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Leukotriene B_4 Biosynthesis in Polymorphonuclear Leukocytes from Blood of Umbilical Cord, Infants, Children, and Adults

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ABSTRACT. The biosynthesis of 5(S), 12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid, leukotriene B_4, by human polymorphonuclear leukocytes was examined in relation to age. The leukotriene B_4 production by polymorphonuclear leukocytes from unbilical cords, infants, and adults was assayed using high pressure liquid chromatography. The specificity of the leukotriene B_4 assay was examined by gas chromatography mass spectrometry. Polymorphonuclear leukocytes from 10 umbilical cords, 24 infants and children, and 10 adults were examined for their ability to synthesize leukotriene B_4, *in vitro* after stimulation by the ionophore A23187 or platelet-activating factor. Among the infants and children, there was a slight age-dependent increase of leukotriene B_4 production by polymorphonuclear leukocytes in response to ionophore A23187, but it was not statistically significant. Leukotriene B_4 production by polymorphonuclear leukocytes in the umbilical cords and infants was not significantly lower than that of polymorphonuclear leukocytes in adults in response to both ionophore A23187 and platelet-activating factor under our experimental conditions. (*Pediatr Res* 20: 402–406, 1986)

Abbreviations

PMN, polymorphonuclear leukocytes
LTB$_4$, leukotriene B_4; 5(S),12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid
GC-MS, gas chromatography mass spectrometry
PAF, platelet-activating factor
fMLP, formyl-methionyl-leucyl-phenylalanine
RP-HPLC, reversed phase high pressure liquid chromatography
SP-HPLC, straight phase high pressure liquid chromatography
RIA, radioimmunoassay
SIM, selected ion monitoring
5S,12S-DHETE, 5(S),12(S)-dihydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid
PGB_2, prostaglandin B_2
$[^2H_4]PGF_{2\alpha}$, tetradeuterated prostaglandin $F_{2\alpha}$
ME-DMiPS, methyl ester dimethylisopropylsilyl

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Newborn infants are prone to develop a variety of infections, especially bacterial infections. The high morbidity and mortality associated with bacterial infections during the neonatal period, despite advanced antibiotic therapy, has been partially attributed to the immature immune system, for example low PMN chemotaxis (1, 2). However, the mechanism of retarded PMN chemotaxis is not well understood (3).

LTB_4 is a lipoxygenase product of arachidonate produced by PMN (4), with a chemotactic activity more than 100 times greater than that of other lipoxygenase and cylooxygenase products of arachidonate (5), and comparable to that of fMLP or the complement fragment C5a (6). And LTB_4 is reported to exist in the synovial fluid of patients with rheumatoid arthritis and gout, and in the skin of patients with atopic dermatitis (7–9). PMN not only show chemotaxis when exposed to LTB_4 but they also produce LTB_4 when stimulated by chemotactic factors such as C5a, fMLP, PAF, and by the calcium ionophore A23187 (10–14). Thus, LTB_4 may serve as an intracellular second messenger in the receptors for the chemotactic factors described above. However, no report has compared the LTB_4 production in neonatal PMN with that of adult PMN, partly because of the great difficulty of separating LTB_4 from its stereospecific isomers, and partly because of the great difficulty in measuring LTB_4 at the low concentrations present in biological fluids (7).

LTB_4 assay has been done most often by HPLC, and recently by RIA and GC-MS (15, 16). We examined the specificity of the HPLC method for LTB_4 using GC-MS, and investigated LTB_4 biosynthesis by PMN from different age groups by a combination of RP and SP HPLC.

MATERIALS AND METHODS

Sampling of blood. Informed consent was obtained from the individuals or their parents. Ten to 20 ml of blood was collected with 20 U/ml of heparin from the placental end of the cut umbilical cords of 10 healthy full-term infants who were delivered vaginally. Six to 12 ml of blood was drawn from the forearm vein of the 14 infants (0–7 yr old), 10 children (7–14 yr old), and 10 adults (20–33 yr old) using a heparinized syringe (20 U/ml). The infants and children had been admitted to the pediatric ward for operations involving noninflammatory orthopedic diseases, or for examination of short stature (−2.0 to −2.5 S.D.). All patients with short stature were well proportioned and none had symptoms that mimicked abnormal hormonal states, or conditions known to affect biosynthesis of LTB_4 by PMN. Subjects with hormonal abnormalities were excluded. All of the subjects were afebrile and had received no medication for at least 4 wk before blood sampling.

