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Deficient Priming Activity of Newborn Cord Blood–Derived Polymorphonuclear Neutrophilic Granulocytes with Lipopolysaccharide and Tumor Necrosis Factor-α Triggered with Formyl-Methionyl-Leucyl-Phenylalanine

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ABSTRACT. Newborn infants are more susceptible to bacterial infections than adults. This susceptibility has been attributed to defects in humoral and cellular activity. Host cellular activity can be modified by factors produced by bacteria or the host in response to infection. We assessed the effect of two factors associated with gram-negative bacterial infection, lipopolysaccharide (LPS) and TNF-α, on polymorphonuclear neutrophilic granulocytes (PMN) obtained from adult or newborns (umbilical cord blood). PMN were primed *in vitro* with LPS (10 μg/L) or TNF-α (10^{-9} M) for 45 min and then assessed, using a chemiluminescence (CL) assay as an indicator of oxidative radical production with formyl-methionyl-leucyl-phenylalanine as the trigger for CL initiation. CL activity of unprimed PMN was similar for adults and newborns (13.3 and 13.7 CL units, respectively). After priming with LPS, CL activity was increased to 43.4 CL units for PMN from adults but to only 17.6 CL units for PMN from newborns ($p < 0.001$, adults *versus* newborn increment). Priming of PMN with LPS was most effective when autologous plasma was present. Using FITC–conjugated LPS and a flow cytometry assay, we could demonstrate no difference between the binding affinity of LPS for adult and newborn PMN. However, formyl-methionyl-leucyl-phenylalanine binding studies indicated that adult PMN had a higher number of binding sites. TNF-α priming of newborn PMN was also ineffective. Adult PMN increased CL activity by 3.9-fold when primed with TNF-α, whereas newborn PMN increased by only 1.75-fold ($p < 0.005$). This priming deficiency was not attributable to TNF-α receptors because phycoerythrin-conjugated TNF-α was associated with PMN from adults and newborns equally. Thus, PMN from newborns are not primed effectively *in vitro* with LPS or TNF-α. This defect may contribute to neonatal susceptibility to bacterial infection. (*Pediatr Res* 34: 243–248, 1993)

Abbreviations

PMN, polymorphonuclear neutrophilic granulocyte
IFN-γ, interferon-γ
TNF-α, tumor necrosis factor-α
LPS, lipopolysaccharide
PT, pertussis toxin
LBP, LPS binding protein
CL, chemiluminescence
FMLP, formyl-methionyl-leucyl-phenylalanine
HBSS, Hanks' balanced salt solution
FACS, fluorescence-activated cell sorter
B_{max}, maximum binding capacity

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Infection of the fetus and newborn continues to be a major cause of morbidity and mortality. Factors that predispose the newborn infant to bacterial sepsis include quantitative and functional deficiencies in immunoglobulin, complement, and phagocytic cells (1–3). For PMN, chemotaxis, phagocytosis, and oxidative burst activity may be decreased in the first few days of life compared with PMN of adults (2, 4, 5).

In adults, bacterial products and host cytokines elaborated during bacterial infection affect PMN activity. IFN-γ (6), granulocyte macrophage colony-stimulating factor (7), TNF-α (6, 8, 9), IL-1 (8) and IL-8 (10, 11) enhance PMN activity *in vitro* and are produced by the host during bacterial infection. In addition, LPS, which is a component of gram-negative bacteria, also enhances PMN activity *in vitro* (12). PMN obtained from adults with bacterial infection have increased oxidative activity (13, 14). In contrast, PMN from newborns with sepsis have decreased bactericidal and oxidative activity (15, 16). Little is known about the response of PMN from newborns to bacterial products or cytokines produced by the host during infection. In one study, recombinant human IFN-γ markedly enhanced the chemotactic responses of PMN from neonates to levels that were not different from adult PMN (4).

