Abnormal Mobility of Neonatal Polymorphonuclear Leukocytes

RELATIONSHIP TO IMPAIRED REDISTRIBUTION OF SURFACE ADHESION SITES BY CHEMOTACTIC FACTOR OR COLCHICINE

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ABSTRACT To determine the mechanism(s) of diminished, stimulated, and directed migration of neonatal (N) polymorphonuclear leukocytes (PMN), chemotactic factor (CF) sensory and PMN effector functions were studied in healthy N and adult or maternal controls (C). N PMN demonstrated high affinity binding for *N*-formyl-methionyl-leucyl-[^{3}H]-phenylalanine (fMLP), which was saturable between 40 and 100 nM as observed with C PMN. The kinetics of binding and the characteristics of dissociation of binding by N PMN were equivalent to control PMN. Both "threshold" and "peak" concentrations (1 and 10 nM, respectively) of fMLP effected comparable PMN chemiluminescence among neonates and controls. An equivalent threshold concentration (0.05 nM) of fMLP effected N and C PMN shape change in suspension, and a maximally effective concentration (5 nM) induced comparable bipolar configuration, although uropod formation was only 38±8% of N PMN, compared with 73±11% of C PMN ($P < 0.01$). Striking abnormalities of N PMN adherence were identified: mean±1 SD base-line (unstimulated) N adherence values (39±8%) were equal to C (38±9%), but diminished increments in response to single CF stimuli were noted among N (fMLP: 42±7% (N), 70±11% (C); C5a: 41±6% (N), 68±6% (C); BCF: 41±6% (N), 63±9% (C), $P < 0.01$ for each CF). On sequential exposure to increasing concentrations of CF, N PMN failed to demonstrate expected decreased adherence values; sequential stimuli with fMLP (0.1 nM, 10 nM) or C5a (8 μg protein/ml, 32 μg protein/ml) effected mean±1 SD values of 51±9% (N), 30±9% (C), and 34±10 (N), 48±14% (C), respectively. As demonstrated with a latex bead-binding technique, N PMN failed to redistribute adhesion sites to the cell's tail under the same experimental conditions; in 21 N samples studied, restricted unipolar binding occurred in 33±8% (fMLP) or 37±7% (C5a) of PMN in contrast to C values of 70% (fMLP), or 71% (C5a), $P < 0.001$. Similar findings were observed when PMN were preincubated with colchicine (25 μg/ml); expected diminished adherence scores (compared with base-line values) were demonstrated with C PMN but not with N cells, $P < 0.01$. Additionally colchicine-induced redistribution of adhesion sites as was observed with C samples (72±14% unipolar binding) was significantly ($P < 0.001$) less among N PMN (31±11% unipolar binding). These investigations indicate that CF sensory mechanisms of N PMN are normal, compared with healthy adult or maternal controls. Diminished stimulated locomotion of the N PMN may be functionally related to reduced modulation of cell adhesiveness by chemotactic stimulation.

INTRODUCTION

Previous investigations of neonatal polymorphonuclear leukocyte (PMN)[1] motility have consistently

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[1] *Abbreviations used in this paper*: ACLB, albumin-coated latex beads; CF, chemotactic factor; CL, chemiluminescence; DPBS, Dulbecco phosphate-buffered saline; f-Met-Leu-[^{3}H]Phe, *N*-formyl-methionyl-leucyl-[^{3}H]phenylalanine; f-Met-Leu-Phe, *N*-formyl-methionyl-leucyl-phenylalanine; PMN, polymorphonuclear leukocyte.

demonstrated defects of both the humoral and cellular contributions to chemotactic function (1–9). Although it is generally agreed that the neonatal PMN is insufficient in its directed migratory response to chemotactic stimuli, the nature of this defect is not understood, and the underlying mechanisms have not been characterized. To allow a better understanding of the biological basis of impaired stimulated locomotion, studies of chemotactic factor (CF) "sensory" function, morphologic and adhesive properties of healthy newborn PMN were carried out with the aid of recently developed techniques (10–13). Investigations reported here suggest that CF sensory functions of the neonatal PMN do not account for their impaired directed migration. In contrast, profound abnormalities of CF or colchicine-induced alterations of PMN adherence and morphology have been identified. Specifically, an impaired redistribution of cell surface adhesion sites and diminished uropod formation by neonatal PMN have been observed and appear to be functionally related to their diminished migratory responses. These findings further extend our previous observations demonstrating the clinicopathologic importance of abnormalities of PMN adherence and morphology (14). In addition, they suggest that interrelated influences of CF on the configurational and adherence properties of PMN are critical functional determinants of their stimulated locomotion.

METHODS

Blood samples. 5–60 ml of heparinized venous blood was drawn from the placentas of normal full term ($n = 50$) or premature ($n = 10$) infants within 5 min of birth. Equivalent or larger samples (100–200 ml) were drawn from healthy maternal pairs or adult laboratory donors. All blood samples were processed for studies of PMN function <10 min after phlebotomy. Informed consent was obtained from all patients before obtaining blood samples.

Preparation of CF. Chemoattractant solutions were prepared for use in PMN mobility, adherence, shape change, latex bead binding, chemiluminescence (CL) and *N*-formyl-methionyl-leucyl-[^{3}H]phenylalanine (f-Met-Leu-[^{3}H]-Phe) binding experiments.

Stock solutions of *N*-formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe) (Sigma Chemical Co., St. Louis, Mo.) and f-Met-Leu-[^{3}H]phe (New England Nuclear, Boston, Mass.) were prepared in Dulbecco phosphate-buffered saline (DPBS) (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.). The radiochemical purity of this product as determined by high performance liquid chromatography before use was >99%. Labeled peptide was demonstrated to be biologically indistinguishable from f-Met-Leu-Phe in dose-response chemotaxis experiments; a concentration of ~10 nM of both labeled and unlabeled peptide effected maximal and equivalent directed migration of PMN in Boyden chambers while lower or higher concentrations effected relatively diminished responses (11, 14).

A low molecular weight chemotactic factor, referred to as C5a in this manuscript, was prepared from activated human sera by the method of Gallin and Rosenthal (15). This reagent as previously demonstrated in our laboratory was shown to exert maximal chemotactic effects at a concentration of 40 μg protein/ml (12, 14).