Leukocyte separation. PMN were separated by the method of Bøyum (17). This preparation resulted in suspensions in which more than 97% of the leukocytes were PMN. PMN purity was

checked with Wright's stain. Cell viability, as determined by trypan blue dye, was more than 95%. The platelet contamination was less than three platelets per PMN. PMN were resuspended in polyethylene tube with balanced Hanks' solution at a concentration of 3×10^6 cells/ml. Preliminary experiment revealed that times up to 5 h from blood sampling until stimulation did not significantly influence LTB_4 biosynthesis by PMN. Thus, PMN were stimulated at approximately 4 h after blood sampling.

PMN stimulation with ionophore A23187. The cell suspension (1.0 ml) was preincubated at 37°C for 5 min and then incubated for another 5 min in the presence of 5 μM ionophore A23187 (Calbiochem-Behring, La Jolla, CA). No arachidonate was added. Reactions were stopped by the addition of 10 ml of ice-cold distilled water to the incubation mixture (18, 19). Preliminary experiments showed no significant difference in LTB_4 biosynthesis between the reactions stopped by ice-cold distilled water and those stopped by methanol. PGB_2 (50 ng) and $[^2H_4]PGF_{2\alpha}$ (150 ng) (MSD, Isotope Division of Merck Frosst Canada Inc., Quebec, Canada) were added as internal standards for HPLC and GC-MS. The resultant mixtures were centrifuged at 3000 × *g* for 20 min.

PMN stimulation with PAF. The cell suspension (2.0 ml) was preincubated for 5 min and then incubated for another 1.5 min in the presence of 900 nM PAF (Calbiochem-Behring). PAF was dissolved in 2.5% bovine serum albumin (12) and added to the suspension after subsequent dilution with 0.15 M NaCl solution. No arachidonate was added. Reactions were stopped as described above. PGB_2 (20 ng) and $[^2H_4]PGF_{2\alpha}$ (60 ng) were added to the incubation mixture, and the mixtures were centrifuged at 3000 × *g* for 20 min.

Sample processing. The supernatant liquid after centifugation was applied to a C18 reversed-phase extraction cartridge (Sep-pak C18; Waters Associated, Milford, MA) which had been first conditioned with 10 ml of methanol and then 10 ml of distilled water. After each sample was loaded on the extraction cartridge, the cartridge was washed successively with 5 ml of distilled water and 5 ml of hexane. The leukotrienes were eluted with 5 ml of ethyl acetate. The ethyl acetate fraction was evaoporated with a nitrogen stream, and the residue was dissolved in 50 μl of methanol and stored under nitrogen gas at −30°C until analysis by HPLC and GC-MS. The solvents used were all of HPLC grade (Nakarai Kagaku, Kyoto, Japan).

Routine HPLC analysis of LTB_4. The samples were first injected into a reversed-phase Cosmosil 5C18 column with a waters Associates Model M6000 Pump and U6K injector. The solvent system used for elution was methanol/water/acetic acid (65/35/0.05, v/v) preadjusted to pH 5.3 with ammonium hydroxide. The flow rate was 1 ml/min, and then eluate was monitored at 270 nm by a variable wavelength spectrometric detector (Shimadzu Model SPD-2A). Fractions corresponding to LTB_4 were collected for further analysis by HPLC on a straight-phase column.

