SPECIFIC BINDING OF LEUKOTRIENE B\textsubscript{4} TO A RECEPTOR ON HUMAN POLYMORPHONUCLEAR LEUKOCYTES\* 

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Human polymorphonuclear leukocytes (PMN)\textsuperscript{1} metabolize arachidonic acid via the lipoxygenase pathway to a number of mono- and di-hydroxyeicosatetraenoic acids (HETE) (1-7). These HETE include 5-D-HETE, 12-L-HETE, 12-L-hydroperoxy-ETE, and at least four isomers of 5,12-diHETE. In addition to arachidonic acid, these HETE have been shown to induce chemotaxis (8-11), chemokinesis (12, 13), degranulation (14-17), and aggregation (13, 15, 18) of human PMN in vitro. The potency of these metabolites to induce chemokinesis is dependent on the position of the substituted hydroxyl groups (5,12-diHETE > 5-HETE > 12-HETE > 12-HPETE > arachidonic acid [12, 19]). Methyl-ester derivatives of 12-HETE specifically inhibit the chemotactic response to the parent molecule (19, 20). Among the four isomers of 5,12-diHETE, 5S,12R-dihydroxy-6(cis),8(trans),10(trans),14(cis)-ETE (leukotriene B\textsubscript{4} [LTB\textsubscript{4}]) is the most active in inducing chemokinesis of human neutrophils, aggregation of rat PMN, and changes in vascular permeability in vivo (21). LTB\textsubscript{4} produces cellular responses in vitro at nanomolar concentrations and is several times more active than the stereoisomers 5S,12R-dihydroxy-6,8,10(all trans)14(cis)-ETE and 5S,12S-6,8,10(all trans),14(cis)-ETE (6-trans-LTB\textsubscript{4} and 12-epi-6-trans LTB\textsubscript{4}, respectively) (3, 21). This difference in potency of LTB\textsubscript{4} from its diastereoisomers suggests the existence of highly specific receptor(s) which are directly involved in modulating PMN responses (21). In this report, we describe the binding of [\textsuperscript{3}H]LTB\textsubscript{4} to human PMN. The binding is shown to be saturable and specific, consistent with the existence of a specific receptor for LTB\textsubscript{4}. The metabolism of LTB\textsubscript{4} on incubation with human PMN is also described.

Materials and Methods

Sources of Materials. Arachidonic acid (Nu Check Prep, Inc., Elysis, MN; N-[phenylalanine-ring-2,6,\textsuperscript{3}H(N)]-formyl-methionyl-leucyl-phenylalanine (47 Ci/mmol), [3-20\textsuperscript{H}] N-arachidonic acid (240 Ci/mmol), a generous gift of New England Nuclear, Boston, MA; [1-\textsuperscript{14}C]arachidonic acid (55 mCi/mmol) (Amersham, Arlington Heights, IL); A23187, lipopoor

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\textsuperscript{1} Abbreviations used in this paper: ETE, 6,8,10,14-eicosatetraenoic acid; HETE, monohydroxy-ETE; diHETE, dihydroxy-ETE; 5-HPETE, 5-hydroperoxy-ETE; LTB\textsubscript{4}, 5S,12R-dihydroxy-6(cis),8,10(trans),14(cis)-ETE; 6-trans-LTB\textsubscript{4}, 5S,12R-dihydroxy-6,8,10,(all trans),14(cis)-ETE; 12-epi-6-trans-LTB\textsubscript{4}, 5S,12S-dihydroxy-6,8,10,(all trans),14(cis)-ETE; SS,12S-DHETE, 5S,12S-dihydroxy-6(cis),8(trans),10(trans),14(cis)-ETE; FMLP, N-formyl-methionyl-leucyl-phenylalanine; IC\textsubscript{50}, inhibitor concentration giving 50% inhibition; PMN, polymorphonuclear leukocyte; RP-HPLC, reverse phase high pressure liquid chromatography; SP-HPLC, straight phase high pressure liquid chromatography; TLC, thin-layer chromatography.
bovine serum albumin (Calbiochem-Behring Corp., La Jolla, CA); Hepes, N-formyl-methionyl-leucyl-phenylalanine (FMLP) (Sigma Chemical Co., St. Louis, MO); Ficoll-Paque, Pharmacia Fine Chemicals, Inc., Piscataway, NJ.