TNF-α was originally identified on the basis of its ability to cause necrosis of some tumors in mice (17). It is now known that TNF-α has diverse physiologic effects and is produced by various cell types and stimuli (18–22). It has been implicated as an endogenous inflammatory mediator induced by LPS (18, 22–24) or other bacterial products (17). In high concentrations, TNF-α is a mediator of vascular collapse and septic shock (25, 26). However, in low concentrations, it enhances host defense mechanisms (27–31). PMN functions that are stimulated by TNF-α *in vitro* include adherence, aggregation, chemotaxis, and hydrogen peroxide and superoxide ion generation (6, 8, 9, 29, 32–34). Although production of TNF-α and its effects on the host have been well characterized for adult animals, little is known about

TNF-α in newborn animals. Production of TNF-α by fetal or neonatal cells *in vitro* has been reported as functionally equivalent to that of adult cells (35, 36), but production *in vivo* in response to infection is unknown. Similarly, the effect of TNF-α on newborn PMN is unknown.

Because TNF-α and LPS are generated during the early phases of bacterial infection and because both may enhance PMN function, we examined the effect of recombinant human TNF-α and LPS on PMN from human neonates using autologous plasma to simulate an *in vivo* situation.

MATERIALS AND METHODS

Reagents. FMLP, lucigenin, phorbol myristate acetate, LPS (serotype O111:B4), LPS-FITC, and the limulus amoebocyte lysate assay kit were obtained from Sigma Chemical Co. (St. Louis, MO.) [^{3}H]FMLP was purchased from NEN Products (Boston, MA). Ficoll-Hypaque cell separation medium was obtained from Flow Laboratories (Montreal, Canada). HBSS was obtained from GIBCO Laboratories (Grand Island, NY). Recombinant human TNF-α was kindly provided by H. Jaffe, Genentech Inc. (San Francisco, CA). It had a sp act of 5×10^7 units/mg protein and contained < 0.06 endotoxin units/mg. Phycoerythrin-labeled recombinant human TNF-α was obtained from R&D Systems (Minneapolis, MN). PT was purified by a modification of the method of Sekura (37, 38).

PMN preparations. PMN for the CL assay were isolated from heparinized (10 U/mL) adult peripheral blood or term neonate umbilical cord blood using density gradients as described (39, 40). Cells were routinely purified to >90% purity and >98% viability in approximately 2 h and were assayed immediately afterward. All experiments were completed within 6 h of obtaining the blood. Contaminating red blood cells were removed by brief hypotonic lysis using 0.15 M NH_4Cl. Cells were then washed and resuspended in HBSS at a concentration of 2×10^6 PMN/mL.

PMN priming. Priming of PMN was done in the presence or absence of autologous plasma using 0.2 mL of PMN suspended in HBSS. To each aliquot, 10 μL of PBS, TNF-α, or LPS was added. Duplicates were incubated at 37°C with shaking (100 rpm). After 45 min, 0.8 mL of lucigenin (0.1 M) was added to each tube. Lucigenin is a CL enhancer with specificity for superoxide (41). In preliminary experiments, the optimal concentrations of TNF-α (10^{-9} M) and LPS (0.01 μg/mL) were determined as well as the optimal time for priming 45 min.

CL assay. A CL assay was used as an indicator of oxidative radical production. After adding lucigenin, background CL activity (measured in mV) was determined using a luminometer (LKB, Wallac, Finland). The oxidative reaction was then triggered by adding 100 μL of FMLP (final concentration 10^{-5} M). The CL reaction was monitored continuously for 4 min. Results were expressed as CL units, which were calculated as the area under the curve defined by mV and time in min. Background area under the curve (*i.e.* CL units before FMLP) was subtracted from the area under the curve after adding FMLP.

FACS assay. Fifty μL of heparinized whole blood (10 U/mL) were incubated for 15 min at 4°C with 10 μL of a 1:200 dilution of TNF-α conjugated with phycoerythrin or LPS-FITC in PBS having 0.1% BSA. After incubation, 2 mL of FACS lysing solution (Becton Dickinson, Mountain View, CA) were added to lyse red blood cells. The cell suspension was washed, then made to 0.3 mL with PBS having 0.5% paraformaldehyde. Suspended cells were analyzed using a Becton Dickinson flow cytometer (FACScan, Becton Dickinson).