For quantitation of the concentration of LTB_4 in the sample, mixtures of authentic LTB_4 (1, 2, 5, 10, and 20 ng/ml) and PGB_2 (20 ng/ml) were made in balanced Hanks' solution. These mixtures of the standards were treated as described above for the cell supernatants, and injected into the RP-HPLC system. The absorption at 270 nm was recorded, as the LTB_4 and PGB_2 eluted, and a calibration curve was made by plotting the ratio of peak heights (LTB_4/PGB_2) as a function of the LTB_4 concentration. The ratio of the absorption peak height of LTB_4 to that of PGB_2 in each RP-HPLC analysis of cell supernatants was referred to this calibration curve to quantify the LTB_4. To determine the extent of contamination of LTB_4 in the samples with 5(S), 12(S)-dihydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid (5(S), 12(S)-DHETE), the RP-HPLC fractions contained LTB_4 were dried with a nitrogen stream, methylated with diazomethane, and applied to a straight-phase Cosmosil 5SL column. The solvent system was hexane/isopropanol/acetic acid (95/5/0.02, v/v) at a flow rate of 1 ml/min, and the eluate was monitored at 270 nm. LTB_4 and 5(S), 12(S)-DHETE were resolved under these conditions, and the contribution of the 5(S), 12(S)-DHETE to the LTB_4 peak on RP-HPLC was corrected for. The recovery from the RP-HPLC of $[^3H]$-LTB_4 added to the incubation mixture was found to be about 60% (Shimizu T, personal communication).

Analysis by GC-MS. Five samples (three umbilical cords and two adults were assayed for LTB_4 by both HPLC and GC-MS to check the specificity and accuracy of the HPLC method. Aliquots to the sample analyzed by HPLC were converted to the corresponding ME-DMiPS derivatives for analysis by GC-MS. Derivatization was performed essentially by the same method described by Miyazaki *et al.* (20) except the methoxymilation step was omitted. The recovery of $[^3H]$-LTB_4 added to the incubation mixture after these extraction and derivatization procedures was found to be constantly about 60% (Shimizu T, personal communication).

GC-MS analysis with SIM was carried out using a JOEL-DX300 instrument equipped with a computer system. The column used was a cross-linked OV-1 fused silica capillary column (25 m × 0.31 mm id, Hewlett-Packard). Helium (1.0 ml/min) was used as the carrier gas. The column temperature was maintained at 250°C, and samples were applied via a Van der Berg type solventless injector. Electron impact ionization was used, at an ionization potential of 70 eV and an ionization current of 300 μA. The ions monitored (SIM) were m/z439 ([M-111, loss of CH_2-CH=CH-$(CH_2)_4$-$CH_3]^+$) and m/z321 ([M-111-DMiPSOH]$^+$) for LTB_4, and m/z483 ([M-C_5H_{11}-DMiPSOH]$^+$) for $[^2H_4]PGF_{2\alpha}$.

For quantitative mass spectrometric analysis of LTB_4 performed in the SIM mode, standard mixtures of authentic LTB_4 and $[^2H_4]PGF_{2\alpha}$ were made in balanced Hanks' solution at the following concentrations: 200 pg/ml, 500 pg/ml, 1 ng/ml, 2 ng/ml, and 5 ng/ml for LTB_4, and 5 ng/ml for $[^2H_4]PGF_{2\alpha}$. These mixtures of the standards were treated as described above for the other samples and analyzed by GC-MS after derivatization to ME-DMiPS derivative. To obtain standard calibration curves for LTB_4, peak intensities were monitored both at m/z321 and m/z439 for LTB_4, and at m/z483 for $[^2H_4]PGF_{2\alpha}$. The peak heights ratios m/z321/483 and m/z439/483 were calculated and plotted against the concentration of LTB_4. The two peak height ratios m/z321/483 and m/z439/483 obtained from cellular supernatant samples were calculated and converted to the values for the content of LTB_4 reference to the standard curves. The amounts of LTB_4 presented in the "Results" section are the means obtained from the two peak height ratios (m/z321/483 and m/z439/483).

Statistical analysis. The unpaired *t* test was used to evaluate the significance of the difference between the means of the production rate of LTB_4 by PMN from different age groups. Linear regression analyses were performed on these data and on the values of LTB_4 measured by HPLC and GC-MS, using the least squares method.

RESULTS

Typical chromatograms from RP-HPLC, SP-HPLC, and GC-MS separations are shown in Figure 1*A* and *B* and Figure 2. The detection limit of authentic LTB_4 was 1 ng by RP-HPLC and 200 pg (at a signal/noise ratio of 3:1) by GC-MS in the SIM mode. The known LTB_4 isomers were clearly separated by both SP-HPLC and GC-MS in the SIM mode. The calibration curves were linear over the ranges being monitored both by GC-MS in the SIM mode and by RP-HPLC (data not shown).