Purification of Cells. Purified human PMN were prepared from heparinized peripheral venous blood by dextran sedimentation of erythrocytes followed by isopycnic centrifugation using a Ficoll-Hypaque gradient (22). Residual contaminating erythrocytes were removed by hypotonic lysis. Platelets were removed by repeated low speed centrifugation to levels of less than one platelet per nucleated cell. More than 98% of the nucleated cells were PMN as determined morphologically (Wrights stain). Human peripheral blood lymphocytes were prepared by the procedure described previously (23). Approximately 95% of the nucleated cells were lymphocytes. Contamination of platelets and erythrocytes was minimal (usually less than one platelet or erythrocyte per nucleated cell). Cells were examined for viability by exclusion of suprivial dye (24). Viability was routinely >95%.

Preparation of [1H]- and [14C]5,12-diHETE. Radiolabeled 5,12-diHETE (LTB₄, 6-trans-LTB₄, and 12-epi-6-trans-LTB₄) were prepared by incubation of human PMN with the ionophore A23187 in the presence of radiolabeled arachidonic acid as described elsewhere (25, 26). In brief, PMN at a concentration of 1 × 10⁶ cells/ml in Dulbecco’s phosphate-buffered saline (27), pH 7.4, were incubated with 10 μg/ml A23187 and either 4.8 μg/ml [3H]arachidonate or 38.3 μg/ml arachidonic acid containing a tracer amount of [14C]arachidonic acid. The cells were incubated with the [3H]ligand for 5 min at 37°C in a final volume of 2 ml. The cells were then added to 3.75 ml of methanol and agitated vigorously. The extraction mixture was centrifuged at 1,200 rpm for 10 min at 4°C, and the supernatant was removed. The pellet was washed with an additional 1.25 ml of methanol and recentrifuged. The supernatants were combined, acidified to an apparent pH of 3, and added to a mixture of 15 ml peroxide-free ether and 10 ml water. After vigorous mixing, the water phase was removed and the ether phase was washed with an additional 2.5 ml water. The ether phase was dried under N₂. The residue was dissolved in a small volume of methanol and applied to a 17 × 1.2-cm column of silicic acid (silicic acid, 100 mesh, Mallinckrodt Inc., Science Products Div., St. Louis, MO) (heated at 110°C just before use, packed in chloroform/methanol 7:3, vol/vol). The 5,12-diHETE eluted in the first fraction with chloroform/methanol, 7:3 (vol/vol). This fraction was dried under N₂ and chromatographed on silica gel G-coated plates (Brinkman Instruments, Inc., Westbury, NY) developed with benzene/diethyl ether/ethanol/acetic acid (50:40:2.0:0.2, vol/vol/vol/vol) (1). The thin-layer chromatography (TLC) plates were autoradiographed, and the 5-HETE and 5,12-diHETE bands were identified by comparison with radiolabeled reference standards prepared and characterized by Dr. William Stenson (Dept. of Medicine, Washington University) or ourselves using previously described methods (1, 5, 28, 29), including in some instances mass spectroscopy. The bands were scraped, eluted in methanol, and further analyzed and purified by high pressure liquid chromatography. Reverse-phase high pressure liquid chromatography (RP-HPLC) was performed on a Varian model 8550 instrument (Varian Associates, Palo Alto, CA) using a μBondapack C-18 column (4 × 250 mm) (Waters Associates, Milford, MA) in a solvent system of methanol/water/acetic acid, 700:300:01 (vol/vol/vol), at 25°C and a flow rate of 1 ml/min. Three of the 5,12-diHETE isomers used in this paper (Table I) gave single peaks on HPLC (UV monitor, 280 nm) that were clearly separated from one another. In this system, 6-trans-LTB₄, 12-epi-6-trans-LTB₄, and

| Identification of [14C]-5,12-diHETE* |
|--------------------------------------|
| rř | Ultraviolet maxima | Retention time HPLC minutes |
| 6-trans-LTB₄ | 55,12R-dihydroxy-6,10(all trans)14-cis-ETE | 0.21 | 258, 268, 279 | 12 |
| 12-epi-6-trans-LTB₄ | 55,12S-dihydroxy-6,10(all trans)14-cis-ETE | 0.19 | 258, 268, 279 | 13.2 |
| LTB₄ | 55,12R-dihydroxy-6 cis,8,10trans,14cis-ETE | 0.17 | 260, 270, 280 | 14.5 |
| 35,12S-8HETE | 55,12S-dihydroxy-6,10trans,14-cis-ETE | 0.16 | 258, 268, 278 | — |