Bacterial chemotactic factor (BCF) was derived from *Escherichia coli* UCLA 0101 as previously described (14); a 1:2 dilution of this reactant effected maximal directed PMN migratory responses.

Isolation of PMN. Purified populations of PMN were obtained by the gradient centrifugation technique of Boyum (16). 5 ml of heparinized (10 U/ml) whole blood was layered over an equal volume of a Ficoll-Hypaque mixture (24 parts of Ficoll, Sigma Chemical Co.; 10 parts Hypaque, Winthrop Laboratories, New York). After centrifugation at 300 *g* for 30 min at 4°C, the interface layer was removed and the PMN/erythrocyte button was resuspended in 10 ml of DPBS. PMN were then isolated by dextran sedimentation, washed twice and resuspended in DPBS. Cell viability as determined by trypan blue dye exclusion was >95%. Final PMN suspensions contained >85% PMN. Minimal platelet contamination in these suspensions was observed and the erythrocyte:PMN ratio was <3:1. Final cell preparations were adjusted to a concentration of 10^7 or 10^8 PMN/ml DPBS. For use in CL or f-Met-Leu-[^{3}H]Phe binding studies, erythrocyte contamination was eliminated by hypotonic lysis of suspensions with distilled water (14).

Preliminary studies were performed to exclude the possibility that significant degranulation was occurring under experimental conditions chosen to evaluate motility, adherence, and morphology. PMN suspensions (5×10^6/ml) were stimulated with the maximum f-Met-Leu-Phe concentration (10 nM) used in our studies for 10 min at room temperature or at 37°C. Following centrifugation, PMN were lysed by suspending the cell button in Triton-X (10% in water) before repeated (five times) freezing and thawing. Supernates of lysed preparations and the supernates of the original cell suspensions were assayed for lysozyme activity, as previously described, with the substrate, *Micrococcus lysodeikticus* (17). For both neonatal and adult control suspensions, minimal (<2 μg/ml) or no lysozyme activity was detectable in supernates of unstimulated or f-Met-Leu-Phe-stimulated cell suspensions. Lysed cell preparations demonstrated 20–30 μg/ml lysozyme activity based on a standardized urine sample obtained from a leukemic patient. In all cases, >90% of total lysozyme of cell suspensions was detected within PMN after f-Met-Leu-Phe stimulation.

Assessment of neutrophil motility. Neutrophil motility into micropore filters was assessed by a modified Boyden technique with blind well chambers (Neuro Probe, Inc., Bethesda, Md.) and 3-μm millipore size filters (Millipore Corp., Bedford, Mass.) as previously described (14). Behavior of neutrophil populations in this system was evaluated in two ways: (*a*) the distribution of cells in the filters was evaluated at various times with an Optimax Image Analyzer, (Optimax, Inc., Colmar, Pa.) after loading the chemotaxis chambers and (*b*) the depths at which only two cells were in focus in one high power (×40) field ("leading front") were measured after incubation periods of 40–60 min. Leukotactic indices were calculated according to the method of Maderazo and Weronick (18) to determine the mean cellular migration of PMN suspensions. Directed migration of neonatal, maternal, or adult control PMN was assessed by placing neutrophil suspensions (2×10^6/ml, 0.2 ml) in 3% human serum albumin (Sigma Chemical Co.) in the cell compartment together with chemoattractants in the stimulant compartment. All experiments were performed in duplicate.

f-Met-Leu-[^{3}H]Phe binding assay. Varying concentrations of f-Met-Leu-[^{3}H]Phe and cells (10^8/ml) were incubated in 200 μl of DPBS at 37°C (unless otherwise indicated) with gentle mixing. Reactions were terminated at designated

time intervals by rapidly diluting incubation aliquots with 2 ml of cold (4°C) DPBS followed by rapid filtration and washing (5 ml, twice) of mixtures through Whatman GFC glass fiber filters (Whatman, Inc., Clifton, N. J.) Washed filters were placed into a 10-ml Insta-Gel scintillation cocktail (Packard Instrument Co., Inc., Downers Grove, Ill.); radioactivity was quantitated in a Packard Tri-Carb Liquid Scintillation Spectrometer. Nonspecific binding was defined as the amount of binding occurring in the presence of a 1,000-fold excess concentration of f-Met-Leu-Phe and was generally 10–30% of total binding values. Specific binding was defined as the total amount of f-Met-Leu-[^{3}H]Phe bound minus that proportion nonspecifically bound (10, 11).

Assessment of changes in neutrophil morphology. A modification of the method of Smith et al. (12) was used. Suspensions of 10^6 neutrophils/ml in DPBS were exposed to chemotactic factors or colchicine under varying conditions and fixed in cold (4°C) 1.5% glutaraldehyde. Neutrophils were examined with a ×100 phase-contrast objective and classified according to shape as previously defined (12).

Assessment of neutrophil adhesiveness. Chambers consisting of two 25-nm round cover glasses, rubber gaskets, and specially milled brass plates were used as previously described (13). Chambers were assembled after pretreatment of one coverglass substrate for 2 min in a solution of 5% pooled human serum in DPBS. Each chamber was filled with a suspension of neutrophils (5×10^5/ml), which was allowed to settle onto the pretreated glass substrate surface undisturbed at room temperature for 500 s. Chambers were then inverted for an additional 500 s. The number of cells remaining attached to the substrate surface was assessed by counting 15–20 randomly selected high power (×40) fields. The chambers were again inverted and the number of cells adhering to the untreated glass surface was also quantitated. Results were expressed as the percentage of neutrophils adhering to the treated surface over the total number of cells counted per high power field.

Assessment of binding of latex beads. Latex beads (0.6 μm) (Sigma Chemical Co.) were suspended in a solution of 1% human serum albumin and DPBS for 2 min at room temperature, washed, and resuspended in DPBS. Control or patient cells were exposed to 1% albumin-coated latex beads (ACLB) suspensions (vol/vol) for 5 min at room temperature as previously described (13, 14). ACLB binding was assessed after exposure of cells to chemotactic factors or colchicine under varying experimental conditions. Cells in suspensions were then fixed in 1.5% glutaraldehyde and examined with phase contrast optics. Results were expressed as the percentage of cells demonstrating unipolar binding (beads restricted to the uropod region of the cell) or generalized (random) binding patterns (13, 14).