The precision of the overall procedure was checked by analyzing one reaction mixture sample with A23187 and 5 ng of authentic LTB_4 in 2 ml of balanced Hanks' solution. Both derivatized samples were subjected to GC-MS analysis four

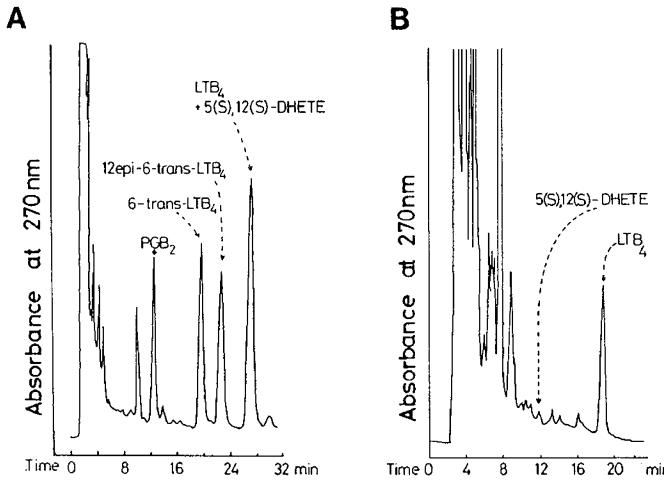

Fig. 1. *A*, RP-HPLC chromatogram of leukotrienes formed in 1 ml of human PMN suspension (3×10^6 cell/ml) incubated for 5 min at 37°C with ionophore A23187 (5 μM). *B*, SP-HPLC chromatogram of leukotriene methyl esters: the extract that was partially purified by RP-HPLC as described in *A* was converted to the methyl ester with diazomethane and subjected to SP-HPLC.

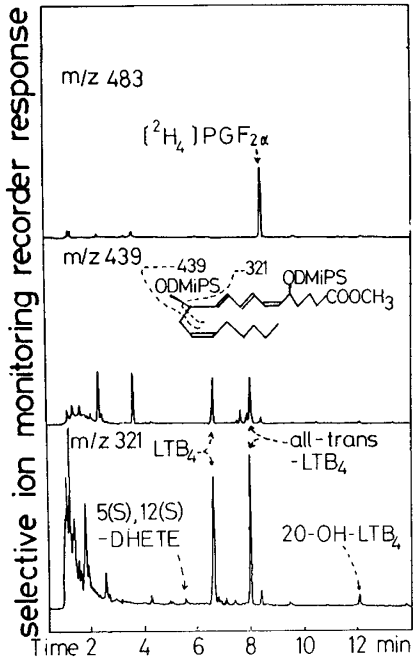

Fig. 2. GC-MS analysis (SIM) of the DMiPS derivatives of leukotriene methyl esters: An aliquot of the reaction mixture described in Figure 1*A* was mixed with the internal standard $[^2H_4]PGF_{2\alpha}$. After extraction by Sep-pak C18 and methylation with diazomethane, the material was further purified by chromatography on a Silica gel Sep-pak, and then derivatized to the DMiPS derivative. Final purification of the derivative was done by chromatography on Sephadex LH-20 (20). The elution of the DMiPS derivative of $[^2H_4]PGF_{2\alpha}$ (*top*) was monitored at m/z483, and the elution of the LTB_4 derivative was monitored both at m/z439 and m/z321 (*middle and bottom*).

times, and the intraassay coefficient of variation was 4% for the reaction mixture sample and 3% for authentic sample.

When known amounts of authentic LTB_4 (1, 2, 3, 4, and 5 ng) in 2 ml of balanced Hanks' solution were analyzed by GC-MS in the SIM mode or by HPLC, the paired values were well correlated, as can be seen from Figure 3 ($r = 0.99$, $p < 0.01$). The two methods for assay of LTB_4, HPLC and GC-MS in the SIM mode, were in good correlation both for cells stimulated by