* See Methods and Materials for synthesis and isolation procedures.
‡ Fractional distance migrated as compared with solvent front on TLC.
LTB₄ eluted at 12, 13.2, and 14.5 min, respectively. In addition, the LTB₄ fraction was examined for the presence of 5S,12S-dihydroxy-6(trans),8cis,10(trans),14(cis)-ETE (5S,12S-diHETE) as described by Borgeat et al. (30). A sample of LTB₄ was methylated by reaction with diazomethane, and the products were evaluated by both reverse-phase and straight-phase (SP) HPLC. On RP-HPLC (~Bondapack C18 column, methanol/water/acetic acid, 75/25/0.1, pH 5.4, flow at 1 ml/min) the derivitized fraction gave a single peak at 15 min. SP-HPLC was performed in an isocratic system of hexane/isopropanol/acetic acid, 94/6/0.1 on a µPorasil column (Waters Associates) with a flow rate of 1 ml/min. In this system, the LTB₄-methyl ester was obtained at 19.0 min with minor peaks at 10 and 14 min (Fig. 2). Greater than 95% of the [³H]LTB₄ counts were associated with the 19-min peak. Fewer than 5% of the counts occurred in the 10-min peak which corresponds to the 5S,12S-DHETE isomer (30). The [¹⁴C]LTB₄ preparation, however, was found to contain ~10–15% of the isomer as counts.
Fig. 2. Time course of [3H]LTB4 binding to human neutrophils at 4°C and 37°C. Cells were incubated at a concentration of 1.5 × 10⁷ cells/ml with 8 nM [3H]LTB4 at 37°C (○) or 4°C (□). After incubation for the indicated time a 100-μl aliquot (1.5 × 10⁶ cells) was centrifuged through a 30% Ficoll-Paque gradient. The cell pellet was then processed for radioactivity and protein content as described in Materials and Methods. (B) Linearity of [3H]LTB4 binding with cell concentration. Human PMN were incubated at varying concentrations with 4 nM [3H]LTB4 for 45 min at 4°C. At the end of the incubation, a 100-μl aliquot of cell suspension was processed as in A. All points represent the mean of triplicate samples ± SD.

recovered in the 10-min peak. Thus, the [3H]ligand was essentially pure, whereas the [14C]ligand contained a small, but significant amount of the 5S,12S-DHETE isomer. The presence of this isomer and its possible effects on the ability of the [14C]LTB4 to block the binding of [3H]LTB4 are discussed below. The 5S,12S-DHETE was prepared by scraping the band immediately below the LTB4, eluting with methanol, and then reapplying to the same TLC system with multiple consecutive runs of solvent. This procedure served to maximize the separation of the LTB4 and 5S,12S-DHETE isomer and allow recovery of either isomer in the form of the free acids, avoiding the necessity of saponifying the material. Further characterization of the 5S,12S-DHETE band showed maxima of the ultraviolet spectrum to be 258, 268, 278 (R. A. Kreisle and C. W. Parker, manuscript in preparation).

Estimates of total recovery and specific activity of the LTB4 as calculated in the original incubation mixture and spectrophotometric measurements at 270 nm with a molar extinction coefficient of 47,000 (4) were in good agreement. Synthesis and isolation of [14C]LTB4 was carried out in a manner identical to the [3H]LTB4, except that the ether extracts were plated directly on thin-layer chromatography plates without separation on a silicic acid column. Products of both the [3H]- and [14C]arachidonic acid incubations appeared to be identical by TLC, HPLC, and ultraviolet spectra. The radio-labeled ligands were stored at -20°C in methanol or chloroform and remained homogeneous to TLC for up to 6 mo.

