Effect of Fibronectin on Adherence of Neutrophils from Newborn Infants

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Neutrophils (polymorphonuclear leukocytes [PMNs]) from infants and adults increased in adherence over a 20-min incubation as measured in a morphological assay. At each time point, PMNs from healthy infants had significantly diminished adherence when compared with those of adults; PMNs from sick newborn infants showed intermediate adherence values. Moreover, PMNs from healthy infants had significantly diminished membrane fluorescence for fibronectin when compared with those of adults. Preincubation with exogenous fibronectin did not augment the adherence of PMNs from infants or adults. Deficient adherence of PMNs from newborns may be related to decreased membrane fibronectin.

Infection remains a significant cause of morbidity and mortality during the newborn period, despite recent advances in infant intensive care and the use of broad-spectrum antimicrobial agents. Several factors appear responsible for the increased susceptibility of the neonate to bacterial sepsis, including the virulence and size of the bacterial challenge, perinatal complications, and prematurity. However, the functional immaturity of neonatal host defense systems is probably the major factor influencing the risk of bacterial infection (17). Previous studies of the immune function in newborn infants have identified functional deficiencies of circulating antibody (16) and complement (8, 18) and defects of cellular function, including chemotaxis (1, 6), phagocytosis (11), oxidative metabolism (24), and intracellular killing (22, 29).

Neutrophil (polymorphonuclear leukocyte [PMN]) adherence is an important premigratory event in the initiation of the inflammatory response. Recent investigations of PMN adherence in newborn infants have produced conflicting results, primarily related to the method of blood preparation (e.g., whole blood or separated PMNs) and the assay system used (e.g., endothelium, glass beads, or nylon wool) (3, 9, 13, 23). The adherence of PMNs from preterm infants and critically ill neonates with respiratory distress syndrome has not been studied.

Fibronectin (FN), an adhesive high-molecular-weight glycoprotein present on cell surfaces and in plasma, is a critical component of cell-to-cell and cell-to-substrate attachment (13). Recent studies in our laboratory (10, 30) and by other investigators (15) have demonstrated diminished plasma FN concentrations in healthy term and premature newborn infants as compared with healthy adults. Further depression of plasma FN has been observed in infants with respiratory distress syndrome, perinatal asphyxia, and bacterial sepsis (10, 30).

The purposes of this study were (i) to compare the adherence of PMNs from sick and healthy newborn infants during the first few days of life, (ii) to investigate the effect of severe illness on the adherence of neutrophils from newborn infants, (iii) to investigate the effects of exogenous FN on

PMN adherence in newborns, and (iv) to determine whether there are quantitative differences in membrane-bound FN between PMNs from healthy infants and adults.

MATERIALS AND METHODS

Patient population. The study population consisted of 13 sick and 25 healthy newborn infants and 30 healthy adult volunteers (Table 1). The sick newborn infant population included nine infants with respiratory distress syndrome, two infants with aspiration syndromes, and two infants with group B streptococcal sepsis, admitted to the Infant Intensive Care units at the Children's Hospital of Philadelphia and the Hospital of the University of Pennsylvania. All sick infants required intermittent mandatory ventilation at the time of study and were investigated within 72 h of birth.

Healthy term infants were enrolled from the Obstetrical Service at the Hospital of the University of Pennsylvania. Blood (15 ml) was obtained from doubly clamped cord segments at delivery. Blood from sick newborn infants was obtained from in-dwelling, central venous or arterial catheters within the first 4 days of life. Written permission was obtained from parents for all blood samples in accordance with the policies of the Human Investigation Committee at the Children's Hospital of Philadelphia and the Hospital of the University of Pennsylvania. Control blood was obtained from healthy adults routinely used as donors for our laboratory. On each occasion, PMNs from healthy or sick newborn infants were tested simultaneously with those from an adult control.

Preparation of leukocytes. Equal volumes of heparinized whole blood from newborn or adult donors and 3% dextran in normal saline were sedimented at 37°C for 30 min. The leukocyte-rich plasma was removed, and the cells were washed twice in phosphate-buffered saline (PBS) at 4°C at 400 × g. The leukocytes were layered on gradients containing Mono-Poly resolving medium (Ficoll-Hypaque, density 1.114; Flow Laboratories, Inc.) and spun at 800 × g for 45 min at room temperature (4). The interface containing PMNs was removed and washed twice in PBS at 400 × g at 4°C. Cells were counted in 2% acetic acid and trypan blue and suspended to 0.5 × 10⁶ or 1 × 10⁶ PMNs per ml for use in