Luminol-enhanced CL assay. The evolution of light or CL by neutrophils after metabolic activation by f-Met-Leu-Phe was quantitated as previously described (19). CL activity was determined over a 12-min interval after the incorporation of 5×10^6 PMN and f-Met-Leu-Phe at concentrations ranging from 0.1 to 10 nM.

Scanning electron microscopy procedures. Unstimulated and chemotactically stimulated neutrophils were fixed in a solution of 2% glutaraldehyde in 0.1 M Pipes buffer (piperazone-*N*, *N' bis*-2-ethane sulfonic acid) at pH 7.4 for 1 h. Cells were then washed three times with Pipes buffer, resuspended for 1 h in this buffer solution containing 1% $OsSO_4$, then dehydrated by a graded series of acetone washes with a final three changes of 100% acetone. After drying out CO_2 in a Bowmar Critical Point Drying apparatus (Bowmar Instrument Corp., Fort Wayne, Ind.), samples were sputter coated with 8 nm gold palladium with a Denton Desk-1 apparatus (Denton Vacuum Inc., Cherry Hill, N. J.), and then examined with a JEOL 100 CX electron microscope equipped with a scanning attachment and operating at 25 kV (JEOL USA, Electron Optics Div., Medford, Mass.). Micrographs were recorded from a 2,000-line cathode ray tube on Polaroid P/N 55 film (Eastman Kodak Co., Rochester, N. Y.).

RESULTS

Assessment of PMN motility. The directed migratory responses of PMN obtained from 22 neonates are illustrated and compared with those of 12 healthy adult controls in Figs. 1 and 2. Leading front values (Fig. 1) of neonatal cells were significantly ($P < 0.01$) diminished, compared with controls in response to C5a, f-Met-Leu-Phe, and BCF, which confirms previous reports (1–3). Despite diminished mean values for neonatal cells, highly variable results were observed among the study population: C5a, 42–98% of control; f-Met-Leu-Phe, 27–89% of control; BCF, 34–95% of control. Representative distributions of migration of neonatal and control PMN as determined with an Optimax Image Analyzer are plotted in Fig. 2. As illustrated, directed migration of the neonatal cell population is significantly diminished, compared with adult control cells; fewer neonatal PMN migrate into micropore filters, and the distance of migration of responding cells is significantly diminished ($P < 0.01$), compared with control cells. Leukotactic indices (mean distance of cellular migration) of cell suspensions assessed were directly proportional to leading front values.

Chemotactic factor sensory mechanisms

CF-induced change of PMN morphology. Dose response experiments as illustrated in Fig. 3 were performed to determine the capacity of neonatal or adult PMN to undergo morphologic changes in response to CF. Cells were exposed to CF for 10 min at 37°C and fixed in suspension. The proportion of cells assuming a bipolar conformation was quantitated. Both neonatal and control cells were insensitive to f-Met-Leu-Phe at concentrations < 0.05 nM. As shown, comparable bipolar activation was observed among the study populations. Although the capacity of neonatal PMN to sense CF stimuli appears normal as evidenced by the shape change, the neonatal cells had a different appearance when compared with adult cells. The tail (region opposite the lamellipodia) did not form into a tightly constricted uropod (12) as often as adult cells. After f-Met-Leu-Phe stimulation (5 nM), definite uropod formation (Figs. 4 and 5) was observed in 68±13% of cells obtained from 12 adult controls, compared with only 28±18% of 14 neonates ($P < 0.001$). Extending the incubation time did not significantly increase the percentage of uropods in neonatal cell suspensions.

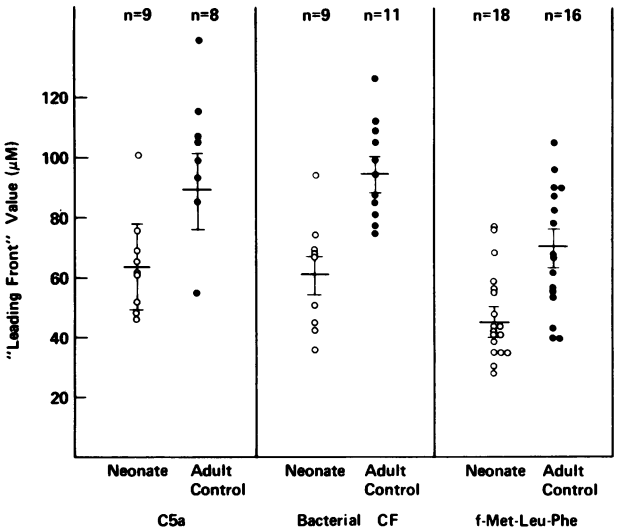

FIGURE 1 Directed migratory responses of neonatal or adult PMN into micropore filters expressed as "leading front" values for C5a, BCF, and f-Met-Leu-Phe. Solid bars designate the mean±1 SD of individual group values.

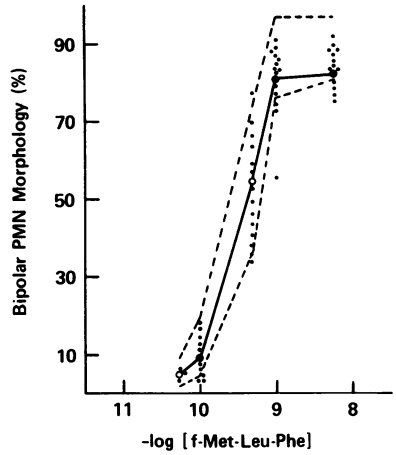

FIGURE 3 The capacity of neonatal PMN to undergo an activated bipolar transformation in response to varying concentrations of f-Met-Leu-Phe. Dotted lines represent the range of values at each concentration for PMN obtained from eight healthy adults. The solid line represents the dose-response curve for PMN obtained from 16 neonates (0, mean of individual values at each concentration).

Uropod formation by neonatal PMN at lower concentrations was also diminished, compared with control values. A mean±1 SD value (n = 6) for neonatal cells exposed to 0.5 nM f-Met-Leu-Phe was 16±9%, compared with 34±18% for control cells (n = 6). Higher concentrations (e.g., 50 nM) did not enhance the percentage of uropod-bearing cells of either neonatal or control suspensions. Sequential CF stimulation elicited cellular responses identical to those observed with single CF stimulus conditions.