Chemokinesis Assay. The chemokinetic activity of [3H]LTB4 and [14C]LTB4 was assayed by the agarose microdroplet assay of Smith and Walker (13, 31) in Gey’s medium (32).

Binding Assay. PMN were incubated in triplicate at a concentration of 1 × 10⁷ cells/ml in Gey’s medium (23) with 25 mM Hepes, pH 7.4 and varying amounts of [3H]LTB4 and blockers at 4°C. Incubations were started by addition of [3H]LTB4 (final concentration 8 nM) with the blocking agent (various concentrations) or buffer, using a final incubation volume of 130 μl. Cells were routinely incubated for 45 min at 4°C (except as noted). At the end of the incubation, 100 μl of the incubation mixture was layered on top of a 200-μl layer of 30% Ficoll-Hypaque in buffer in a 400-μl polyethylene microtest tube (Bio-Rad Laboratories, Richmond, CA). The cells were pelleted by centrifugation for 2 min in a Beckman model 152 microfuge (Beckman Instruments, Fullerton, CA). This method allowed for the rapid separation of the cells from the incubation mixture with minimal contamination by free ligand (<2% in the 50 μl of gradient immediately above the pellet area in control experiment without cells). The supernatant and gradient were removed by aspiration, and the bottom of the tube containing the pellet was excised (33). The pellet was solubilized in 0.5 ml 1 N NaOH at 50°C for 2 h. The solubilized pellet was neutralized with 0.5 ml 1 N HCl, and 100 μl was processed for protein measurements as described below. The remaining 900 μl was counted for 10 min or longer in Scintiverse
Recovery of cells in the pellet was monitored by protein determinations (34) of a fraction of the solubilized pellets. The amount of protein was found to be linearly related to the number of cells layered on top of the 30% Ficoll-Hypaque gradient. For both PMN and lymphocytes, recovery of protein from cells pelleted through the gradient was >95% of that for an equal number of cells pelleted through buffer alone. Apparently complete recovery of cells occurred under all of the conditions tested including the highest concentrations of [\(^{14}\)C]5,12-diHETE. All conditions were carried out in triplicate and major experimental conclusions were verified on multiple occasions.

**Results**

**Characterization of \[^{3}\text{H}]\text{LTB}_4 \text{ and } [^{14}\text{C}]\text{LTB}_4.** To screen for the possible presence of high affinity receptors on human PMN for LTB4, \[^{3}\text{H}]\text{LTB}_4 \text{ of high specific activity was prepared biosynthetically from } [^{3}\text{H}]\text{arachidonic acid (240 Ci/mmol, see Methods and Materials). This } ^{3}\text{H} \text{ ligand permitted the study of binding of LTB}_4 \text{ at nanomolar concentrations. } [^{14}\text{C}]\text{LTB}_4 \text{ of much lower specific activity was used as a blocking ligand. The } [^{3}\text{H}]\text{LTB}_4 \text{ and } [^{14}\text{C}]\text{LTB}_4 \text{ molecules behaved identically on TLC and HPLC, gave identical ultraviolet spectra, and gave nearly identical dose response curves in an agarose chemokinetic assay (not shown) with peak potency at 1 nM. The chemokinetic activity of our labeled LTB}_4 \text{ agree well with previously published reports from other laboratories (12, 13, 21) and indicate that our radiolabeled ligand preparations have the expected level of biological activity.}\)**

**Binding of \[^{3}\text{H}]\text{LTB}_4 \text{ to human PMN.** Fig. 2A shows typical time courses for the binding of \[^{3}\text{H}]\text{LTB}_4 \text{ at } 4^\circ\text{C and } 37^\circ\text{C. At } 4^\circ\text{C, total binding increased rapidly over the first 5 min, increased more slowly over the next 40 min, had leveled off by 60 min (Fig. 2), and remained constant for up to 120 min (not shown). Binding of } [^{3}\text{H}]\text{LTB}_4 \text{ at } 37^\circ\text{C, however, showed a very different time course. Total binding was maximal at 5 min and decreased thereafter. This decrease in the cell-associated counts was associated with the appearance of a } [^{3}\text{H}]-\text{labeled metabolite of LTB}_4 \text{ in the supernatant as discussed below. To minimize this effect, all experiments were carried out at } 4^\circ\text{C. Total binding was linear with cell concentration as shown in (Fig. 2B). A cell concentration of } 1.5 \times 10^7 \text{ cells/ml was routinely used, which bound 5–10% of the total ligand (range 3–13% for eight experiments).}\)**