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TABLE 1. Study population^a

Group	Birth wt (g)	Gestational age (wk)	Apgar score at min:		Study age (days)	Leukocyte count (10 ³) per mm ³
			1	5		
Healthy newborn infants	3,285.3 (±122.4)	38.8 (±2.0)	8.1 (±1.4)	8.9 (±0.7)	Birth ^b	— ^c
Sick newborn infants	2,144.8 (±889.8)	33.8 (±4.1)	6.9 (±3.1)	7.9 (±1.8)	2.6 (±1.5)	17.4 (±4.4)

^a All data are mean values (±standard deviations).^b Samples were obtained from cord blood at delivery.^c Data not obtained.

adherence or FN fluorescence procedures. Cell suspensions characteristically contained greater than 95% viable PMNs.

Preincubation with exogenous FN. Neutrophils (10⁶/ml) were suspended in 500 µl of FN (Sigma Chemical Co.) at various concentrations (10, 75, 125, or 250 µg/ml) and tumbled at 37°C for 30 min. Control cells were tumbled in 500 µl of PBS. Cells were washed, counted, suspended in PBS at 10⁶ PMNs per ml, and adhered to glass cover slips for 5, 10, and 20 min. Time points beyond 20 min were not studied because of technical limitations of the assay system (drying of cover slips and loss of PMN monolayer viability).

Binding of protein-coated MS to PMNs. The method used for binding of microspheres (MS) to PMNs was a modification of the method of Pommier et al. (21). Fluorescent MS (Covaspheres-MX; Covalent Technology Corp., Ann Arbor, Mich.) with diameters of 1.0 µm were bound to FN (FN-MS) or bovine serum albumin (BSA-MS). MS suspension (10 µl) was mixed with 10 µg of protein (FN or BSA) in 100 µl of PBS, sonicated, and incubated at room temperature for 1 h. The coated spheres were washed twice in RPMI 1640 with 1% BSA, suspended to 0.5 ml, and sonicated. Preparations were examined microscopically to ensure a suspension of unaggregated particles. The beads were washed and sonicated before incubation with neutrophils. PMNs (5 × 10⁵/ml) were microfuged for 30 s, suspended in 100 µl of PBS–0.05% azide to inhibit endocytosis of MS, and tumbled with MS for 30 min at 37°C. PMNs were then separated from free beads by centrifugation through 1 ml of 6% BSA in RPMI 1640 for 25 s at 12,000 × g. The cell pellet was suspended in PBS, adhered to cover slips coated with poly-L-lysine, and examined with a Zeiss fluorescence microscope with an epi-illuminator. Cells binding at least three MS were considered positive, and the percentages of PMNs positive for MS bearing each protein were determined by counting 100 to 200 cells under blinded conditions.

Neutrophil adherence. Neutrophils (2 × 10⁵) in 200 µl of PBS were incubated on glass cover slips (diameter, 15 mm) at 37°C for 5, 10, and 20 min. Cover slips were washed and

stained with Wright's solution, and the number of adherent PMNs was calculated by using a 10-mm square grid. Duplicate cover slips were counted blindly at each time point.

FN fluorescence. PMNs (5 × 10⁵) from healthy infants and adult controls were tumbled in 100 µl of paraformaldehyde at 4°C for 15 min. After fixation, the cells were washed in PBS (4°C at 400 × g) and tumbled in 100 µl of a 1:2 dilution of fluorescein-conjugated F(ab')₂ fragment of goat anti-human FN (Cappel Laboratories) for 30 min at 4°C. Neutrophils were washed three times in PBS, adhered to cover slips coated with poly-L-lysine, and examined with a Zeiss fluorescence microscope. Under blinded conditions, 100 PMNs were counted and classified as positive or negative for membrane fluorescent staining.

In addition, because heparin interacts with FN and neutrophil membranes, FN fluorescence was compared on three occasions by using adult PMNs collected into heparin or EDTA.

Statistics. Data between patient groups were analyzed by two-way repeated-measure analysis of variance. Multiple time comparisons within groups were analyzed by a one-way analysis of variance. Data from FN preincubation and FN fluorescence experiments were analyzed by dependent and independent Student's *t* tests, respectively.

RESULTS

Neutrophil adherence. Neutrophils from healthy and sick newborn infants demonstrated a significant increase in adherence through 10 min (*P* < 0.001); however, there was no further increase in PMN adherence at the 20-min time point.