Lymphocyte, PMN, and monocytes were identified on the basis of front scatter and side scatter of laser beam, confirmed on tests using a CD14 cell marker to identify monocytes and a CD3 marker to identify lymphocytes. In experiments described here, fluorescence intensity was determined for PMN, monocyte, and lymphocyte populations of cells. In these studies, the intensity of cell associated fluorescence is directly related to the number of cellular receptors for LPS-FITC or TNF-α phycoerythrin.

FMLP equilibrium binding assays. [^{3}H]FMLP binding assays were conducted using the silicone oil method of Mackin *et al.* (42) with modifications. Forty-μL aliquots of PMN suspended in HBSS (10^7 PMN per mL) were primed with 10 μL of either PBS (PBS with 0.1% BSA) or LPS (final concentration 10 μg/L). Duplicates were incubated with shaking for 30 min at 37°C, then cooled for 5 min in an ice bath. [^{3}H]FMLP (0.3 to 300 nM) then was added to tubes containing unlabeled FMLP (10^{-4} M) to a final volume of 150 μL in HBSS. After a 30-min incubation at 4°C, the tubes were centrifuged in a microfuge for 3 min at 13 000 rpm. A 15-μL aliquot of the supernatant was removed and counted in scintillation cocktail to determine the free [^{3}H]FMLP concentration. Then 65 μL of PBS and 100 μL of silicone oil were added to each tube, and the centrifugation step was repeated. The tubes then were immediately frozen at −70°C, and the tip of the tube containing PMN was cut off to allow measurement of bound [^{3}H]FMLP with standard liquid scintillation techniques. The total bound [^{3}H]FMLP was plotted as a function of free [^{3}H]FMLP and fitted with computer-assisted nonlinear curve fitting techniques (Inplot, GraphPAD software, San Diego, CA).

Statistical analysis. For comparison between adult and newborn neutrophil samples, the *t* test was used, using a two-tailed test; a p value < 0.05 was considered significant. Statistical determinations were done using a computer statistics program (Statistix, Analytical Software, Minneapolis, MN).

RESULTS

LPS priming of PMN. The conditions for reproducible and optimal priming of adult PMN were determined using FMLP-triggered CL as an indicator of oxidative burst activity. The concentration of LPS chosen for further studies (10 μg/L) was selected after assessing the priming effects of a range of concentrations (Fig. 1). As with adult PMN, newborn PMN showed a dose response to LPS over a range 1 to 100 μg/L. However,

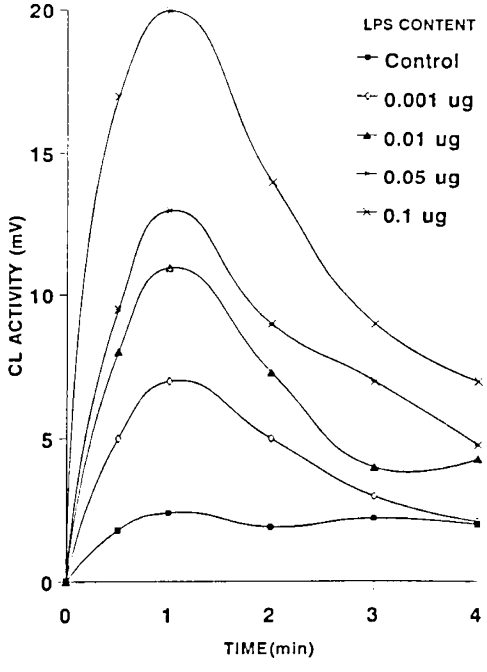

Fig. 1. Effect of LPS concentration on priming of PMN. PMN were primed with various concentrations of LPS (μg/mL or 1 to 100 μg/L) as indicated for 45 min, then triggered with FMLP to initiate an oxidative burst of activity measured by CL. CL was measured continuously for 4 min.

priming activity always was markedly decreased compared with adult PMN. When PMN were primed in the presence or absence of 10% autologous plasma for 45 min, differences were observed; plasma enhanced CL activity ($p < 0.018$, Fig. 2) when paired samples were assessed.

