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Alterations in neonatal neutrophil function attributable to increased immature forms

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ABSTRACT

At birth neonatal neutrophil composition differs from that of adults due to a higher number of circulating immature forms. To date only a single study has evaluated neutrophil performance based on cell maturity. For this study, we examined functional differences in chemotaxis and phagocytosis between neonatal and adult neutrophils based on cell development and labor exposure. Methods: Neutrophils were obtained by venipuncture from adults and cord blood from healthy term neonates delivered vaginally or by cesarean section. Transwells and the chemoattractant fMLP were used to evaluate chemotaxis. Phagocytosis assays were performed using GFP-labeled *E.coli* (RS218) and whole blood. Neutrophil maturation was measured by an accurate and verified flow cytometry technique using the markers CD45, CD11b, and CD16. QuantiGene Plex and Procarta immunoassays were used to determine cytokine and chemokine gene expression and protein concentration, respectively. Results: Labor exposure did not alter neonatal neutrophil function in this study. Neonatal and adult mature neutrophils performed chemotaxis and phagocytosis equally well, while immature forms showed marked impairments. Neonatal immature granulocytes, though, completed chemotaxis more proficiently than those of adults. Although cytokine and chemokine levels varied between neonatal and adult groups, no differences were detected in neonates based upon labor exposure. Conclusion: Historically documented functional impairments of neonatal neutrophils may be due to the increased number of developmentally immature forms at birth rather than absolute global deficiencies.

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1. Background

Neonates, especially very low birth weight premature infants, are at an increased risk for infection after birth. Because of a relative deficiency in adaptive immune responses from lack of antigen exposure in utero, researchers have focused on innate immune responses with particular attention to neutrophils, since they are the first immune cells to respond to infection or inflammation. Numerous well-conducted studies have characterized global deficiencies in neonatal neutrophil function following birth. For example, impairments of transmigration through the vascular endothelium has been shown to result from (1) a reduced number of key membrane surface receptors, i.e. Mac-1 (CR-3, CD11b/CD18) and selectin [1–4,5,6–10], (2) impaired competency of signal transduction [5,11], (3) decreased mobilization of intracellular calcium [10], and (4) diminished concentrations of chemokines and cytokines from resident tissue macrophages and neutrophils [1]. Moreover, clinical stressors resulting from both infectious and non-infectious etiologies, such as premature birth and respiratory distress syndrome, have also been shown to further hinder neutrophil function [1–4,12,13].

The composition of the circulating neutrophil pool also differs significantly between neonates at birth and adults, but its impact on overall neutrophil performance remains mostly unexplored. Multiple studies have documented an increased number of the earliest developmental forms (including promyelocytes, myelocytes, and metamyelocytes), termed immature granulocytes, in neonates compared to adults but methods used for measurements have varied [14,15]. Using flow cytometric techniques to measure neutrophil composition, we have previously demonstrated that these early neutrophil precursors comprise 12% of circulating neutrophils in newborns, irrespective of labor exposure, but only 5% in healthy adults [15]. To date, however, only a single

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study has investigated differences in neutrophil function based on cell maturity. In 1982, using microscopic evaluations of cell morphology to classify neutrophils as bands, bilobed, or multilobed, Boner and colleagues calculated that 30% of neonatal and 9% of adult neutrophils are immature and concluded that chemotactic ability was directly related to cell maturity with neonatal immature cells performing the least well [16].

Neutrophil development is reflected in the production of granule constituents [17–20]. Granulopoiesis, or the formation of granules within the maturing neutrophil, begins with azurophilic granules that form between the myoblast and promyelocyte stages of development and are rich in bactericidal proteins. These initial granules are followed by the formation of specific granules (myelocyte to metamyelocyte stage), gelatinase granules (band stage), and, finally, secretory vesicles that appear in mature, segmented neutrophils [18–21]. Secretory vesicles and gelatinase granules are important in the earliest stages of neutrophil-mediated inflammatory responses because they serve as the reservoirs for membrane-associated receptors [18]. Expulsion of granule substances, or granule exocytosis, occurs in reverse order with secretory vesicles being extruded first and azurophilic last. Thus, immature granulocytes have reduced concentrations of cell substrates necessary to mount a robust, effective response in the early stages of infection. Additionally, immature neutrophil numbers increase during times of stress or infection, which may explain variances in "healthy" and "ill" neonatal patients. For this study, we hypothesized that functional differences between neonatal and adult neutrophils, including chemotaxis and phagocytosis, would be directly related to differences in neutrophil composition and not generalized global deficiencies.

We investigated differences in chemotaxis and phagocytosis for immature granulocytes (promyelocytes, myelocytes, and metamyelocytes) and mature neutrophils (bands and mature, segmented forms) in healthy newborns (≥37 weeks' gestational age) born either by spontaneous vaginal delivery or by primary cesarean section without labor, and compared them to cells from healthy adult volunteers. Additionally, we evaluated a focused subset of pro-inflammatory cytokines and chemokines to determine if variations in their expression profiles would correlate to any identified functional differences.