PMNs from healthy newborn infants demonstrated significantly diminished adherence when compared with PMNs from adults at 5, 10, and 20 min. Neutrophils from sick newborn infants, however, demonstrated adherence values that were intermediate between healthy infants and adults (Table 2). At 10 and 20 min, the adherence of neutrophils from sick newborn infants was significantly greater than that from healthy infants (*P* < 0.02 and *P* < 0.005, respectively). There were no significant differences in PMN adherence between sick newborn infants and healthy adult controls at any time point. Neutrophil adherence did not correlate with birth weight, gestational age, study age, Apgar scores, or peripheral leukocyte count for the sick infant population.

FN preincubation. After preincubation with 250 µg of FN per ml, there was a minimal increase in the adherence of PMNs from adults (13%; *P* < 0.05) only at the 10-min time point. This concentration of FN did not affect the adherence of PMNs from newborn infants (*P* not significant). Preincubation with 10, 75, or 125 µg of FN per ml did not significantly increase the adherence of neutrophils from healthy infants or adults (data not shown).

Binding of protein-coated MS to PMNs. Examination of neutrophils from adults (*n* = 5) demonstrated that 59.0 ± 5.5% (mean plus or minus the standard error of the mean) of

TABLE 2. Neutrophil adherence

Time (min)	Mean ± SEM PMN adherence (10 ⁵) in group ^a :		
	1	2	3
5	1.09 (±0.04) ^b	0.82 (±0.06)	0.93 (±0.06)
10	1.43 (±0.07) ^b	1.10 (±0.06) ^c	1.35 (±0.06)
20	1.53 (±0.06) ^d	1.14 (±0.06) ^b	1.48 (±0.09)

^a Groups: 1, adults; 2, healthy neonates; 3, sick neonates.^b *P* < 0.005 (*P* values refer to comparisons between groups at single time points); group 1 versus 2, 5 min; group 1 versus 2, 10 min; and group 2 versus 3, 20 min.^c *P* < 0.02; group 2 versus 3, 10 min.^d *P* < 0.001; group 1 versus 2, 20 min. There was no significant difference in PMN adherence between sick newborn infants and adults.

PMNs specifically bound FN-MS. In contrast, less than 25% of PMNs from adults bound BSA-MS.

FN fluorescence. PMNs from healthy newborn infants demonstrated significantly diminished membrane fluorescence for FN when compared with those of adults ($73.7 \pm 4.9\%$ fluorescent cells versus $88.7 \pm 6.7\%$ [$P < 0.02$]). There was no significant difference in the percentage of fluorescent-positive cells in heparin or EDTA samples.

DISCUSSION

This study compared PMN adherence for newborn infants and adults, investigated the effects of exogenous FN on granulocyte adherence, and determined quantitative differences in membrane-bound FN between PMNs from healthy newborn infants and adults. Neutrophils from sick and healthy infants and healthy adults demonstrated an increase in adherence between the 5- and 10-min time period, which did not increase significantly at 20 min. At each time point, neutrophils from healthy infants had significantly diminished adherence when compared with those from adults; PMNs from sick newborn infants showed intermediate adherence values. Moreover, neutrophils from healthy infants had significantly diminished membrane fluorescence for FN when compared with those of adults. Preincubation with exogenous FN had little effect on the adherence of PMNs from infants or adults.

Neutrophil adherence is an important cell event which is essential for subsequent margination on vascular endothelium, diapedesis, and chemotaxis. Defects in PMN chemotaxis have been associated with abnormalities of adhesion in neonates (1) and older children (7) and may enhance susceptibility to severe bacterial disease. Previous investigations of the adherence of PMNs from healthy infants have yielded conflicting results. Fontan et al. demonstrated equivalent adherence of neutrophils from neonates and adults by using glass bead columns and heparinized whole blood (9), whereas other investigators have found diminished (glass and nylon wool assays [13]) or increased granulocyte adherence (nylon fiber [23]). Recent studies have investigated the role of FN in the adherence of PMNs from adults. Brown and Lackie (5) found that FN inhibited PMN adherence to glass cover slips, both when FN was present as a coat on the cover slip and when it was present in the fluid phase in the medium. In contrast, Wall et al. demonstrated enhanced granulocyte adherence to washed endothelial cells, preincubated with FN treated with dithiothreitol (26) to stabilize sulfhydryl groups. The discrepancy between these studies may be related to the choice of substrate (cover slip versus vascular endothelium), the FN preparation (untreated or dithiothreitol treated), or the methods of cell and substrate preparation after FN treatment.

Using immunofluorescence and immunoferritin localization, Weissmann et al. (27) and Hoffstein et al. (12) concluded that PMNs display FN on their surfaces and transfer it to substrates to which they adhere. These studies suggested that neutrophils have the capacity to both synthesize FN and take up exogenous FN from the surrounding medium. Pommier et al. (21) have used FN bound to MS to demonstrate that PMNs have specific binding sites for FN on the plasma membrane. However, unlike mononuclear cells which develop an increase in C3b, C3bi, and Fc receptors in the presence of FN (2, 20, 28), neutrophils do not become actively phagocytic (21) or release FN (25) unless they are preexposed to C5a or formyl peptides.