LPS effect on newborn and adult PMN. To compare the effect of LPS on PMN from newborns and adults, we used paired PMN primed with and without LPS in the presence or absence of plasma. Duplicate samples of all four possible conditions were assessed. When the number of cells was limited, samples were assessed as single pairings of control and test-primed cells.

For control PMN (no LPS present in priming media) incubated with 10% autologous plasma, CL activity was similar for adult and newborn cells. After addition of FMLP, the median CL activity for adult and newborn PMN was similar (Fig. 3, $p > 0.05$). Adult PMN primed with LPS had greater CL activity than unprimed adult cells (44.3 CL units ± 23 and 16.3 CL units ± 10 for LPS- and PBS-primed PMN, respectively; $p < 0.0001$). For newborn PMN, CL activity was also greater for cells primed with LPS (19.9 CL units ± 10.3 and 12.7 CL units ± 7.4 for LPS- and PBS-primed cells, respectively; $p < 0.018$). The increment of CL activity (LPS-primed/control) was significantly greater for adult (3.96-fold) compared with newborn PMN 1.69-fold ($p < 0.004$). PMN primed with LPS in the absence of plasma had similar but less marked difference in increment of CL activity compared with PBS-primed PMN (3.61-fold *versus* 2.50-fold for adult and newborn, respectively; $p > 0.05$).

The differences noted in LPS priming between adult and newborn PMN can be attributed to any number of extracellular or intracellular factors: affinity of LPS to PMN receptors, transcription, RNA protein translation, electron transport for oxidative radicals, or other factors. We assessed the affinity of LPS to PMN using flow cytometry as a potential explanation for the differences noted between newborn and adult PMN activation. When LPS-FITC was incubated with whole blood (autologous plasma present), FITC activity was found in association with the PMN and monocyte but not the lymphocyte fractions, indicating a specific cellular attachment. Intensity of fluorescence (mode) was similar for adults and newborns (5.7 ± 2.2 and 5.02 + 2.3 fl units for adults and newborns, respectively; $p > 0.05$). Also, the percentage of PMN with greater than 10 fl units of activity were similar (90.9 ± 10.6% and 86.7 ± 11.9% for adults and newborns, respectively; $p > 0.05$). PMN from newborn cord blood appeared similar to PMN from adults by light microscopy and by flow cytometry (both front and side scatter).

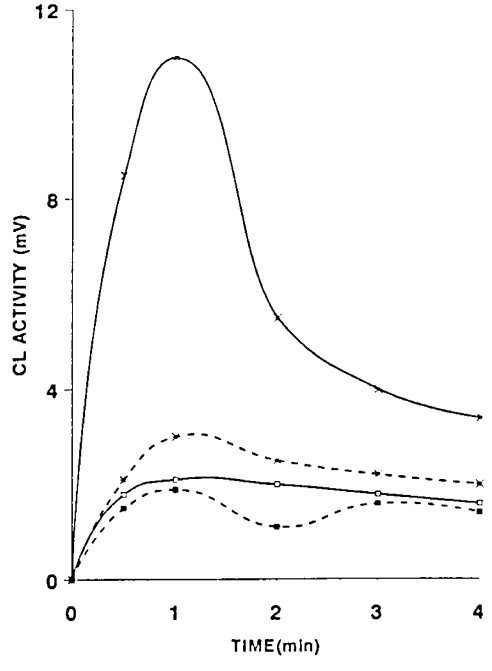

Fig. 2. Role of autologous plasma. The role of 10% autologous plasma and LPS in priming PMN was assessed. Adult PMN were incubated with (———) or without (---) plasma. In addition, LPS (10 μg/L) was either present (*) or absent (□). PMN were primed for 45 min and then triggered with FMLP to initiate an oxidative burst of activity measured by CL. CL was then measured continuously for 4 min. Representative result of six separate experiments is shown. ($p < 0.018$ for presence or absence of plasma using paired t test.)

