NADPH oxidase FAD binding site [6]. In our study, CL revealed no response to stimulation of

granulocytes from this CGD patient. To our surprise, a small amount of ROS could be detected by flow cytometry after PMA stimulation. This fluorescence was not due to autofluorescence induced by PMA stimulation because no fluorescence was detected in granulocytes from normal subjects or the CGD patients without addition of the H_2DCF probe (data not shown). This result suggests that the flow cytometric method is more sensitive in measuring intracellular ROS than the chemiluminescent method.

Given that flow cytometry is a widely available cellular assay in clinical settings where newborn infants are cared for, we chose flow cytometric analysis with H_2DCF to evaluate respiratory burst ability of granulocytes in premature neonates. Our study revealed that premature neonates with lower birth body weights and less advanced gestational ages tend to have lower ROS production in respiratory burst after stimulation with PMA. The increased strength of respiratory burst with increase in birth body weight was most significant when comparing neonates with birth body weight more than 2000 g with those with lower birth body weights. We also found a similar increase in level of ROS response in neonates with gestational ages more than 34 weeks. Infants with birth body weights of 2000 g and gestational ages of 34 weeks are both in the category of moderate prematurity based on our standard curve. This data thus suggests that both birth body weight and gestational age should be considered as significant clinical factors for pinpointing neonates with low ROS-producing ability. Due to the fact that gestational age and birth body weight may significantly deviate from each another, the maturation state of ROS production ability by granulocytes in each neonate should be determined individually.

We conclude that premature neonates have lower respiratory burst ability of granulocytes than mature neonates. This may explain in part the greater sensitivity to bacterial infection in this group of smaller newborn infants, underscoring the importance of preventive measures in premature infants. Flow cytometric assay for leukocyte respiratory burst correlates with chemiluminescent measurements in the clinically relevant range and is a feasible method for identifying high-risk patients in this high-risk population.

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