DEFECTIVE MEMBRANE POTENTIAL CHANGES IN
NEUTROPHILS FROM HUMAN NEONATES

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A number of studies have been carried out to determine the functional activity of polymorphonuclear leukocytes (PMN) from human neonates in an attempt to explain their unusual susceptibility to infection. Although neonatal PMN are capable of ingesting and killing microorganisms (1, 2), chemotaxis of these cells is markedly impaired in vitro and in vivo (3-7). The mechanism(s) involved in the pathogenesis of abnormal neonatal PMN chemotaxis are unknown. Miller (6) has reported that neonatal PMN have a decreased ability to deform following exposure to chemotactic stimuli, while Anderson and co-workers (7) found that neonatal PMN fail to form uropods and to redistribute adhesion sites. The decreased ability of these cells to change shape and form surface projections suggests that the functional defect may be at the level of the cell membrane. There does not appear to be a significant abnormality in the interaction between chemotactic factor and cell membrane-bound chemotactic factor receptors on resting neonatal PMN at 37°C (7, 8). The defect may be, therefore, at a level beyond this initial interaction.

The recent introduction of spectrofluorometric techniques for measuring critical ionic changes at the membrane and in the intracellular milieu has led to a better understanding of the events associated with phagocyte activation (9-14). Such probes include the cyanine dye 3-3 dipentyloxacarbocyanine [DiOC₂(3)], which is sensitive to changes in membrane potential (11, 12, 15), and Quin 2/AM (13, 16), which assesses changes in intracellular free calcium. We have used these two fluorescent probes to assess the cellular events following chemotactic factor-receptor interaction in PMN from human neonates and compared the results with those obtained in PMN from adults.

Materials and Methods

PMN Preparations. Whole blood was obtained from peripheral veins of healthy adults and from the umbilical cords of normal-term neonates in acid citrate dextrose (Becton Dickinson and Co., Rutherford, NJ). The blood was allowed to settle in 1% dextran (Pharmacia, Piscataway, NJ) and the PMN were separated on Ficoll-Hypaque (Pharmacia) gradients. Contaminating erythrocytes were hypotonically lysed, and the PMN suspensions (98%) were washed in phosphate-buffered saline (PBS, pH 7.4) and suspended in a modified Hanks' balanced salt solution (HBSS) containing 124 mM NaCl, 4 mM KCl, 0.64 mM NaH₂PO₄, 0.66 mM K₂HPO₄, 15.2 mM NaHCO₃, 10 mM Hepes buffer, 5.56 mM glucose, and 1.6 mM CaCl₂ or 2 mM EGTA. The pH was adjusted to 7.4.

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Membrane Potential Changes. Membrane potential changes were monitored using a spectrofluorometric technique that employs the fluorescent dye DiOC₅(3) (15). 100 μ l of 10^{-5} M DiOC₅(3) (Molecular Probes, Junction City, OR) were added to 2 ml of the cell suspension containing 5×10^6 PMN/ml. Fluorescence emission was measured over time using an SLM Aminco spectrofluorometer (Urbana, IL) with the excitation wave length set at 470 nm and the emission wavelength at 495 nm. The cells were kept in suspension using a magnetic stirrer, and changes in fluorescence were recorded at 1, 5, and 10 min after FMLP stimulation.

Changes in Intracellular Free Calcium. Changes in intracellular free calcium were estimated using the calcium-sensitive dye Quin 2/AM (Calbiochem, La Jolla, CA) (13, 16). The dye was diluted in dimethyl sulfoxide (Mallinckrodt, St. Louis, MO) to a concentration of 10^{-2} M; 2 μ l of this solution was then added to 1 ml of a PMN solution containing 10^8 PMN/ml in calcium-containing HBSS. The cells were incubated for 10 min in a rotary shaker and then diluted 10-fold in HBSS with or without calcium and allowed to incubate for one hour at 37°C. At the end of this incubation period the cells were washed and resuspended (10^7 PMN/ml) in calcium-containing HBSS (1.6 mM) or calcium-deprived HBSS to which 2 mM EGTA had been added. Fluorescence was recorded using the SLM Aminco spectrofluorometer with the excitation wavelength set at 339 nm and the emission wavelength set at 492 nm. Changes in fluorescence were recorded at 1, 2, 3, and 5 min after stimulation with FMLP.

