Impaired Leukotriene B₄ Release by Neonatal Polymorphonuclear Leukocytes

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ABSTRACT

Leukotriene B₄ (LTB₄) is a potent mediator of inflammation generated by polymorphonuclear leukocytes (PMN) in response to an appropriate stimulus. It acts as a chemoattractant and stimulates PMN functions, amplifying their inflammatory response. Newborn infants show an increased susceptibility to infections in which PMN dysfunctions play the main role. In this work, LTB₄ release from neonatal polymorphonuclear cells was assessed to investigate whether a defect was detectable. Blood was obtained from the umbilical cord of 10 full-term healthy neonates and 10 adult controls. The LTB₄ production from purified PMN suspensions was induced by three different stimuli: the calcium ionophore A23187, serum-treated zymosan, and formyl-methionyl-leucyl-phenylalanine at final concentrations of 2 μ M, 10 mg/mL, and 10 μ M, respectively. The kinetics of LTB₄ release were studied for up to 30 min by assaying the supernatants of the stimulated cells with a specific RIA. The LTB₄ release, undetectable in resting PMN, was strongly stimulated by the A23187, peaking at 5 min, with significantly higher levels (*t* test, *p* < 0.01) in newborn than in adult PMN preparations (means $\pm$ SD: 12.46 $\pm$ 2.96 and 6.21 $\pm$ 2.09 ng/10⁶ cells, respectively). In comparison, serum-treated zymosan-stimulated PMN released smaller amounts of LTB₄. The levels peaked at 10 min

and were significantly (*t* test, *p* < 0.01) lower in newborn than in adult samples (means $\pm$ SD: 0.71 $\pm$ 0.22 and 3.19 $\pm$ 1.06 ng/10⁶ PMN, respectively). Finally, when the PMN were stimulated by formyl-methionyl-leucyl-phenylalanine, the release of LTB₄ was highly variable both in newborn and in adult samples, as previously reported. In conclusion, the PMN response to A23187 provides evidence that in neonatal PMN the lipoxygenase pathway for LTB₄ synthesis is already fully developed at birth. However, the neonatal cells were defective when triggered by a receptor-mediated stimulus, suggesting the presence of a limiting step in transmembrane signal transduction that results in a low response in neonatal PMN. (*Pediatr Res* 36: 60–63, 1994)

Abbreviations

LTB₄, leukotriene B₄
PMN, polymorphonuclear leukocyte
N-PMN, neonatal PMN
A-PMN, adult PMN
fMLP, formyl-methionyl-leucyl-phenylalanine
STZ, human serum-treated zymosan
HBSS, Hanks' balanced salt solution
GC-MS, gas chromatography-mass spectrometry
HETE, 12-hydroxyicosatetraenoic acid

Human PMN are capable, with appropriate stimulation, of cleaving arachidonic acid from membrane-bound phospholipids and converting it, by the 5-lipoxygenase pathway, into leukotrienes. LTB₄ is mainly produced by PMN (1), and acts as a potent chemoattractant for neutrophils, both *in vitro* (2–4) and *in vivo* (5, 6). It also enhances PMN aggregation (7), induces lysosomal enzyme and oxygen radical release (8, 9), and increases the membrane expression of complement receptors (10). Stimulation of these functions is elicited through specific plasma-membrane receptors (11, 12). Therefore, LTB₄ could be considered a potent mediator of inflammation,

amplifying the recruitment and the activation of leukocytes into the inflammatory focus.

Newborn infants are prone to developing serious and overwhelming infections, especially bacterial ones (13). This leads to a high morbidity and mortality, despite the advances in antibiotic therapy. Although abnormalities have been described in different compartments of the neonatal host defense system (14), altered PMN functions are considered the main factor in infection susceptibility (15).

One of the hypotheses formulated (15) to account for the biochemical and functional impairment of N-PMN was a reduced release of LTB₄ from these cells. In this work, we compared the LTB₄ release from adult and cord blood PMN in response to three different compounds: 1) the calcium ionophore A23187, a soluble stimulus pro-

Received April 19, 1993; accepted January 25, 1994.

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ducing an increase in intracellular Ca²⁺ concentration; 2) a synthetic tripeptide, fMLP, with both structural and chemotactic characteristics similar to naturally occurring bacterial oligopeptides (16), which binds to specific plasma-membrane receptors on PMN (17); 3) zymosan particles opsonized with serum, which induce stimulatory signals in PMN as a result of prevalently binding to complement plasma-membrane receptors (18).