Recovery of cells through the gradient was measured by protein determination of the pellet and was linear with cell concentration (not shown). Although LTB4 is known to cause agglutination and changes in cell density and volume (35, 36), no changes in the recovery of cells through the gradient occurred under any of the conditions tested. Recovery of lymphocytes was identical to that of PMN as determined by cell surface iodination studies (data not shown).

**Blocking of \[^{3}\text{H}]\text{LTB}_4 \text{ Binding to PMN by } [^{14}\text{C}]\text{LTB}_4.** The effect of various concentrations of \[^{14}\text{C}]\text{LTB}_4 \text{ on the binding of cells incubated with } 8 \text{ nM } [^{3}\text{H}]\text{LTB}_4 \text{ is shown in Fig. 3. Inhibition of } [^{3}\text{H}]\text{LTB}_4 \text{ binding by } [^{14}\text{C}]\text{LTB}_4 \text{ demonstrates competition for a saturable number of sites and represents specific binding. From Fig. 3 it is evident that } [^{3}\text{H}]\text{LTB}_4 \text{ binding changed very little as the concentration of } [^{14}\text{C}]\text{LTB}_4 \text{ was increased from } 1 \times 10^{-6} \text{ M to } 3 \times 10^{-5} \text{ M. Maximal inhibition ranged from 65 to 80% in this concentration range. Based on the assumption of reversibility (see below), the 50% inhibitory concentration (IC}_{50} \text{ of the blocking gives an apparent affinity constant } (K_D) \text{ of } 2 \times 10^{-7} \text{ M for the specific binding sites. From the difference between total }}\)
and nonspecific binding at $1 \times 10^{-6}$ M $^{[14}C]LTB_4$, the minimal number of specific binding sites per cell is $3.86 \times 10^5$ (±0.72 SEM, n = 6). Because the amounts of LTB$_4$ available were limited, repeated experiments at higher LTB$_4$ concentrations could not be performed, and the presence of additional low affinity binding sites is not excluded.

Fig. 4 shows the binding of $[3H]LTB_4$ as a function of increasing concentration of free LTB$_4$. Nonspecific binding, as determined by the binding of $[3H]LTB_4$ in the presence of $1 \times 10^{-5}$ M $^{[14}C]LTB_4$, is linear over the concentration range tested and represents 2.5% of the free ligand concentration. Specific binding is determined by subtracting the nonspecific binding from the total binding and is approximated by the solid circles in Fig. 4. Although the specific binding curve is not quantitatively determined by these points, it can be seen that the curve is approaching saturation at concentrations above 150 nM.

**Ability of $^{[14}C]LTB_4$ to Block the Binding of $^{[3}H]FMLP$ to PMN.** Binding by $^{[14}C]LTB_4$ at 4°C was also studied for its ability to block a second chemokinetic agent, FMLP. Excess LTB$_4$ (1 μM) at a concentration of $^{[14}C]LTB_4$ that reduces $^{[3}H]LTB_4$ binding to PMN by 70-80% does not affect $^{[3}H]FMLP$ binding to these cells at any point in the time course. At 37°C, blocking was seen only at 10 μM LTB$_4$ and represented <35% of the total FMLP binding (data not shown, n = 3 experiments). These data suggest that LTB$_4$ and FMLP react with different receptors and that most of the self inhibition by LTB$_4$ does not involve nonspecific cellular aggregation or surface membrane changes.