In this study, we have demonstrated that PMNs from adults bind FN-coated MS at the cell membrane. However,

despite FN-MS binding, neutrophil adherence to cover slips is not significantly increased after preincubation with fluid-phase FN. Pearlstein and Gold (19) have suggested that fluid-phase FN will not bind to cells and that a conformational change in FN, induced by binding to other molecules such as MS, is required for cell binding. In contrast, Pommier et al. (21) have demonstrated that fluid-phase FN will bind to cell membrane receptors; however, they speculate that aggregated FN may have greater avidity.

We have investigated PMN adherence in sick, premature infants with acute respiratory illnesses requiring mechanical ventilation. Under these conditions, adherence was significantly enhanced when compared with that of healthy term neonates and was not significantly different from adult controls. In a similar fashion, Lentnek et al. (14) have shown augmented granulocyte adherence which is mediated by a plasma factor in adults with inflammatory processes. Increased adherence during severe neonatal illness may be related to neutrophil activation or the maturational stage of the PMN. Boner et al. (3), however, have recently shown that the adherence of granulocytes from cord blood is unaffected by the stage of neutrophil differentiation. Investigations of FN receptors on neutrophils may elucidate further the mechanisms for improved PMN adherence in sick newborn infants.

The mechanism responsible for the diminished neutrophil adherence response of healthy newborn infants is unknown. It is possible, however, that the diminished adherence of neutrophils from newborn infants may be related to FN deficiency, since healthy term and preterm infants have diminished plasma FN levels (30) and FN has been shown to be an important factor in PMN adhesion (27). The current study demonstrates that neutrophils from healthy newborn infants have significantly diminished membrane fluorescence for FN, although PMN adherence is not increased after FN preincubation. We speculate that PMNs from newborn infants are deficient in the uptake of exogenous FN or that the number or function of FN-binding sites is decreased. Deficiencies of neutrophil adherence in newborn infants may diminish the magnitude of the inflammatory response and increase susceptibility to infection.

ACKNOWLEDGMENTS

We thank Dorothy Zurlo for editorial assistance.

This study was supported by the Thrasher Research Fund and by Public Health Service grants 1 RO1 HL 27068 and 1 PO1 NS 17752 from the National Institutes of Health.

LITERATURE CITED

1. Anderson, D. C., B. J. Hughes, and C. W. Smith. 1981. Abnormal motility of neonatal polymorphonuclear leukocytes. *J. Clin. Invest.* **68**:863-874.
2. Bevilacqua, M. P., D. Amrani, M. W. Mosesson, and C. Bianco. 1981. Receptors for cold-insoluble globulin (plasma fibronectin) on human monocytes. *J. Exp. Med.* **153**:42-60.
3. Boner, A., B. J. Zeligs, and J. A. Bellanti. 1982. Chemotactic responses of various differentional stages of neutrophils from human cord and adult blood. *Infect. Immun.* **35**:921-928.
4. Boyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97):9-29.
5. Brown, A. F., and J. M. Lackie. 1981. Fibronectin and collagen inhibit cell-substratum adhesion of neutrophil granulocytes. *Exp. Cell Res.* **136**:225-231.
6. Christensen, R. D., and G. Rothstein. 1980. Efficiency of neutrophil migration in the neonate. *Pediatr. Res.* **14**:1147-1149.
7. Crowley, C. A., J. T. Curnette, R. E. Rosen, J. A. Schwartz, J. I. Gallin, M. Klempner, R. Snyderman, F. S. Southwick, T. P. Stossel, and B. M. Babior. 1980. An inherited abnormality of