METHODS

Sampling of blood. Heparin at a concentration of 20 U/mL was used as an anticoagulant. Venous blood (10–20 mL) was collected from the placental vessels of 10 full-term infants who were delivered vaginally. Newborns were the product of uncomplicated pregnancies and deliveries; neither general nor local anesthesia was administered to the mothers. No perinatal complications occurred in any of the babies studied, and they all had an Apgar score > 8 at 1 and 5 min. Blood of 10 healthy adult donors was drawn from the forearm vein and run simultaneously with the cord blood samples as a control. The following steps of leukocyte separation were started within 2 h of the blood collection.

PMN purification. Blood was layered over Ficoll-Hypaque and centrifuged at 1250 × *g* for 35 min to remove the lymphomonocytes. The lower phase was mixed with a 6% dextran solution (molecular weight 480 000; ratio 2:1) and sedimented at room temperature for 45 min. Contaminating erythrocytes were removed by hypotonic lysis with 0.87% ammonium chloride. Isolated PMN were washed twice in HBSS buffered to pH 7.4 with *N*-2-hydroxyethylpiperazine-*N'*-pr-2-ethanesulfonic acid. PMN suspensions obtained with this technique were ≥95% pure and 98% viable as determined by exclusion of trypan blue. The cells were suspended in HBSS at a final concentration of 5 × 10⁶ cells/mL and were incubated for 10 min at 37°C before the next steps of stimulation.

PMN stimulation procedures. A23187, fMLP, and cytochalasin B (Sigma Chemical Co., St. Louis, MO) were dissolved in DMSO and diluted in HBSS to give a final concentration of DMSO < 0.3%, which did not affect viability or activation of PMN (19).

Zymosan-A particles (Sigma Chemical Co.) were boiled for 30 min, washed with PBS (pH 7.2), and incubated with serum from adult donors of the AB group (10 mg/mL) at 37°C for 30 min. After centrifugation of STZ at 100 × *g* for 10 min, the pellet was washed twice and resuspended in HBSS at a final concentration of 20 mg/mL. The stimulation of PMN was performed at 37°C by adding either A23187, STZ, or fMLP to the PMN suspensions at final concentrations of 2 μM, 10 mg/mL, and 10 μM, respectively. The incubation was stopped at fixed times by rapid cooling at 4°C in an ice bath; then the mixture was centrifuged at 8000 × *g* for 2 min at 4°C. Cells were preincubated with cytochalasin B (5 mg/mL) for 15 min at 37°C before the addition of fMLP. The supernatants were stored for 3–4 wk at –20°C until anal-

ysis. No breakdown of LTB₄ occurred during the storage of the samples.

LTB₄ determination. LTB₄ was quantitated by a specific RIA (Amersham, Milano, Italy). Briefly, antiserum against LTB₄ was diluted in 50 mM Tris-HCl buffer (pH 8.6) and 100-μL aliquots were transferred to tubes containing: 100 μL of the diluted samples, 100 μL of the ³H-LTB₄ and 100 μL of Tris-HCl buffer. The tubes were incubated overnight at 4°C. Then 200 μL of dextran-coated charcoal were added, and the tubes were strongly mixed by vortexing and centrifuged for 15 min at 2000 × *g* at 4°C. Finally, the radioactivity in the supernatant was counted in a Packard 1600 TR liquid scintillator analyzer, Packard Instrument Co., Meriden, CT. LTB₄ concentration was expressed as ng/10⁶ cells.

The cross-reactivities of antiserum specific for LTB₄ were LTB₄ = 100%; diastereoisomers of 5,12-dihydroxy-6,8,10 trans-14-cis-eicosatetraenoic acid = 3.3%; 12-HETE = 2%; diastereoisomers of 5,6-diHETE = 1.6%; 5(S),12(S)-diHETE = 0.14%; and leukotriene C₄, leukotriene D₄, 5-HETE, 11-HETE, 15-HETE, prostaglandins, and arachidonic acid = 0.04% (19, 20).

Statistical analysis. The unpaired *t* test was used to evaluate the significance of difference between the two groups. Nonparametric analysis, such as the Mann-Whitney *U* test, gave similar results.

RESULTS

A23187 stimulation. Release of LTB₄ by cord PMN and A-PMN was studied for up to 30 min. LTB₄ production was undetectable in resting PMN, and its release was found to be strongly induced at early time points of A23187 stimulation (Fig. 1A) both in adults and in newborns. LTB₄ release peaked at 5 min, with a mean value significantly higher in newborns than in adults (means ± SD: 12.46 ± 2.96 and 6.21 ± 2.09 ng/10⁶ cells, respectively; *p* < 0.01; Fig. 2).