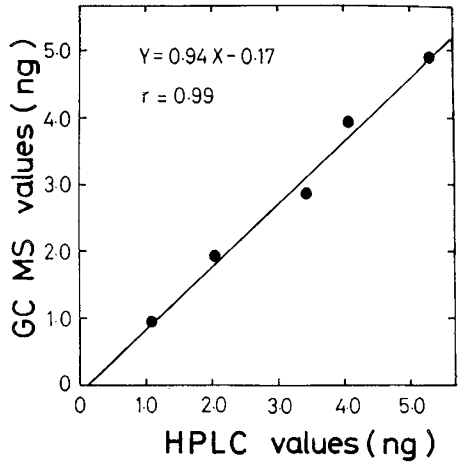

Fig. 3. Correlation of GC-MS and HPLC measurements of authentic LTB_4: authentic LTB_4 (1, 2, 3, 4, and 5 ng) in 2 ml of balanced Hanks' solution were measured by the GC-MS method and by the HPLC method as described in Figures 1 and 2.

A23187 ($r = 0.94$, $p < 0.01$) and by PAF ($r = 0.95$, $p < 0.01$), as can be seen from Figure 4*A* and *B*. However, the amounts of A23187-stimulated and PAF-stimulated LTB_4 production found by GC-MS were about two-thirds and nine-tenths of those found by HPLC, respectively.

The diurnal variation of LTB_4 production stimulated by A23187 was also examined. PMN were isolated from one individual five times during a 4-wk period and the LTB_4 production was assayed. The range of values was small, from 39 to 50 ng/10^6 cells/5 min. The mean (± SD) was 43.6 ± 8.1 ng/10^6 cells/5 min.

There were no significant differences between the production rate of LTB_4 by PMN in the umbilical cords and those of the adults, either in response to A23187 (43 ± 17 *versus* 37 ± 12 ng/10^6 cells/5 min, means ± SD, $p > 0.1$, Fig. 5*A*), or in response to PAF (1.1 ± 0.80 *versus* 0.84 ± 0.30 ng/10^6 cells/1.5 min, $p > 0.1$, Fig. 5*B*). There was a slight tendency for A23187-stimulated LTB_4 biosynthesis by PMN to increase with age, the mean value being 34 ± 8.8 ng/10^6 cells/5 min for infants 0 to 1 yr of age, 41 ± 24 ng/10^6 cells/5 min for infants 1 to 7 yr of age, and 52 ± 24 ng/10^6 cells/min for children 7 to 15 yr of age (Fig. 5*A*). However, there was no significant difference among these age groups, and these values were also similar to those of the umbilical cords ($p > 0.1$). As another check for an age dependence, the values of LTB_4 production by PMN from different ages were plotted against the age of the donor, but linear regression analysis of these data revealed no apparent correlation of LTB_4 production with ages ($r = -0.11$, $Y = -0.19 \times +43.61$).

DISCUSSION

The GC-MS method is generally thought to be more specific than any other assay methods for prostaglandins, including RIA, bioassay, HPLC and enzyme-immunoassay. In the present studies, when LTB_4 values obtained by the GC-MS method were compared with those by HPLC, there was almost no discrepancy for authentic LTB_4. However, discrepancies were evident with reaction mixture samples, especially with those in response to ionophore A23187. These discrepancies in LTB_4 values might be due to the low specificity of the HPLC method. For example, there is the possibility of contamination of the LTB_4 peak by HPLC with other UV absorbing material. However, there are only a few reports on prostaglandins concentrations in biological samples studied by two independent analytical methods such as GS-MS and RIA (21, 22). From these reports, the discrepancies encountered in the present investigation do not seem unreason-