2. Methods

2.1. Neutrophil isolation

Neutrophils were purified from the cord blood of healthy term infants, delivered vaginally (n = 18) or by scheduled cesarean section without labor (n = 18), and from the peripheral blood of healthy adults (n = 16), using PolymorphPrep™ (Axis Shield, Oslo, Norway) according to the manufacturer's instructions. Informed consent was obtained from healthy, adult volunteers and mothers who were expected to deliver a healthy term infant at the Women and Newborn Pavilion of the Children's Hospital at the University of Oklahoma (OU) Medical Center. Participants were enrolled in accordance with an approved protocol by the University of Oklahoma Health Sciences Center's (OUHSC) Institutional Review Board (IRB). In brief, blood was collected into sterile tubes containing sodium citrate as an anticoagulant (Becton Dickinson, Franklin Lakes, NJ). Peripheral neutrophils were prepared from the anti-coagulated blood using gradient separation layering on PolymorphPrep™ in a one-to-one ratio. Adult samples were centrifuged at 450 ×*g* for 35 min at 20 °C in a swing-out rotor. PolymorphPrep™ was diluted to 90% with double distilled water for isolation of neonatal samples. The samples were then centrifuged at 400 ×*g* for 30 min at 20 °C in a swing-out rotor. Neutrophils were collected from the interphase, washed in Ca^{2+} and Mg^{2+}-free Hanks's balanced salt solution (Life Technologies, Grand Island, NY), and collected by centrifugation at 400 ×*g* for 12 min at 20 °C. The supernatant was aspirated and contaminating red blood cells were lysed with the addition of 10 ml ice cold double distilled water via 15 s vortex, followed by the addition of 5 ml 3.6% NaCl and raising the volume to 50 ml with Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS; Life Technologies, Grand Island, NY).

2.2. Labeling for flow cytometry, flow cytometry, and cell sorting

Purified neutrophils were collected by centrifugation at 400 ×*g* for 12 min at 20 °C and resuspended in 100 μl of RPMI (Life Technologies, Grand Island, NY) with 10% native serum and gently vortexed for 3 s. Neutrophils were labeled with the addition of 15 μl of the following: CD16 (clone: NKP15) fluorescein isothiocyanate (FITC); CD11b (clone: D12) phycoerythrin (PE); and CD45 (clone: 2D1) (Becton Dickinson, Franklin Lakes, NJ). These were then incubated for 30 min in the dark at room temperature. After incubation, we added 400 μl of RPMI with 10% native serum and samples were placed at 4 °C until analyzed by flow cytometry. No samples were stored for >12 h.

Flow cytometric analysis and cell sorting was performed on an Influx cell sorter (Becton Dickinson, Franklin Lakes, NJ) located in the Flow and Image Cytometry Laboratory at OUHSC. Forward scatter (FSC), side scatter (SSC), and two-color fluorescence signals (FITC and PE at 531/40 and 572/21 nm, respectively) were collected and stored in list mode data files. A total of 10,000 neutrophil events were recorded for each sample, and a total of 50,000 cells were collected from each of the following groups: adult total neutrophils, adult mature neutrophils (bands and segmented forms), neonatal total neutrophils, neonatal mature neutrophils (bands and segmented forms), and neonatal immature granulocytes (promyelocytes, myelocytes, and metamyelocytes). The instrument settings were fixed for all data collection. Throughout the study, we performed quality control on the instrument before each measurement was taken using Flow Check beads (Beckman Coulter, Miami, FL). List mode data files were analyzed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

2.3. Chemotaxis

One day before chemotaxis assays were performed, a Transwell 12-mm membrane containing 3-μm-pores (Corning Life Sciences, Tewksbury, MA) was coated with 2.5 μg/ml fibrinogen (Sigma-Aldrich Corp., St. Louis, MO) for each sample. To coat, 600 μl was added to the bottom of the Transwell and 200 μl was added to the top, and the membrane was incubated at 37 °C for 1 h. After incubation, the Transwells were washed twice with PBS. The PBS wash was aspirated off and the Transwells were dried overnight in a laminar flow hood. Purified neutrophils were counted and resuspended at a concentration of 4×10^5 cells in 200 μl Gey's Buffer (Life Technologies, Grand Island, NY). Cells were added to the top of the Transwell. Chemoattractant, 1×10^{-7} M of *N*-formyl-methionine-leucine-phenylalanine (fMLP; 600 μl; #F-3506, Sigma-Aldrich, St. Louis, MO) or buffer alone for control, was added to the bottom of the Transwell. Cells were allowed to migrate for 2 h at 37 °C and 5% CO_2. Post incubation, 60 μl of 0.5 M EDTA was added to the bottom chamber and the plate was incubated for 15 min at 4 °C. The number of cells was then quantified with an automated cell counter (Bio-Rad, Hercules, CA). Chemotaxed neutrophils were stained with 10 μl anti-human CD11b allophycocyanin (APC) and anti-human CD16 phycoerythrin (PE) (eBioscience, San Diego, CA) and incubated 30 min at room temperature in the dark. Neutrophils were pelleted at 500 ×*g* for 5 min and the media aspirated. Cells were resuspended in 500 μl 1-step Fix/Lyse Solution (eBioscience, San Diego, CA) for 30 min at room temperature in the dark. Cells were then pelleted at 500 ×*g* for 5 min and the media aspirated and resuspended in 500 μl PBS for assessment of neutrophil composition by flow cytometry. Immature granulocytes included promyelocytes, myelocytes, and metamyelocytes, whereas mature neutrophils comprised band and segmented forms.

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