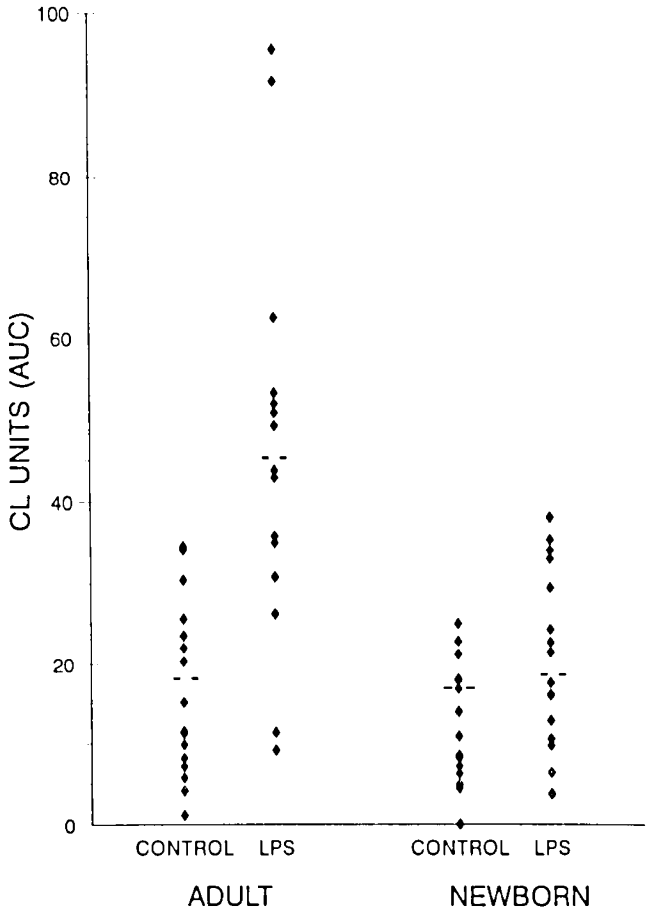

Fig. 3. PMN primed with LPS. Adult or newborn PMN were primed with LPS (10 μg/L) in the presence of 10% autologous plasma. After 45 min of incubation at 37°C, background CL was measured and FMLP was added to trigger the CL burst. Results were expressed as CL units that were calculated as the area under the curve (*AUC*) defined by mV and time in min. Median is indicated by *horizontal bar*. LPS-primed adult PMN had greater CL activity ($p < 0.0001$) than control (no LPS priming) adult PMN. For newborn LPS-primed PMN, a modest increase in CL activity was found compared with control PMN ($p = 0.018$). For adult LPS-primed PMN, the 3.96 fold increase was greater than that of newborn PMN (1.69-fold; $p = 0.0004$, adult *vs* newborn fold increase using two-sample t tests).

In some experiments, the effect of PT on LPS priming of PMN was assessed. The optimal conditions for the effect of PT on unprimed PMN were determined. When PT (1.6 mg/L) was incubated with PMN for 120 min before FMLP stimulation, CL activity decreased by 62% compared with controls incubated with PBS. To confirm that PT was not toxic to the cells, we used PMA as a trigger of CL. As reported by Christiansen (43) PMA-triggered PMN were not affected by PT. Next, we assessed the effect of PT on LPS priming of PMN. LPS from a smooth (0111:B5) and from a rough (J5) serotype of *Escherichia coli* were used. In two separate experiments, PMN incubated with PT for 120 min and LPS for 45 min showed a decrease in CL activity by 76 and 61% for J5- and 0111:B5-stimulated PMN,

respectively, compared with LPS controls not incubated with PT. Therefore, PT appeared to block LPS priming of PMN.