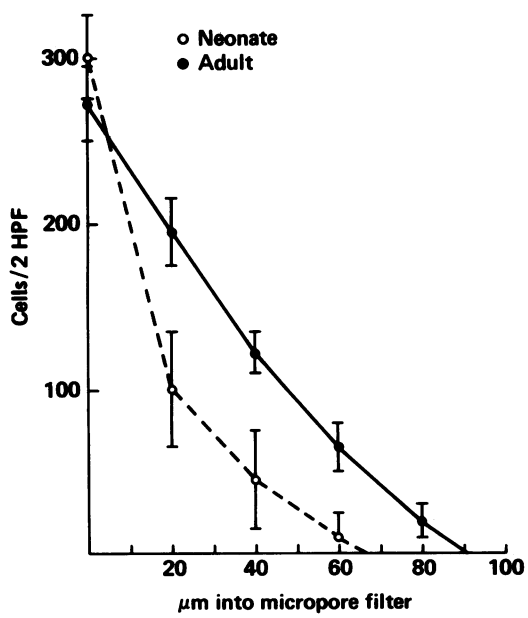

FIGURE 2 Representative distributions of neonatal or adult PMN migrating into micropore filters (60 min) toward a gradient of f-Met-Leu-Phe (10 nM). Solid bars designate the mean±1 SD of individual group values (n = 3).

Uropod formation by neonatal PMN after colchicine preincubation was also significantly diminished (P <0.01), compared with control PMN. For 12 neonates studied, a mean±1 SD% of uropod-bearing cells was 30±14%, compared with 71±18% for 13 adult control PMN suspensions.

CF-induced CL. A luminol-enhanced CL assay was used to evaluate the threshold and maximally effective concentrations of f-Met-Leu-Phe necessary to stimulate PMN oxidative metabolism (14). As shown (Fig. 6), no CL stimulation was observed for both neonatal and adult PMN suspensions when concentrations of ≤1 nM were used. A concentration of 10 nM effected maximal and equivalent responses among neonatal and control populations ($P > 0.05$ for CL integrals, maximum initial slopes and peak CL values).

Binding characteristics of f-Met-Leu[^{3}H]-Phe to neonatal or control PMN

Kinetics of f-Met-Leu-[^{3}H]Phe binding. The kinetics of total and specific binding of f-Met-Leu-[^{3}H]Phe (12 nM, 37°C) by neonatal or adult PMN are shown in Fig. 7. Under these experimental conditions, no significant differences were observed with respect to PMN binding characteristics among neonatal or control populations. PMN binding was rapid with a $t_{1/2} \simeq 2$ min for one-half maximal binding for both

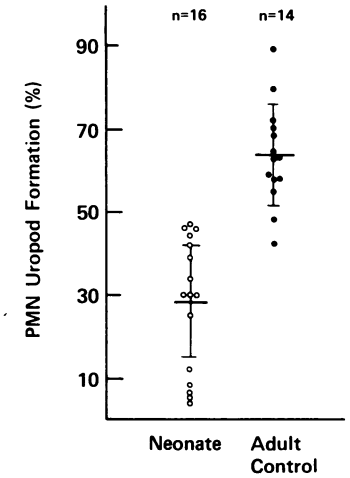

FIGURE 4 The capacity of neonatal or adult PMN to form uropods in suspension in response to f-Met-Leu-Phe (5 nM). Solid bars designate the mean±SD of individual values.

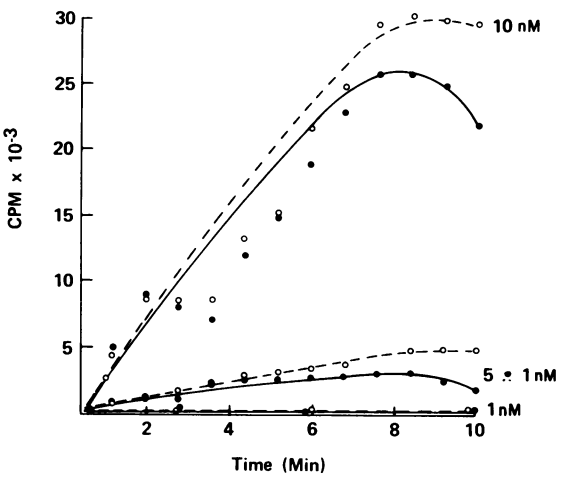

FIGURE 6 The temporal evolution of CL by neonatal (○ - - - ○) or adult (○ – ○ – ○) PMN after exposure to f-Met-Leu-Phe (1, 5 and 10 nM).

neonatal and control PMN. Binding reached equilibrium within 10–12 min and specific binding values were generally 70–90% of total binding values. The reversibility of total f-Met-Leu-[^{3}H]Phe binding at 37°C was tested by chasing incubation mixtures with a 1,000-fold excess unlabeled ligand after equilibration; slow dissociation of labeled peptide consituting ~25% of the total amount bound was observed for PMN of both study populations. These findings are consistent with recent reports demonstrating similar dissociation characteristics of f-Met peptides binding to intact cells at 37°C (10, 20). These observations suggest that the numbers of molecules bound per cell reflect those irreversibly bound (possible internalized ligand) in addition to those occupying specific membrane receptors.

Determinations of total and specific binding of 12 nM f-Met-Leu-[^{3}H]Phe (12 min, 37°C) by PMN suspensions of 21 neonates and 16 adult controls are shown in Fig. 8. Although no significant differences were observed with respect to mean, total, or specific binding values among the two study populations, considerable variance was observed among individual neonates or controls studied.