**Specificity of $^{[3}H]LTB_4$ Binding to PMN.** Preliminary experiments examining the ability of different lipids to block the binding of LTB$_4$ to PMN demonstrated a relative inhibitory capacity of $^{[14}C]LTB_4 > 5$-HETE > arachidonic acid = 12-OH stearic acid (data not shown). LTB$_4$ was at least 3-10-fold more effective than 5-
Fig. 4. Total and nonspecific binding of [3H]LTB4 to PMN. Cells were incubated at a concentration of 1.4 × 10^7 cells/ml with increasing concentrations of [3H]LTB4 in the presence (Δ) or absence (□) of 1 × 10^{-5} M [14C]LTB4 for 45 min at 4°C. Specific binding (○) was determined by the difference between the two curves.

Fig. 5. Ability of the 5,12-diHETE stereoisomers, 6-trans-LTB4, and 12-epi-6-trans-LTB4, to block [3H]LTB4 binding to PMN. Cells were incubated at a concentration of 3 × 10^7 cells/ml for 25 min at 4°C with 6 nM [3H]LTB4 and varying concentrations of [14C]LTB4 (□), 6-trans-LTB4 (○), and 12-epi-6-trans-LTB4 (●). Aliquots containing 3 × 10^6 cells were processed as in Fig. 2. Specific binding was determined as the difference between binding in the absence of any blocking agent and the [3H]LTB4 binding in the presence of 10^{-5} M [14C]LTB4. The binding at any given blocker concentration was adjusted for nonspecific binding, and the percent of control binding was calculated. Each point represents mean ± SD for triplicate determinations.

HETE in blocking the binding of the [3H] ligand. Further specificity of the system is demonstrated by a comparison of the ability of the stereoisomers 6-trans-LTB4 and 12-epi-6-trans-LTB4 to block the binding of [3H]LTB4 (Fig. 5). The inhibition curves for 6-trans-LTB4 and 12-epi-6-trans-LTB4 were shifted to the right (less effective inhibition) as compared with the curve for [14C]LTB4. This represents an approximate threefold increase in the concentration of 6-trans-LTB4 and 12-epi-6-trans-LTB4 required to
Fig. 6. Reversibility of [3H]LTB₄ binding to human PMN. In panel A, PMN were incubated at a concentration of 1.4 × 10⁷ cells/ml with 3.6 nM [3H]LTB₄ for 30 min at 4°C in a total volume of 400 µl. At 30 min, 50 µl of buffer (○) or [14C]LTB₄ (●) (final concentration of 700 nM) was added. Aliquots were removed at 0, 15, 30, and 45 min after addition of the buffer or ligand and compared with a simultaneous time control (□) in which 50 µl of buffer was added at the beginning of the incubation. In panel B, PMN at a concentration of 3 × 10⁷ cells/ml were incubated with 4 nM [3H]LTB₄ in the presence (△, □) or absence (○, ●) of 1 µM [14C]LTB₄ in a final volume of 1 ml for 45 min at 4°C. At the end of this incubation, the cells were pelleted at 2,000 rpm for 2 min, the supernatant was removed, and the pellets were resuspended at twice the incubation volume in either buffer alone (○, △) or the original supernatant (□, □), and aliquots were removed at the indicated time after resuspension. Aliquots were processed as in Fig. 4 and each point represents mean ± SD of triplicate samples.

reduce [3H]LTB₄ binding by 25–75% as compared with [14C]LTB₄. The maximal amount of blocking seen by all three isomers is the same. Although LTB₄ was distinguishable from both 6-trans-LTB₄ and 12-epi-6-trans-LTB₄ as an inhibitor of [3H]LTB₄ binding, 6-trans-LTB₄ and 12-epi-6-trans-LTB₄ were quite comparable to one another in this regard. We also looked at the ability of the 5S,12S-DHETE to block the binding of [3H]LTB₄. At 1 × 10⁻⁶ M, this isomer was able to block nearly as well, but certainly not better, than the LTB₄ itself (R. A. Kreisle and C. W. Parker, manuscript in preparation).