In preliminary experiments, the effect of LPS priming on FMLP binding for adult and newborn PMN was assessed. As shown in Figure 4, the maximum number of FMLP binding sites was lower in newborn PMN and this was not affected by LPS priming. In contrast, the maximum number of binding sites was greater for adult PMN, and LPS altered the characteristics of FMLP binding from a two-site model to a single-site binding model having a B_{max} of 0.29 fmol/1000 cells.

TNF-α priming of PMN. The optimal conditions for TNF-α priming of adult PMN were determined. TNF-α (10^{-9} M) incubated with PMN for 45 min produced a reproducible increase in CL activity with adult PMN. In contrast to the findings with LPS, plasma did not enhance the priming effect of TNF-α for adult or newborn PMN (data not shown). Comparison of TNF-α priming of adult and newborn PMN was made with plasma present. As shown in Figure 5, TNF-α had no significant priming effect on newborn PMN, whereas adult PMN demonstrated an increase in CL activity when primed with TNF-α. TNF-α–primed adult PMN increased CL activity by 3.9-fold to a median of 35.5 CL units, whereas newborn PMN increased only 1.75-fold to a median of 19.0 CL units ($p < 0.005$ fold increase in CL activity newborn *versus* adult).

Because TNF-α priming of PMN can occur by a number of mechanisms, we explored the possibility that newborn and adult PMN may have a different number of receptors for TNF-α or that the receptors have different affinity from each other. TNF-α receptors were assessed by flow cytometry as described for LPS, using phycoerythrin-labeled TNF-α. In this experiment, TNF-α association was similar for PMN derived from adult and cord blood (90% ± 7 and 87% ± 13.4, respectively; $p > 0.05$).

DISCUSSION

Bacterial infections are more common and more severe during the first few days of life than in older children or adults. Deficiencies of PMN function have been reported as a factor contributing to infection among newborn infants (1–3, 44). In most of these reports, defects in PMN chemotaxis or phagocytosis have been described. PMN from full-term or premature infants generate levels of oxidative radicals (which cause bacterial damage or death) only marginally lower than adults (45, 46). In these latter experiments, PMN were not primed before being assessed. However, PMN are exposed to bacterial products and cytokines *in vivo* as they migrate from the circulatory system to the site of infection. Because LPS and TNF-α are plentiful at inflammatory sites and enhance PMN oxidative activity (12, 29, 34, 47, 48), we studied PMN after exposure to these products. CL with lucigenin as the enhancer was used to assess superoxide production by PMN (49).

We found that both TNF-α and LPS are priming agents for adult PMN. Thus, preincubation of PMN with LPS or TNF-α led to enhanced oxidative radical generation when these cells were subsequently stimulated with FMLP. For LPS, the presence of autologous plasma enhanced its priming activity; however, even in the absence of plasma, PMN priming still occurred. In contrast, neither TNF-α or LPS was as effective at priming PMN from newborn infants. These *in vitro* experiments mirror the clinical observation in humans where it is recognized that septic or stressed newborns have depressed CL activity compared with PMN from normal newborn infants or adults (15, 16, 46).

The difference between adult and newborn PMN may relate to a deficiency of LBP in the plasma of newborns. As described by Vosbeck *et al.* (47), priming of PMN by LPS is enhanced by this humoral factor. Although LBP has not been assessed in newborns, we cannot explain our finding on this basis alone because the defect in newborn PMN compared with adult PMN was evident both in the presence and absence of plasma. A second explanation for the effect of LPS is that it induces the production of TNF-α from monocytes that may be contaminating the PMN. Therefore, the difference between newborn and adult PMN may be explained by the differences in TNF production. However, the time used for PMN priming (45 min) is much