Saturability of f-Met-Leu-[^{3}H]Phe binding sites. The specific binding of 0.12-100 nM f-Met-Leu-[^{3}H]Phe to neonatal or control PMN (12 min. 4° or 37°C) is illustrated in Fig. 9. The saturating concentration of PMN suspensions of both study populations was between 30 and 100 nM, with one-half maximal binding occurring at a concentration of ~10–30 nM. As determined in additional studies, binding was tem-

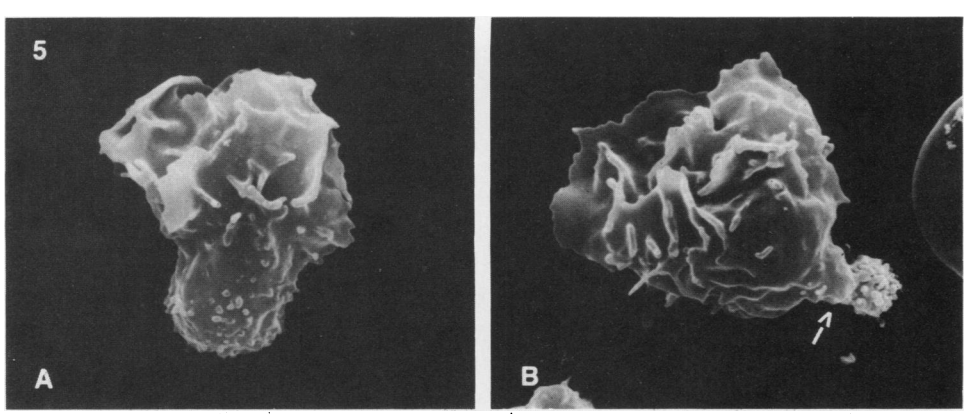

FIGURE 5 Scanning electron micrographs of neonatal and adult PMN following f-Met-Leu-Phe stimulation. (A) This example shows a neonatal cell with a bipolar configuration but lacking a uropod. (B) A bipolar adult cell showing the typical appearance of a uropod (arrow).

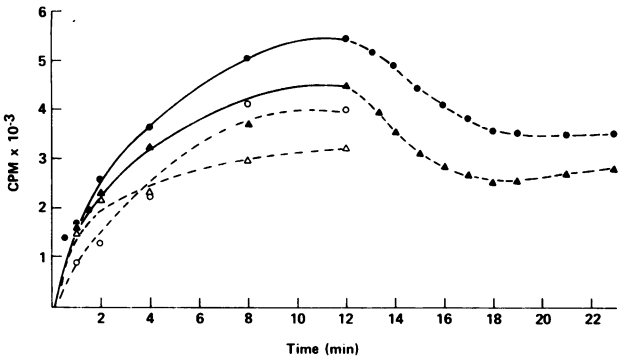

FIGURE 7 The kinetics of binding of f-Met-Leu-[^{3}H]Phe (12 nM, 37°C) by neonatal or adult PMN. Values as shown represent the mean of determinations performed on six individuals. Solid lines represent the total binding of neonatal (●) or adult (▲) PMN. Specific binding patterns are designated by (– – ○ – –) for neonates and (– – △ – –) for adult control PMN suspensions. The reversibility of f-Met-Leu-[^{3}H]Phe binding was evaluated after the incorporation of excess unlabeled f-Met-Leu-Phe (10 μM final concentration) at time = 12 min. The proportion of irreversibly cell-associated f-Met-Leu-[^{3}H]Phe is shown for neonatal (● – – ●) and adult (▲ – – ▲) cells incubated for up to 22 min.

perature dependent; saturable binding at 37°C for both neonatal and control cells was ~ eight-fold that at 4°C. Finally, PMN of both study groups incubated at saturating concentrations (100 nM) of f-Met-Leu-[^{3}H]Phe for 12 min at 37°C demonstrated an equivalent extent and slow rate of dissociation of bound peptide when "chased" with an excess of unlabeled peptide; as was also observed with less than saturating concentrations (Fig. 7), ~ 70% of bound peptide remained cell associated.

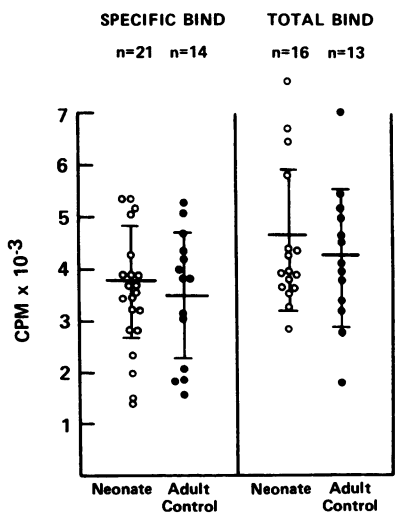

FIGURE 8 The total and specific binding of f-Met-Leu-[^{3}H]Phe (37°C, 12 nM) by PMN suspensions obtained from 21 neonates (○) and 16 adults (●). Solid bars represent the mean±1 SD of individual values.

Assessment of PMN adherence. PMN adherence was assessed under experimental conditions that allow an evaluation of the possible relationship of impaired leukocyte motility to abnormalities of PMN adhesive function or morphology, as previously described (12, 13). Assessments of base-line (unstimulated) adherence as well as adhesive characteristics under conditions of chemotactic stimulation or restimulation were performed on PMN suspensions obtained from 21 neonates and adult controls. As shown (Fig. 10 and Table I), mean±1 SD base-line adherence values for neonates (37±13%) and controls (38±11%) were comparable, but under conditions of chemotactic stimulation, striking abnormalities of PMN adherent function were observed among neonates. Expected enhancement of control PMN adherence was observed after single CF exposures; mean±1 SD values of 63±9%, 70±11%, and 68±8% were observed for BCF, f-Met-Leu-Phe, and C5a stimuli, respectively. For each CF tested by control PMN, single stimuli values were significantly ($P < 0.01$ greater than base-line mean values (t test). In contrast, a single CF stimulus of neonatal PMN effected only minimal or no adherence increments above base-line values. Mean±1 SD values of 47±7%, 42±7%, and 41±6% for BCF, f-Met-Leu-Phe, and C5a, respectively, were observed for neonatal cells. For each chemotactic factor tested, adherence values of neonatal PMN were significantly ($P < 0.001$) less than control values (paired t test). Furthermore, no statistically significant differences were observed between neonatal base-line and CF stimulated values. In additional studies, PMN from four maternal pairs were also studied; both base-line and CF-stimulated adherence

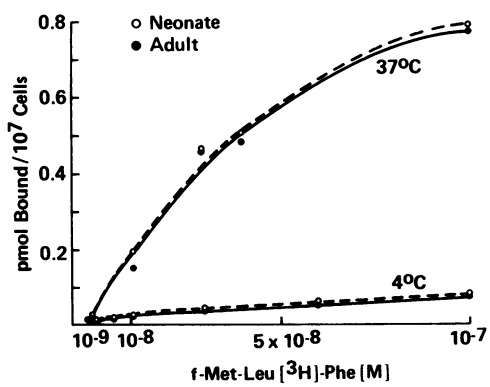

FIGURE 9 Specific binding of f-Met-Leu-[^{3}H]Phe by neonatal (○) or adult (●) PMN incubated at 37°C for 12 min. Values shown represent the mean of determinations performed in six individuals. Saturation of f-Met-Leu-[^{3}H]Phe binding sites of both neonatal and control PMN is demonstrated. Approximately one-half maximal binding was achieved for each cell population with 20 nM f-Met-Leu-[^{3}H]Phe at 37°C and 10-20 nM at 4°C.