Reversibility of [3H]LTB₄ binding to PMN. To examine the reversibility of [3H]LTB₄ binding, cells were incubated for 30 min at 4°C with 3.6 nM [3H]LTB₄, at which time 700 nM [14C]LTB₄ or buffer was added in a small volume (Fig. 6 A). Little or no reversibility was demonstrated on addition of 200-fold excess of [14C]LTB₄ over the original total LTB₄ present, although further uptake of [3H]LTB₄ was inhibited. In the experiment shown in Figure 6 B, cells were incubated with 4 nM [3H]LTB₄ with or without 1 µM [14C]LTB₄ for 45 min at 4°C. The cells were then pelleted and resuspended in twice the original volume of supernatant (containing label) or buffer alone. Again, no reversibility was seen over a period of 90 min. Thus, neither the
addition of excess [\(^{14}\text{C}\)]LTB\(_4\) nor resuspension in ligand-free medium convincingly promoted the release of cell-bound counts. The lack of evidence for dissociation could be due to covalent binding of LTB\(_4\), its internalization and unavailability to surface ligand, or a low kinetic constant for dissociation of a reversible binding site at the cell membrane. The first possibility is unlikely, since neither we (W. Stenson and C. W. Parker, unpublished data) nor others (37) have been able to demonstrate covalent binding of LTB\(_4\) to cellular lipids, in marked contrast to the monohydroxy-eicosa-tetraenoic acids (1, 37). Moreover, when LTB\(_4\) is bound to cells at 4°C and the cells are then washed and incubated further at 37°C, the radiolabel is rapidly eluted into the medium, so there is no doubt that association with cells is reversible (75% by 45 min, data not shown).

**Binding of [\(^{3}\text{H}\)]LTB\(_4\) to Lymphocytes.** In addition to PMN, we examined the ability of human peripheral blood lymphocytes to specifically bind [\(^{3}\text{H}\)]LTB\(_4\). The ability of 2 × 10\(^6\) lymphocytes to bind [\(^{3}\text{H}\)]LTB\(_4\) was compared with an equal number of PMN in the presence and absence of 1 μM [\(^{14}\text{C}\)]LTB\(_4\). Both the total number of counts bound (156 ± 36 vs. 73 ± 8) and the percentage of binding seen with the [\(^{14}\text{C}\)]LTB\(_4\) (55% vs. 37%) was greater with the neutrophils than with the lymphocytes. The time course of binding and recovery of cells through the 30% Ficoll-Hypaque gradient was similar for the two cell populations (data not shown).

**Metabolism of [\(^{3}\text{H}\)]LTB\(_4\) on Incubation with PMN.** In view of the striking loss of cell-associated counts on incubation of PMN with [\(^{3}\text{H}\)]LTB\(_4\) at 37°C (Fig. 2) and the

| Band | rf | 4°C | 37°C |
|------|-----|-----|-----|
|      |     | No cell (control) | 10 min (142,780 cpm) | 40 min (101,925 cpm) | No cell (control) | 10 min (67,660 cpm) | 60 min (73,555 cpm) |
| 1    | 0.02-0.03 | 0.5 | 1.3 | 3.4 | 2.0 | 3.5 | 4.2 | 9.5 | 3.7 | 13.4 | 1.0 |
| 2    | 0.05-0.07 | —   | 1.1 | 7.7 | 1.3 | 8.6 | 3.7 | 23.0 | 42.0 | 63.0 | 3.6 |
| 3    | 0.13-0.15 | —   | —   | —   | —   | —   | 5.4 | 3.2 | 2.2 | 5.9 | <1 |
| 4    | 0.16-0.19 | 87.8 | 47.0 | 20.5 | 39.8 | 26.5 | 69.7 | 12.5 | 3.8 | 11.8 | <1 |
| 5    | 0.19-0.24 | 8.1 | 10.9 | 3.4 | 7.3 | 4.3 | —   | —   | —   | —   | —   |
| 6/7  | 0.38-0.45 | 2.0 | —   | —   | —   | —   | 3.1 | —   | —   | —   | —   |
| Total |       | 98.4 | 60.3 | 35 | 53.5 | 42.9 | 100.0 | 48.2 | 51.7 | 94.1 | 4.6 |

* 2.5 × 10\(^7\) cells were incubated with 20 nm [\(^{3}\text{H}\)]LTB\(_4\) at either 4°C or 37°C. After incubation for the indicated time the supernatants and pellets were separated and extracted with ethanol/ether. The extracts were then analyzed by TLC (Silica gel G-coated plates; benzene/diethyl ether/ethanol/acetic acid, 50:40:2:0.2, vol/vol/vol/vol).