Figure 1. A, Time course of A23187-stimulated LTB₄ production by A-PMN and N-PMN. B, Time course of STZ-stimulated LTB₄ production by A-PMN and N-PMN. Solid line, neonates; dashed line, adults. Each point is the arithmetic mean of 10 samples. Vertical bars represent the SD. The difference between the means of production rate of LTB₄ at each different fixed time of stimulation was significant at the 1% level (*t* test). (*), Significant at the 5% level; (**), not significant.

Figure 2. Peak LTB₄ production by N-PMN and A-PMN in response to A23187 and STZ. Vertical bars represent the SD.

STZ stimulation. The release of LTB₄ by cord PMN and A-PMN in response to STZ was studied for up to 30 min. Results are depicted in Figure 1B. This stimulus induced a release of LTB₄ lower than the one induced by the soluble ionophore, reaching the peak at a late time point. STZ, unlike the A23187, stimulated a significantly lower amount of LTB₄ release in newborn PMN than in A-PMN (mean $\pm$ SD: 0.71 ± 0.22 and 3.19 ± 1.06 ng/10⁶ cells, respectively; $p < 0.01$; Fig. 2).

fMLP stimulation. fMLP-induced LTB₄ release was found to be highly variable in both newborn and A-PMN and was negligible in most samples.

DISCUSSION

The analysis of arachidonic acid metabolites has been carried out by a variety of techniques, but it is generally accepted that GC-MS and RIA are the most specific and sensitive methods (21, 22). Even though GC-MS is the preferred method to assay LTB₄ and its metabolites in biologic samples, RIA offers important practical advantages over other assays (23). HPLC is the technique most frequently used for LTB₄ analysis; however, it shows a lower sensitivity and specificity than GC-MS and RIA (21, 24).

N-PMN and A-PMN were stimulated in initial experiments by the calcium ionophore A23187, which increases the cytosolic free calcium concentration, inducing activation of the enzymatic pathways via the calcium-dependent protein kinases. A23187 is the strongest known stimulus of the 5-lipoxygenase pathway and LTB₄ production by human neutrophils. Our results clearly show that N-PMN have an efficient enzymatic pathway for LTB₄ synthesis. We also found that N-PMN released higher amounts of LTB₄ than A-PMN (Fig. 1A). This metabolic hyperactivity has been reported by others and could be caused by either a maturational defect in leukocyte metabolism (25) or a "priming" effect on neonatal neutrophils by parturition (26).

Kikawa *et al.* (24) reported no differences between the A-PMN and N-PMN LTB₄ production. This could be caused by the different extraction or assay methods used,

as well as by the high ionophore concentrations used (5 μ M). In fact, it has been reported that amounts of A23187 greater than 3.2 μ M are cytotoxic for PMN (19).

However, our data conflict only partially with those of Kikawa *et al.* (24). Similar to their results, our findings provide evidence that in N-PMN the 5-lipoxygenase pathway for LTB₄ synthesis is fully developed at birth.

We also studied the LTB₄ release in response to more physiologically relevant stimuli primarily acting at the plasma-membrane level (fMLP and STZ). fMLP is a synthetic tripeptide structurally and functionally similar to bacterial N-formyl-methionyl-oligopeptides (16), with a potent chemotactic action on PMN, mediated through specific membrane receptors (17). STZ is made up of zymosan particles, derived from yeast polysaccharide membranes, on which complement proteins are adsorbed. Interaction of opsonized zymosan with human PMN is prevalently mediated by receptors for C3 fragments (mainly CR-1 and CR-3) (18). Both fMLP and STZ stimulate LTB₄ release by human PMN. However, we noticed a high variability in LTB₄ release using fMLP by both N-PMN and A-PMN; in most samples LTB₄ was undetectable, as reported by others (19). This is probably because of the incapacity of fMLP to mobilize arachidonic acid from membrane-bound phospholipids (19).

When N-PMN were triggered by STZ, they released significantly lower amounts of LTB₄ than A-PMN.

Therefore, N-PMN showed an impaired LTB₄ release in response to a phagocytic stimulus but a normal capacity to produce and release LTB₄ in response to A23187.

This suggests the failure of a specific event during the signal transduction process. One of the events that occurs during LTB₄ synthesis is the translocation of the phospholipase A₂ onto membranes in a Ca²⁺-dependent manner, with the consequent release of arachidonic acid from the pools of endogenous arachidonate. This, in turn, is metabolized by the 5-lipoxygenase pathway to LTB₄ (27).

We have data showing an impaired D-myo-inositol 1,4,5-triphosphate (IP₃) generation from N-PMN (unpublished data). This second messenger is responsible for the rise in intracellular Ca²⁺ concentration.