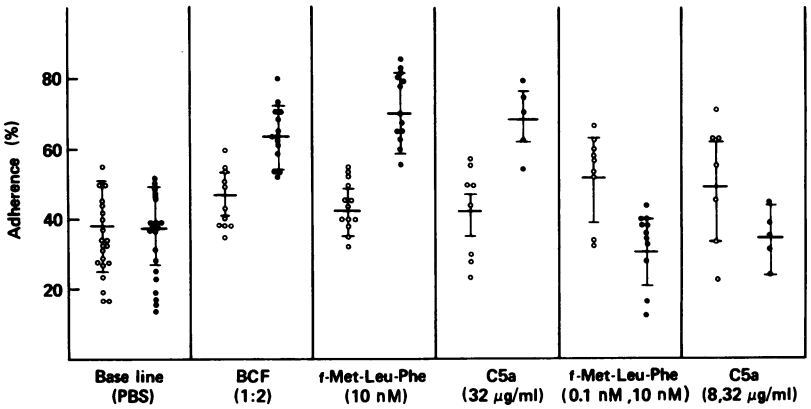

FIGURE 10 Effects of chemotactic stimulation or restimulation on PMN adherence. The adhesive characteristics of neonatal or adult PMN suspensions are demonstrated. Solid bars designate the mean±1 SD of individual group values for base-line conditions or those after single or sequential CF stimuli.

values were comparable to adult control values (data not shown).

As previously reported (13, 14), adherence of normal PMN diminishes sharply after sequential exposure (restimulation) to increasing concentrations of CF. This phenomenon appears to be the result of a restricted redistribution of adhesion sites on the cell uropod (13). These observations were confirmed in the adult control population studied here (Fig. 10 and Table II). After a sequential exposure to f-Met-Leu-Phe (0.1 nM, 5 min; 10 nM, 15 min) or C5a (8 μg/ml, 5 min; 32 μg/ml, 15 min), adherence was diminished, compared with base-line ($P < 0.05$) or single CF stimuli values ($P < 0.001$). In striking contrast, sequential stimulation of neonatal PMN enhanced their adherence in almost all cases, compared with base-line or single stimuli values. For both CF, these values were significantly ($P < 0.001$) greater than sequential CF stimulus control PMN values and significantly greater than base-line values of both neonates and adult controls. In most cases, double CF stimulus values of neonatal cells were greater than those following a single stimulus, although mean differences among the study population were not significant ($P > 0.05$). Thus, in contrast to normal control cells, increases in adherence were observed for neonatal PMN but only after sequential CF stimuli. Therefore, sequential (double) CF stimulation of neonatal PMN altered (enhanced) cell adherence as normally observed after a single exposure of control PMN.

Adhesive function was further evaluated after pretreatment (37°C, 10 min) of cells with colchicine (50 μM). As previously reported (21), normal control PMN studied under these conditions demonstrate significantly diminished adherence ($P < 0.01$) for protein-coated glass substrates (Table II). In contrast, colchicine treatment of neonatal PMN did not generally alter their adhesive properties; no significant differences were observed between mean base-line and colchicine-treated adherence values among the neonates studied. The mean calculated relative change (decrease) in adherence was significantly greater (p

TABLE I
Effect of CF on PMN Adherence

Preincubation conditions	Adult control PMN			Neonatal PMN		
	n	Mean±SD	Δ in mean% from base line*	*n*	Mean±SD	Δ in mean% from base line‡
		%			%	
DPBS (base line)	20	37±13	—	22	38±11	—
BCF (1:2)	11	63±9	+70	7	47±7	+23
f-Met-Leu-Phe (10 nM)	11	70±11	+89	13	42±7	+11
C5a (32 μg-N/ml)	4	68±8	+83	8	41±6	+8

* Refers to cells remaining adherent to the serum-coated glass substrate.
‡ The relative increase in adhesion caused by the addition of CF [(stimulated − base line)/base line] × 100 = %Δ.

TABLE II
Effect of Sequential CF Stimuli or Colchicine on PMN Adherence

Preincubation conditions	Adult control PMN				Neonatal PMN			
	n	Mean±2 SD*	Δ in mean% from baseline‡	Δ in mean% from single stimulus value‡	*n*	Mean±2 SD*	Δ in Mean% from base line‡	Δ in mean% from single stimulus value‡
	%					%		
f-Met-Leu-Phe, *0.1 nM, 5 min; 10 nM, 15 min*	11	30±9	−19	−57	13	51±12	+34	+18
C5a, *8 μg/ml, 5 min; 32 μg/ml, 10 min*	4	34±10	−8	−50	5	48±14	+26	+13
Colchicine, *25 μg/ml, 20 min*	14	27±16§	−39	—	11	34§±11	−15	—

* Refers to cells remaining adherent to the serum-coated glass substrate.
‡ The relative change in adherence caused by the sequential CF stimulus or colchicine pretreatment [(stimulated − base line)/base line] × 100 = %Δ.
§ Mean±1 SD of base-line values for colchicine experiments = 44±11% (adult control) and 39±12% (neonates).

< 0.05) for colchicine-treated adult control cells (−39) than with neonate cells (−15).