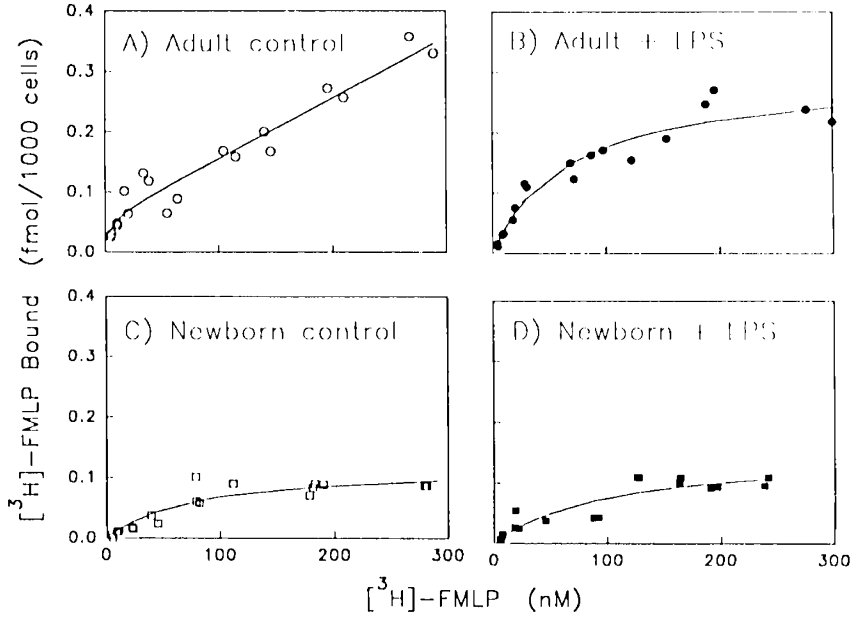

Fig. 4. Effects of LPS priming on [^{3}H]FMLP binding. Saturation binding of [^{3}H]FMLP to adult or newborn PMN was compared with control cells and with cells primed with LPS (10 μg/L) for 30 min at 37°C. Representative examples of saturation binding are illustrated in this figure. *A*, In adult PNM in the absence of priming, two populations of [^{3}H]FMLP binding sites were revealed, a high-affinity site with apparent k_d = 334 μM. B_{max} values were 0.056 fmol/1000 cells and 209 fmol/1000 cells for high- and low-affinity sites, respectively. The fit to a two-site model was significantly better than to a one-site model ($p < 0.01$). *B*, In adult PMN primed with LPS, [^{3}H]FMLP bound to a single population of binding sites with k_d = 68 nM and B_{max} = 0.296 fmol/1000 cells. *C*, In newborn PMN in the absence of priming, [^{3}H]FMLP bound to a single low-affinity site (k_d = 76 nM and B_{max} = 0.120 fmol/1000 cells). *D*, Priming newborn PMN with LPS appeared to have no effect on [^{3}H]FMLP binding (k_d = 101 nM and B_{max} = 0.151 fmol/1000 cells). Similar results were obtained in two separate experiments for each condition.