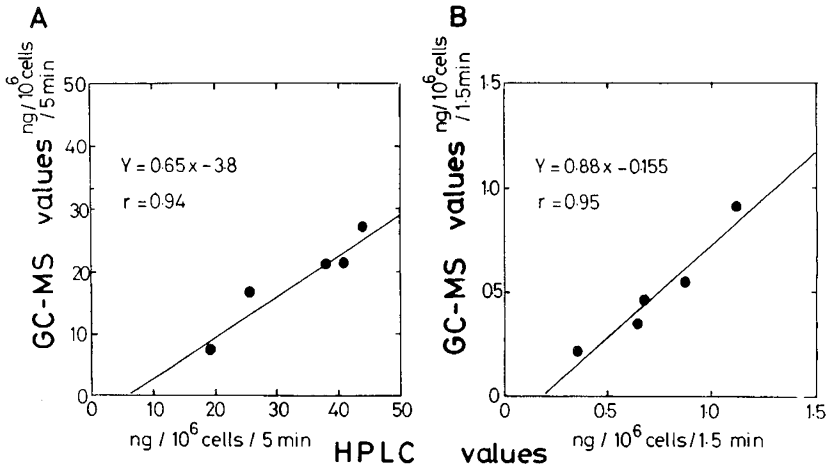

Fig. 4. Correlation of GC-MS and HPLC measurements of LTB_4 production by PMN: PMN preparations (three cord bloods and two adult bloods) were stimulated by ionophore A23187 (*A*) or PAF (*B*) and the LTB_4 were measured by the GC-MS method and by the HPLC method as described in Figures 1 and 2.

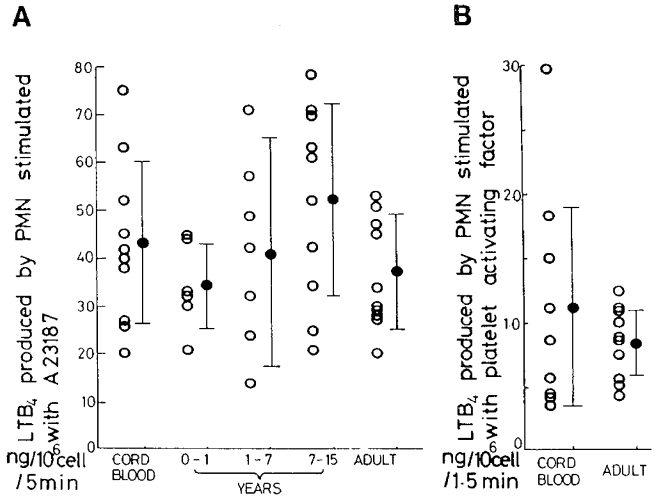

Fig. 5. The ionophore-stimulated (*A*) and PAF-stimulated (*B*) LTB_4 production of PMN from cord blood and subjects in the different age groups: *brackets* indicate the mean ± SD.

able, given the fact that nanogram amount of prostaglandins in biological samples were measured by two very different methods.

The values for LTB_4 production in response to A23187 and in response to PAF obtained by HPLC were well correlated with the values obtained by GC-MS. Therefore, the LTB_4 assay by HPLC in this investigation combined with reversed and straight phase columns seems to be sufficiently specific to examine the relative abilities of PMN from different age groups to produce LTB_4 in response to A23187 or PAF.

Recently, the metabolism of LTB_4 to 20-OH-LTB_4 has been reported to be quite rapid (23), but the metabolism of LTB_4 to 20-OH-LTB_4 in this investigation was negligible in PMN from both umbilical cords and adults, as judged from the GC-MS data in Figure 2.

Several kinds of PMN stimulants for PMN are known to induce quite different metabolic responses in PMN (24, 25). We have experimented using ionophore A23187 and PAF. Therefore, our conclusions probably must be limited to ionophore A23187 and PAF. But ionophore A23187 seems to be one of the best stimulants for production of LTB_4 in PMN, because it represents a maximal stimulus for PMN. So it would appear that the ability of PMN to produce and release LTB_4 in newborns, infants, and children is as well developed as in adults.

The present data did not support our expectation that the increased susceptibility to infection in the neonate and the mechanism of retarded PMN chemotaxis in the neonatal period might be partially explained by the developmental alteration of LTB_4 biosynthesis in PMN. However, if we consider the developmental aspects of the inflammatory response, neonatal infections such as meningitis, pneumonia, necrotizing enterocolitis, and peritonitis may initially involve the release of LTB_4 from tissue effector cells such as macrophages. These phagocytes are likely to be more immature than end stage cells such as PMN. Developmental change of LTB_4 production be tissue phagocytic cells remains to be examined, but might be significant enough to explain the increased susceptibility to infection in the neonate.

Another possibility is that the defective chemotaxis of neonatal PMN is due to alteration of PMN responsiveness to LTB_4. The high affinity LTB_4 binding site of PMN appears to be associated with chemotaxis and degranulation (26). It is also possible that the high affinity receptor binding of LTB_4 is not fully developed in the neonatal PMN. Further study is necessary to clarify the involvement of LTB_4 in the host defense against bacterial infections in the perinatal period.

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