Distribution of PMN adhesion sites. To explore the relationship of impaired motility of neonatal PMN to observed abnormalities of adhesive function, the distribution of adhesion sites was assessed under identical experimental conditions by means of a previously described technique (13). Essentially no binding of ACLB to unstimulated cells was observed for any of the study populations. After a single CF exposure, binding of several ACLB per cell was generally observed. Among the three study groups, a comparable generalized (random) distribution of ACLB binding was seen on ~75% of the cells. Unipolar binding of ACLB (Fig. 11) was identified in only a small percentage of cells exposed to C5a or f-Met-Leu-Phe: for neonates, mean±SD = 25±6% (C5a), 21±13% (f-Met-Leu-Phe); for adult control, mean = 27±3% (C5a), 23±9% (f-Met-Leu-Phe); for maternal control, mean = 19±3% (f-Met-Leu-Phe). Since neonatal cells had a reduced incidence of uropods, unipolar binding was considered present if ACLB were clustered at the pole of the cell opposite the lamellipodia. This was quite distinct in stimulated bipolar neonatal cells (Fig. 11b).

Striking abnormalities were observed for neonatal PMN under conditions of sequential CF exposure (Fig. 12). As shown, a second (higher) CF stimulus markedly enhanced the unipolar binding of adult or maternal control PMN. But, a redistribution of binding sites for ACLB to the uropod of neonatal cells was

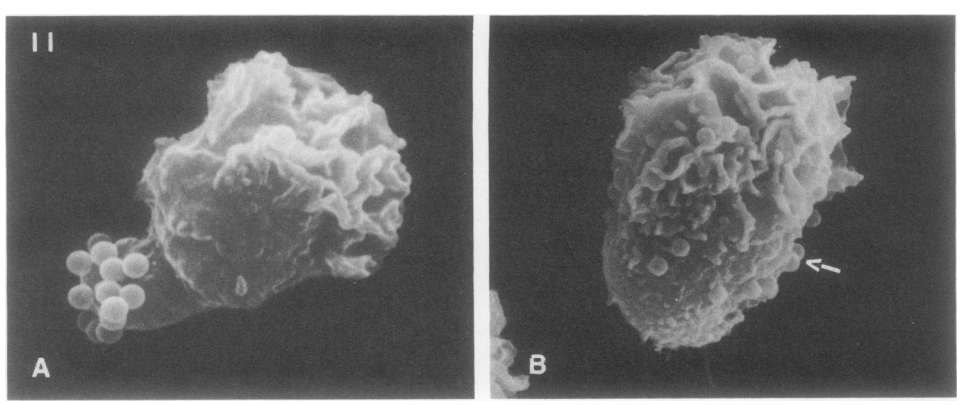

FIGURE 11 These scanning electron micrographs depict binding of ACLB to PMN exposed in suspension to chemotactic factors. (A) Predominate pattern seen in adult cells: unipolar binding elicited by sequential f-Met-Leu-Phe stimulation (0.1 nM, 10 nM). (B) Predominate pattern seen in neonatal cells: random binding following sequential f-Met-Leu-Phe stimulation (0.1 nM, 10 nM). The arrow indicates two ACLB. Several other ACLB are readily visible on this cell. ×5400.

generally not observed. Significant ($P < 0.001$) differences were determined (paired t test) for both C5a and f-Met-Leu-Phe among neonatal and control groups under these conditions. Unipolar binding distributions were observed for a mean±1 SD of 72±8% (C5a) and 71±11% (f-Met-Leu-Phe) of control cells assessed, and comparable values were apparent in maternal controls. In contrast, restricted binding was observed for only 38±8% (C5a) and 31±11% (f-Met-Leu-Phe) of neonatal PMN. Prolonged incubation (up to 50 min) of neonatal cells in the CF did not significantly alter these percentages. Remarkable differences among ACLB binding distributions are emphasized by the findings that among 20 neonatal and 21 control PMN suspensions studied with sequential f-Met-Leu-Phe stimulation, no overlap of values was observed. These findings are in close agreement with differences in PMN adherence observed among neonatal and control populations after CF restimulation. Clearly, an impaired redistribution of adhesion sites to the uropod of the neonatal PMN accompanies its failure to demonstrate diminished adherence to serum-coated glass substrates after sequential CF stimulation.

A similar relationship between ACLB binding distributions and glass adherence was apparent when neonatal or control PMN were pretreated with colchicine (Fig. 12). As previously reported (21), colchicine incubation effectively redistributes the ACLB binding sites to the uropod of normal PMN and, concomitantly, diminishes their adherence to protein-coated glass substrates. As shown (Fig. 12), an impaired redistribution of ACLB binding sites of neonatal cells was generally observed when compared with adult or maternal control cells. Mean percentage ±1 SD unipolar binding values with 50 μM colchicine were 34±11% for neonates ($n = 15$), compared with 69±17% for adult controls ($n = 21$, $P < 0.001$) and 68±14% for maternal controls ($n = 3$, $P < 0.001$). As little as 1 μM colchicine would induce these changes in adult cells (unipolar binding, 65±12%, $n = 12$) though it did not affect neonatal cells. Thus, as also observed with CF, impaired colchicine-induced redistribution of ACLB binding by neonatal cells is consistent with our findings that colchicine fails to reduce neonatal PMN adherence for serum-coated glass substrates.

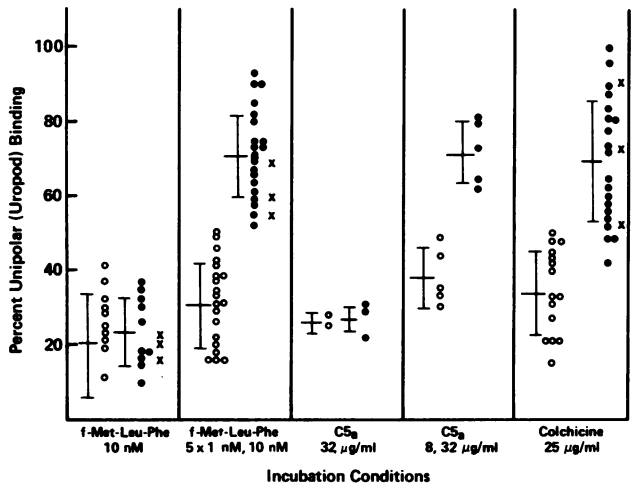

FIGURE 12 Latex bead binding distributions of neonatal (○), adult control (●), or maternal (×) PMN under conditions of single or sequential chemotactic stimulation. Solid bars represent the mean±1 SD of individual group values. Results are expressed as the percentage of cells demonstrating bead binding restricted to the uropod region (percentage of unipolar binding).