One hypothesis that may account for our results is that low levels of the calcium ion were responsible for the lack of activation of phospholipase A₂ and the consequent reduced synthesis of LTB₄. However, inasmuch as it has been reported that the release of LTB₄ in response to unopsonized zymosan may be an event that is regulated separately from its generation, a defect in the release mechanism cannot be excluded (28). Again, it has been reported that resting N-PMN have as many CR-1 and CR-3 as do A-PMN (29, 30). When triggered, expression of CR-1 increases to the same extent in both N-PMN and A-PMN, whereas expression of CR-3, which mainly mediates PMN responses to STZ (18), is lower in N-PMN (29, 30). This may explain our results if up-regulation is critical for the PMN response, which is not in agreement with other results previously reported (31). Other altered

stimulus-response coupling mechanisms in N-PMN, such as the changes in membrane potential after chemotactic stimulation (32), could play a role in the low STZ-stimulated LTB₄ production.

We cannot state whether this impaired response is really significant *in vivo* and whether it has a causal role in the defective N-PMN activation, which is important to limit the infections typical in the neonatal age. However, several studies have shown that LTB₄ has an important role in initiating and amplifying the inflammatory response *in vivo*. Shortly after a tissue is exposed to LTB₄, the granulocytes can be seen to adhere to the endothelium and subsequently migrate to the extravascular space (33, 34). When a skin window is exposed to LTB₄, an increased migration of PMN into the skin window chamber can be observed (6). LTB₄, in addition to being a potent activator of PMN functions *in vitro*, probably also has an important role *in vivo*, stimulating the emigration of leukocytes from the bloodstream into the inflammatory focus and activating PMN phagocytosis (1). In conclusion, even though N-PMN have a normal LTB₄ synthetic pathway, they release low amounts of LTB₄ in response to a phagocytic stimulus (STZ), suggesting the presence of a limiting step in the signal transduction mechanism.