Chemotactic Factor Preparation. Formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma Chemical Co., St. Louis, MO) was diluted in PBS to 10^{-5} M and stored in aliquots at -70°C until use. Before each set of experiments, the solution was diluted appropriately in HBSS and 50 μ l was added to 2 ml of the cell suspension. Tritium-labeled FMLP was obtained from New England Nuclear, Boston, MA. Zymosan-activated serum was prepared by incubating 10 ml of fresh human serum with 100 mg of wash zymosan (Schwarz-Mann, Orangeburg, NY) for 60 min at 37°C in a rotating rack. Following centrifugation, the clarified activated serum was stored in aliquots at -70°C . For use the activated serum was diluted in HBSS to the appropriate concentration.

Calcium Antagonist. Verapamil, a calcium antagonist capable of blocking calcium channels as well as interfering with the release of stored intracellular calcium, was diluted in HBSS to a final concentration of 10^{-4} M (17-19).

FMLP Binding. Total and specific FMLP binding to PMN was performed exactly as described by Anderson and co-workers (7).

Results

PMN from adults had a marked change in membrane potential following stimulation with FMLP in concentrations ranging from 10^{-9} to 10^{-7} M. As shown in Fig. 1, DiOC₅(3) fluorescence emission increased 25% ($P < 0.001$) 1 min after exposure of PMN from 11 adults to 2.5×10^{-8} M FMLP, and the response persisted at 5 and 10 min. In striking contrast, PMN from 10 normal-term neonates failed to show any significant change in DiOC₅(3) fluorescence after FMLP stimulation. Similar results were obtained when 10% zymosan-activated serum containing activated C5a was employed. It should be pointed out that the level of fluorescence in resting, unstimulated neonatal cells exposed to DiOC₅(3) was essentially the same as that in resting adult PMN. This indicates that dye penetration and resting membrane potential were probably equivalent in the cells of neonates and adults. Binding of tritiated FMLP (2.5×10^{-8} M) to PMN from 9 adults and 10 neonates in the presence or absence of an excess of unlabeled FMLP (10^{-5} M) at 37°C was not significantly different. (Total binding in the absence of unlabeled FMLP was 12,123 cpm $\pm$ 1,735 SE in adults vs. 9,165 cpm $\pm$ 905 in neonates; NSD. Specific binding was 8,600 cpm $\pm$ 1,459 with adult PMN vs. 7,854 $\pm$ 967 in neonatal PMN; NSD.)

FIGURE 1. Membrane potential changes induced in PMN from 11 adults and 10 neonates by exposure to 2.5×10^{-8} M FMLP as determined by DiOC₅(3) fluorescence emission. Data includes results from five experiments in which adult PMN were exposed to 10^{-4} M verapamil for 15 min before FMLP stimulation.

There was no significant difference in the responses of adult cells to FMLP when calcium-deprived media was employed (Fig. 2). In contrast, when adult cells were exposed to the calcium antagonist verapamil (10^{-4} M), the response to FMLP was very similar to that of the neonatal PMN. The results were similar when 10% zymosan-activated serum was employed as the chemoattractant. These data suggested to us that calcium plays a role in the membrane potential changes observed in FMLP-stimulated PMN.

Additional studies were carried out using the calcium-sensitive probe Quin 2/AM. This dye enters the cytoplasm of cells, after a period of incubation, where it is sensitive to changes in the concentration of free calcium (13, 16). As shown in Fig. 2, the PMN from 10 adult donors showed a marked change in Quin 2/AM fluorescence following FMLP stimulation (47–51%). A change in Quin 2/AM fluorescence was also detected following FMLP stimulation of PMN from 10 healthy, term neonates (Fig. 2). The response was significantly lower ($P < 0.01$ – 0.05) than that of the adult PMN at each of the time intervals studied, however. Again, the baseline level of fluorescence in neonatal cells was essentially the same as that in adult cells, indicating normal penetration of the dye and comparable levels of free calcium. The responses to zymosan-activated serum were similar to those using FMLP with adult and neonatal PMN.