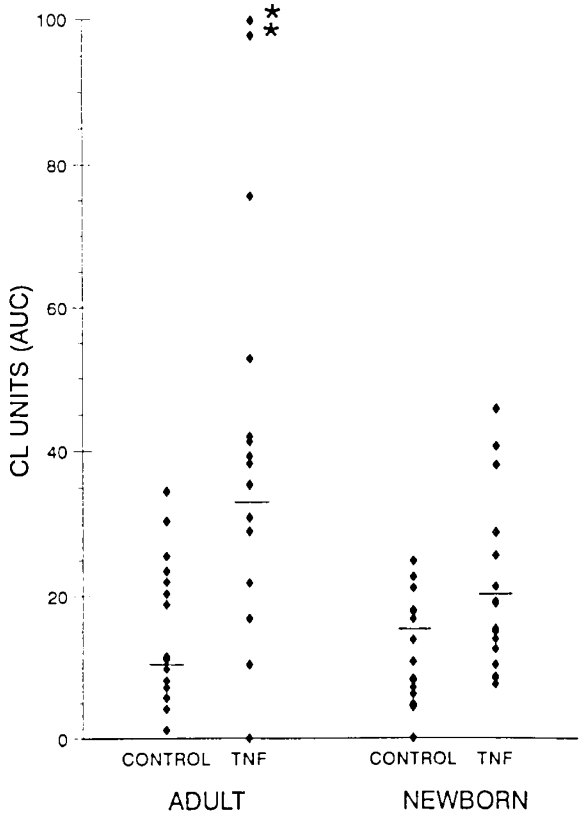

Fig. 5. Priming of adult and cord blood PMN with TNF. Priming of PMN was done in the presence of 10% autologous plasma. Either TNF-α (10^{-9} M final concentration) or PBS (control) was added to each PMN aliquot. After 45 min of incubation at 37°C, background CL activity was measured and oxidative burst was triggered with FMLP as previously described in Figure 4. Median is indicated by *horizontal line*; * indicates two values that were off the scale. Increment of CL activity was significantly greater for adult PMN (3.9-fold) compared with PMN for newborns (1.75-fold, $p = 0.013$).

shorter than the time required for monocytes to produce this cytokine (50). In addition, Moore *et al.* (48) showed that endotoxin and TNF cause PMN priming through separate pathways. In their experiments, LPS effect on PMN was not explained by contaminating monocytes; antibody to TNF in LPS-stimulated PMN did not alter the effect on PMN. Moreover, the method we used for preparation of PMN provides cells virtually free of contaminating monocytes.

Because we used FMLP to trigger the CL response, another explanation for our findings may be that LPS induces more receptors to FMLP. Although there may be a difference between FMLP receptors in newborn and adult PMN, the induction of FMLP receptors on adult PMN is not yet settled; both increased (47) and unaltered (12) PMN cell-surface receptors for FMLP are reported with PMN exposed to LPS. We, therefore, assessed FMLP receptor affinity and density before and after LPS priming using an [^{3}H]FMLP equilibrium binding assay (42). In preliminary experiments, we found that B_{max} was greater in adult PMN than newborn PMN. Also, B_{max} and k_d were modified by exposure of adult PMN to LPS, whereas newborn PMN were unaffected. After LPS exposure, adult PMN bound FMLP to a single population of binding sites with a relatively low affinity. It is noteworthy that Atkinson *et al.* (51) have demonstrated that low-affinity FMLP receptor binding sites, which are associated with superoxide-triggering mechanisms, are increased with TNF-α exposure, whereas high-affinity FMLP receptor sites, which are associated with chemotaxis, are decreased.

PT has been shown to inhibit oxidative generation in PMN stimulated with FMLP. PT is believed to act through ADP ribosylation of G-proteins (43). In PMN, PT blocks the activation of protein kinase C (43). In our experiments, PT blocked FMLP-triggered CL activity for both LPS-primed and unprimed PMN. This suggests that LPS priming involves pathways occurring before protein kinase C activation.

Finally, we assessed whether our findings can relate to a deficiency of PMN receptors for LPS (or LBP) and TNF on newborn cells. This possibility was assessed using FACS to measure FITC-LPS and phycoerythrin–TNF-α binding to PMN. No significant differences in LPS or TNF association with PMN were seen: Approximately 90% of PMN bound TNF-α for both newborn and adults. However, less than 10% of PMN bind LPS in this assay. We could not demonstrate any major differences in receptor to LPS or TNF for newborn PMN to explain these findings. Other mechanisms such as signal transduction or translation also can be considered.

These results provide an explanation for the observation previously made that PMN from septic newborns do not have enhanced oxidative or bactericidal activity (52, 53). Overall, these results suggest that newborn PMN are not responsive to priming action of TNF-α or LPS that can be present at an inflammatory site. This defect in PMN priming may further compromise the ability of newborn infants to mount an effective defense against microorganisms that produce LPS or induce TNF-α at the site of infection.

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