REFERENCES

- Samuelsson B, Dahlén SE, Lindgren JA, Rouzen CA, Serhan CN 1987 Leukotrienes and lipoxins: structures, biosynthesis and biological effects. *Science* 237:1171-1176
- Palmblad J, Malmsten C, Uden AM, Radmark O, Engstedt L, Samuelsson B 1981 Leukotriene B₄ is a potent and stereospecific stimulator of neutrophil chemotaxis and adherence. *Blood* 58:658-667
- Dahinden CA, Clancy RM, Hugli TE 1984 Stereospecificity of leukotriene B₄ and structure-function relationships for chemotaxis of human neutrophils. *J Immunol* 133:1477-1482
- Czarnetzki BM, Rosenbach T 1986 Chemotaxis of human neutrophils and eosinophils towards leukotriene B₄ and its 20-*omega*-oxidation products *in vitro*. *Prostaglandins* 31:851-858
- Bray MA, Ford-Hutchinson AW, Smith MJ 1981 Leukotriene B₄: an inflammatory mediator *in vivo*. *Prostaglandins* 22:213-222
- Bisgaard H, Helkvist S, Bondet L, Venge P, Dahl R, Sondergaard J 1986 Chemotactic activity of LTB₄ in man. *Allergy* 41:365-372
- Ford-Hutchinson AW, Bray MA, Doig MV, Shipley ME, Smith JH 1980 Leukotriene B₄, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature* 286:264-265
- Hafstrom I, Palmblad J, Malmsten CL, Radmark O, Samuelsson B 1981 Leukotriene B₄: a stereospecific stimulator for release of lysosomal enzymes from neutrophils. *FEBS Lett* 130:146-150
- Sumimoto H, Takeshige K, Manakami S 1984 Superoxide production of human polymorphonuclear leukocytes stimulated by leukotriene B₄. *Biochim Biophys Acta* 803:271-276
- Nagy I, Lee TH, Goetzl EJ, Pickett WC, Kay AB 1982 Complement receptor enhancement and chemotaxis of human neutrophils and eosinophils by leukotrienes and other lipoxygenase products. *Clin Exp Immunol* 47:541-547
- Goldman DW, Gifford LA, Olson DM, Goetzl EJ 1985 Transduction by leukotriene B₄ receptors of increases in cytosolic calcium in human polymorphonuclear leukocytes. *J Immunol* 135:525-530
- O'Flaherty J, Kosfeld S, Nishihira J 1986 Binding and metabolism of leukotriene B₄ by neutrophils and their subcellular organelles. *J Cell Physiol* 126:359-370
- Miller ME, Stiehm ER 1983 Immunology and resistance to infection. In: Remington JS, Klein JD (eds) *Infectious Disease of the Fetus and Newborn Infants*. Saunders, Philadelphia, pp 27-68
- Wilson CB 1986 Immunological basis for increased susceptibility of the neonate to infection. *J Pediatr* 108:1-12
- Hill HR 1987 Biochemical, structural and functional abnormalities of polymorphonuclear leukocytes in the neonate. *Pediatr Res* 22:375-382
- Schiffmann E, Corcoran BA, Wohl SM 1975 N-formyl-methionyl peptides as chemoattractants for leukocytes. *Proc Natl Acad Sci USA* 72:1059-1064
- Sklar LA, Jesaitis AJ, Painter RG 1984 The neutrophil N-formyl peptide receptor: dynamics of ligand-receptor interactions and their relationship to cellular responses. In: Snyderman R (ed) *Regulation of Leukocyte Function*. Plenum Press, New York, pp 29-82
- Ezekowitz RAB, Sim RB, MacPherson GG, Gordon S 1985 Interaction of human monocytes, macrophages and polymorphonuclear leukocytes with zymosan *in vitro*. *J Clin Invest* 76:2368-2376
- Palmer RMJ, Salmon JA 1983 Release of leukotriene B₄ from human neutrophils and its relationship to degranulation induced by N-formyl-methionyl-leucyl-phenylalanine, serum-treated zymosan and the ionophore A23187. *Immunology* 50:65-73
- Salmon JA, Simmons PM, Palmer RMJ 1982 A radioimmunoassay for leukotriene B₄. *Prostaglandins* 24:225-230
- Izumi T, Shimizu T, Kosama T, Seyama Y, Susimoto H, Takeshige K, Minakami S, Wetterholm A, Radmark O 1986 A simultaneous quantitation of leukotriene B₄ and its *omega*-oxidized products by gas chromatography-mass spectrometry. *Biochem Biophys Res Commun* 134:512-518
- Mathews WR, Bundy GL, Wynalda MA, Guido DM, Schneider WP, Fitzpatrick FA 1988 Development and comparative evaluation of radioimmunoassay and gas chromatographic/mass spectrometric procedures for determination of leukotriene B₄. *Anal Chem* 60:349-353
- Salmon JA 1984 Analysis of cyclo-oxygenase and lipoxygenase products in inflammatory exudate. *Prostaglandins* 27:364-366
- Kikawa Y, Shigematsu Y, Sudo M 1986 Leukotriene B₄ biosynthesis in polymorphonuclear leukocytes from blood of umbilical cord, infants, children and adults. *Pediatr Res* 20:402-406
- Anderson DC, Pickering LK, Feigin RD 1974 Leukocyte function in normal and infected neonates. *J Pediatr* 85:420-425
- Ambruso DR, Stork LC, Gibson BE, Thurman GW 1987 Increased activity of the respiratory burst in cord blood neutrophils: kinetics of the NADPH oxidase enzyme in subcellular fractions. *Pediatr Res* 21:205-210
- Cockcroft S 1992 G-protein-regulated phospholipase C, D, and A₂-mediated signalling in neutrophils. *Biochim Biophys Acta* 1113:144-146
- Williams JD, Lee TH, Lewis RS, Austen F 1985 Intracellular retention of the 5-lipoxygenase pathway product, leukotriene B₄, by human neutrophils activated with unopsonized zymosan. *J Immunol* 134:2624-2630
- Bruce MC, Baley JE, Medvik KA, Berger M 1987 Impaired surface expression of C3bi but not C3b receptors on neonatal neutrophils. *Pediatr Res* 21:306-311
- Anderson DC, Becker Freeman KL, Heerdt B, Hughes BJ, Jack RM, Wayne Smith C 1987 Abnormal stimulated adherence of neonatal granulocytes: impaired induction of surface MAC-1 by chemotactic factors or secretagogues. *Blood* 70:740-750
- Philips MR, Buyon JP, Winchester R, Weissmann G, Abramson SB, 1988 Upregulation of C3bi receptor (CR3) is neither necessary nor sufficient to promote neutrophil aggregation. *J Clin Invest* 82:495-501
- Sacchi F, Hill HR 1984 Defective membrane potential changes in neutrophils from human neonates. *J Exp Med* 160:1247-1252
- Hoover RL, Karnowsky MJ, Austen KF, Corey EJ, Lewis RA 1984 Leukotriene B₄ action on endothelium mediates augmented neutrophil-endothelial adhesion. *Proc Natl Acad Sci USA* 81:2129-2193
- Lindbom L, Hedqvist P, Dahlen SE, Lindren JAA, Arfors KE 1982 Leukotriene B₄ induces extravasation and migration of polymorphonuclear leukocytes *in vivo*. *Acta Physiol Scand* 116:105-108