- neutrophil adhesion. *N. Engl. J. Med.* **302**:1163-1168.
8. Fireman, P., D. A. Zuchowski, and P. M. Taylor. 1969. Development of human complement system. *J. Immunol.* **103**:25-31.
 9. Fontan, G., F. Lorente, J. Rodriguez, and J. A. Ojeda. 1979. Granulocyte adherence in umbilical cord blood. *J. Pediatr.* **94**:969-970.
 10. Gerdes, J. S., M. C. Yoder, S. D. Douglas, and R. A. Polin. 1983. Decreased plasma fibronectin in neonatal sepsis. *Pediatrics* **72**:877-881.
 11. Harris, M. C., J. Stroobant, C. S. Cody, S. D. Douglas, and R. A. Polin. 1983. Enhanced phagocytosis of group B streptococcus from newborn infants. *Pediatr. Res.* **17**:358-361.
 12. Hoffstein, S., G. Weissmann, and E. Pearlstein. 1981. Fibronectin is a component of the surface coat of human neutrophils. *J. Cell Sci.* **50**:315-327.
 13. Krause, P. J., E. G. Maderazo, and M. Scroggs. 1982. Abnormalities of neutrophil adherence in newborns. *Pediatrics* **69**:184-187.
 14. Lentnek, A. L., A. D. Schreiber, and R. R. MacGregor. 1976. The induction of augmented granulocyte adherence by inflammation. *J. Clin. Invest.* **57**:1098-1103.
 15. McCafferty, M. H., M. Lepow, T. M. Saba, E. Cho, H. Meuwissen, J. White, and S. F. Zuckerbrod. 1983. Normal fibronectin levels as a function of age in the pediatric population. *Pediatr. Res.* **17**:482-485.
 16. Miller, M. E. 1969. Phagocytes in the newborn infant: humoral and cellular factors. *J. Pediatr.* **74**:255-259.
 17. Miller, M. E. 1978. Phagocytic cells, p. 59-71. In T. K. Oliver (ed.), *Host defenses in the human neonate*. Grune and Stratton, New York.
 18. Notarangelo, L. D., G. Chirico, A. Chiara, G. Colombo, G. Rondini, A. Plebani, A. Martini, and A. G. Ugazio. 1984. Activity of classical and alternative pathways of complement in preterm and small for gestational age infants. *Pediatr. Res.* **18**:281-285.
 19. Pearlstein, E., and L. I. Gold. 1978. High-molecular-weight glycoprotein as a mediator of cell adhesion. *Ann. N.Y. Acad. Sci.* **312**:278-292.
 20. Pommier, C. G., S. Inada, L. F. Fries, T. Takahashi, M. M. Frank, and E. J. Brown. 1983. Plasma fibronectin enhances phagocytosis of opsonized particles by human peripheral blood monocytes. *J. Exp. Med.* **157**:1844-1854.
 21. Pommier, C. G., J. O'Shea, T. Chused, K. Yancey, M. M. Frank, T. Takahashi, and E. J. Brown. 1984. Studies on fibronectin receptors of human peripheral blood leukocytes. *J. Exp. Med.* **159**:137-151.
 22. Quie, P. G., and E. L. Mills. 1979. Bactericidal and metabolic function of polymorphonuclear leukocytes. *Pediatrics* **64**(Suppl.):719-721.
 23. Rao, S., R. Olesinski, U. Doshi, and D. Vidyasagar. 1981. Granulocyte adherence in newborn infants. *J. Pediatr.* **98**:622-624.
 24. Shigeoka, A. O., R. P. Charette, M. L. Wyman, and H. R. Hill. 1981. Defective oxidative metabolic responses of neutrophils from stressed neonates. *J. Pediatr.* **98**:392-398.
 25. Spagnuolo, P. J., M. M. Lederman, J. Schiltz, J. Ellner, and L. Culp. 1982. A possible role for cold insoluble globulin in chemotactic factor mediated polymorphonuclear leukocyte adherence to plastic. *Biochem. Biophys. Res. Commun.* **106**:471-478.
 26. Wall, R. T., S. L. Cooper, and J. C. Kosek. 1982. The influence of exogenous fibronectin on blood granulocyte adherence to vascular endothelium in vitro. *Exp. Cell Res.* **140**:105-109.
 27. Weissmann, G., E. Pearlstein, H. D. Perez, S. Falkow, I. M. Goldstein, and S. T. Hoffstein. 1980. Neutrophils synthesize and deposit fibronectin on surfaces to which they will attach. *Trans. Assoc. Am. Physicians* **93**:72-84.
 28. Wright, S. D., L. S. Craigmyle, and S. C. Silverstein. 1983. Fibronectin and serum amyloid P component stimulate C₃b and C₃bi mediated phagocytosis in cultured human monocytes. *J. Exp. Med.* **158**:1338-1343.
 29. Wright, W. C., B. K. Ank, J. Herbert, and E. R. Stiehm. 1975. Decreased bactericidal activity of leukocytes of stressed newborn infants. *Pediatrics* **56**:579-584.
 30. Yoder, M. C., S. D. Douglas, J. Gerdes, J. Kline, and R. A. Polin. 1983. Plasma fibronectin in healthy newborn infants: respiratory distress syndrome and perinatal asphyxia. *J. Pediatr.* **102**:777-780.