DISCUSSION

Our observations confirm previous reports demonstrating diminished migration of the neonatal PMN in chemotactic gradients. One possible basis for this deficit not previously investigated could involve quantitation of functional abnormalities of cellular CF sensory mechanisms. Recent investigations support the concept that CF receptor ligand interactions at the cell surface initiate a sequence of events leading to stimulated or directed migration (22). The precise nature of the interaction of CF with membrane receptors has received considerable attention (10, 11, 22–24). Our data do not support the possibility that sensory function is deficient in the neonatal cell. Neonatal PMN suspensions demonstrated high affinity binding of f-Met-Leu-[^{3}H]Phe. The kinetics of binding, the dissociation of bound ligand, and the saturation of binding sites of PMN of a large population of neonates was comparable to that of healthy adult controls. As was also demonstrated in previous investigations of rabbit and adult human PMN (10, 23, 24), the majority of peptide bound at 37°C was irreversibly cell associated. Approximately 70% of total binding of peptide was not dissociable when PMN suspensions were "chased" with an excess (1,000-fold) of unlabeled peptide after 12 min of incubation at 37°C. Neonatal and adult cells reacted comparably, which further supports the contention that the kinetics of f-Met peptide-receptor interactions are normal in the neonate. Further studies will be necessary, however, to completely characterize the CF receptors of neonatal cells such as the CF modulation of receptor function (e.g., down regulation) and the activity of receptors for other chemotactic factors.

Further confirmation of normal CF sensory function of neonatal PMN is suggested by our observations that similar threshold concentrations of f-Met-Leu-Phe were required to initiate oxidative metabolism (CL) (>1 nM) and effect activated bipolar morphology of cells in suspension (0.1 nM), when compared with

controls. Dose-response experiments also demonstrated comparable concentrations effecting maximal CL evolution or bipolar morphology. Slightly increased peak CL values were noted for neonatal cells exposed to 10 nM f-Met-Leu-Phe ($P > 0.05$, compared with adult PMN). This may reflect the well-recognized enhanced metabolic activity of neonatal PMN (25).

Although neonatal cells exhibited some normal CF-stimulated functions (such as shape change), others were distinctly unlike adult cells. Uropod formation was low and cellular adhesiveness was only marginally influenced by CF stimulation. Interrelated influences of CF on PMN morphology, adhesive properties, and motility have been strongly supported by recent studies (12–14, 26–34). The neonatal PMN appears to represent an example of an abnormality in these interrelationships. Whereas unstimulated neonatal PMN exhibit adherence comparable to adult cells (Table I, Fig. 10) (35), the expected increments of PMN adherence for protein-coated glass substrates after a single CF exposure were generally not observed with neonatal cells. Single CF stimulus values were essentially unchanged from base-line values in contrast to the increased adherence of adult cells. The neonate thus is a naturally occurring example of dissociation of CF-induced shape change and adherence. This dissociation can be produced pharmacologically with 0.2 nM *N*-Tosyl-Lysine chloromethyl ketone, which blocks the increase in adherence but not shape change, and with cytochalasin B, which blocks shape change but not the increase in adhesiveness (12, 13). It would be of considerable interest to determine whether the decreased response of neonatal cells to CF also extends to other factors shown to modulate PMN adherence such as thromboxane A2 (36) or cyclic AMP (37).

The precise nature of cell-substrate interactions have been shown to influence the characteristics of cell translocation in vitro, and the clinical significance of PMN adherence properties in host response to infection in gaining increased recognition (12–14, 33, 38). It seems plausible that the increased adhesiveness caused by CF plays a role in the mobilization of PMN at an inflammatory site. The overall importance of disturbed PMN function in clinical disorders of leukocyte motility is largely unknown. Studies of PMN adherence in some clinically relevant reports have not specifically included evaluations of adhesive function under conditions of chemotactic activation. A failure to evaluate CF-induced alterations of PMN adherence in this investigation would have precluded detection of the profound abnormalities of adherence observed.

Recent studies have shown that an initial CF stimulus modifies the response of adult PMN to subsequent CF exposure (13, 14). An increase in CF stimulation causes a drop in cell adhesiveness. This appears to be due to a restricted distribution of adhesion sites, since cells treated in this manner attach to protein-coated surfaces only by the tip of the uropod, and binding sites for ACLB are restricted to the cell uropod (13). The CF-induced adhesion sites flow to the uropod with an increased CF concentration. These events appear to be permitted by the disassembly of microtubules, since colchicine causes essentially the same effect as a single-step increment in the concentration of CF (21, 38–40). We attempted to determine whether this mechanism was normal in neonatal cells, and found clear evidence that it is not. Neonatal cells showed little change in adhesiveness with either sequential increments in CF stimulation or colchicine treatment. They also exhibited a significantly reduced ability to move binding sites for ACLB to the cell's tail. Our findings suggest a basic cellular defect involving the movement of surface moieties to the uropod of neonatal PMN as well as an abnormality in CF modulation of adherence. If one can assume that the colchicine levels used in our study disrupted the microtubule-linked anchoring of surface moieties, it appears that neonatal cells have an abnormality in the movement of these substances in the membrane. The basis for this decreased fluidity is not clear but may be linked to the decreased deformability observed in immature neutrophils (5, 41).

We recently reported similar investigations in a child with severe recurrent staphylococcal infections and profoundly diminished leukocyte motility in vitro (14). In contrast to neonatal PMN, his cells showed normal shape change and decrease in adherence after exposure to colchicine, as well as normal movement of binding sites for ACLB for one pole of the cell. Like the neonatal cells, however, CF stimulation failed to activate this mechanism, although it evoked a normal CL response. Thus, the mobility of membrane moieties was normal but CF stimulation was defective and did not permit this mobility to occur.

Our current study indicates that the healthy neonate provides a readily accessible clinical model in which to explore possible mechanisms of impaired PMN migration as well as functional requirements for normal PMN locomotion. Diminished leukocyte mobilization in neonates is thought to contribute significantly to their enhanced susceptibility to microbial invasion (5). Although it presumably occurs on a developmental basis, studies to further define the mechanism of the abnormalities of cell locomotion will be necessary to allow future approaches to the pharmacologic modulation of PMN mobilization in these and other high risk pediatric populations.

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