The alteration observed in Quin 2/AM fluorescence emission following stimulation of adult and neonatal PMN with FMLP was not appreciably changed when the experiment was carried out in calcium-deprived media to which 2 mM EGTA had been added (Fig. 2). Addition of the calcium antagonist verapamil (10^{-4} M) to the PMN 15 min before FMLP stimulation essentially abolished the change in free calcium levels observed in untreated adult or neonatal cells exposed to this chemoattractant (Fig. 2).

FIGURE 2. Changes in intracellular free calcium in FMLP-stimulated (2.5×10^{-8} M) PMN from 10 adults and 10 neonates as determined by Quin 2/AM fluorescence emission. Calcium-containing (1.6 mM) and calcium-depleted (no added calcium + 2 mM EGTA) media were employed as indicated. The bottom lines represent the responses of PMN from 10 adults and 10 neonates that were incubated with 10^{-4} M verapamil for 15 min before FMLP stimulation.

Discussion

Adult PMN responded to FMLP stimulation with a marked change (31%) in membrane potential as measured by DiOC₅(3) fluorescence. In striking contrast, PMN from neonates showed essentially no change in membrane potential following stimulation with FMLP. Since calcium is required for chemotaxis, and this cation contributes to changes in membrane potential (11–14), we next assessed the effects of FMLP on changes in intracellular free calcium in neonatal and adult PMN. In each instance, PMN from adults and neonates showed significant increases in free intracellular calcium, as indicated by Quin 2/AM fluorescence. The response of neonatal PMN was markedly decreased, however, when compared to that of PMN from adults.

Chemotaxis is a function at least partially dependent upon calcium (20, 21). Korchak and associates (22) have recently indicated that FMLP produces a rise in cytosolic calcium that is one of the earliest events in the process of PMN activation. In fact, they reported that chemotactic levels of FMLP elicited optimal increments in cytosolic calcium without triggering degranulation or superoxide production. We found that the level of extracellular calcium did not directly affect membrane potential changes (data not shown) and, surprisingly, did not alter the increase in intracellular free calcium induced by FMLP. This suggests that the increase is due mainly to mobilization of membrane or intracellular calcium stores. The calcium antagonist verapamil blocks calcium channels responsible for entry of calcium into cells, but also interferes with the release of intracellular calcium (17–19). This agent completely abolished the change in

membrane potential observed in adult PMN exposed to FMLP, making their response identical to that of the cells from neonates. Clearly, calcium is not the only factor involved, as other ionic events such as sodium and potassium exchange may also affect membrane potential and may be abnormal in neonatal PMN.

Summary

In an attempt to determine the mechanism of the profound defect in chemotaxis observed in the polymorphonuclear leukocytes (PMN) of human neonates, we have examined membrane potential changes and alterations in free intracellular calcium following chemotactic factor stimulation. Following exposure to formyl-methionyl-leucyl-phenylalanine (FMLP), PMN from adult donors (11) showed a marked change in membrane potential (31%) as determined by fluorescence emission using the cyanine dye, 3-3-dipentylloxacarbocyanine [DiOC₅(3)]. In marked contrast, FMLP-stimulated PMN from 10 human neonates failed to show any significant change in membrane potential (1–2%). Using the calcium-sensitive probe Quin 2/AM, FMLP induced an increase in fluorescence of up to 51% in adult PMN (10). In contrast, the change in intracellular free calcium induced in neonatal PMN was much less (32%; $P < 0.01$). These results suggest that the profound defect in chemotactic responsiveness of PMN from human neonates may result from an inability of these cells to undergo changes in membrane potential following inflammatory mediator stimulation.

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