‡ The figures in the table represent the % of the original CPM recovered for that incubation (total pellet plus supernatant). The overall recovery of radioactivity was ≥90%.

§ Total amount of counts recovered (pellet + supernatant) for an aliquot of cells taken at the given time (mean of two).

¶ Bands were visualized by autoradiography and identified by comparison with LTB\(_4\) standard. Band 4 co-migrated with [\(^{3}\text{H}\)]LTB\(_4\).

† Supernatant.
apparent lack of reversibility at 4°C but not at 37°C, we looked to see if any differences existed in the cell-bound vs. free ligand at both temperatures. Briefly, we incubated the PMN with 20 nM [3H]LTB4 for 10 or 40 min at 4°C (10 or 60 min at 37°C). The incubation mixtures were centrifuged, and the supernatants and pellets were extracted separately with ethanol/ether (7). These extracts were then subjected to TLC as described in Materials and Methods. A total of seven major and minor bands appeared on radioautography of the TLC plates (Table II). A no-cell control was run for comparison in which [3H]LTB4 was incubated in buffer at each temperature and extracted in the same way as the cell suspensions.

Band 4 represents unaltered [3H]LTB4 and is the major form of the [3H] label at both temperatures on incubation in the absence of cells. At 4°C, the total amount of unchanged ligand recovered in the supernatant and pellet together was 67 and 66% for 10 and 40 min, respectively. At both of these times about 30% of the remaining LTB4 was cell bound. Nearly 25% of the cell-bound ligand is in the form of band 2. Band 2 is probably the 20-hydroxy derivative of LTB4 (5,12,20-trihydroxy-ETE) which has recently been characterized by Hansson et al. (38) in human leukocyte preparations.

After only 10 min at 37°C, almost 50% of the original counts are cell-bound and >80% of these are in band 2, but 7-8% are in LTB4. By 60 min, <7% of the total counts are associated with the pellet. This agrees well with the loss of cell-associated counts in the time course study of binding at 37°C (Fig. 2). After 60 min at 37°C there is no longer any cell-associated LTB4, providing further evidence for reversibility of binding at this temperature. In addition, at 10 min 23% of the total counts are present in the supernatant as band 2. At 60 min, this fraction has increased to 63% and corresponds quantitatively to the loss of band-2 counts associated with the cell pellet. Band 5 is a doublet and corresponds to the stereoisomers, 6-trans-LTB4 and 12-epi-6-trans-LTB4, which also occur in small quantities in the cell-free control (but not in the LTB4 standard, not shown). Bands 1, 3, 6, and 7 are as of yet unidentified but are quantitatively minor in any case.

Discussion

Several lines of evidence support a role for arachidonic acid metabolites in the chemotaxis, aggregation, and degranulation of PMN (1, 8-18, 35-36, 39, 40). In this study we have demonstrated the ability of PMN to specifically bind [3H]LTB4, the most potent of the arachidonic acid metabolites in inducing PMN activity: (a) Maximal or near maximal blocking of [3H]LTB4 binding by [14C]LTB4 occurs at concentrations of 1 μM or greater and represents 65-80% of the total binding. Thus, at low molar concentrations of [3H]LTB4 nonspecific binding may represent as little as 20% of the bound ligand. Reversibility of binding is readily demonstrable at 37°C although not at 4°C. (b) Due to the slow dissociation rate seen at 4°C, a Scatchard plot of these data is inappropriate for determining receptor affinity (KD) or number of sites (n). A minimum number of specific binding sites, however, can be determined from maximal amounts of inhibition seen at concentrations of 1 μM [14C]LTB4 or greater (3.9 × 10⁵ specific sites per cell, n = 6). In addition, affinity can be approximated by the concentration of IC50 of [14C]LTB4 which blocks half the specific sites (KD = 1-2 × 10⁻⁷ M for Figs. 3 and 5). The specific binding shown in Fig. 4 is consistent with these values, although saturation of binding is not shown. Because the
maximal LTB₄ concentration we were able to use in our binding studies was 10 × 10⁻⁶ M; the presence of additional binding sites of lower affinity is not excluded. (c) Competition for [³H]LTB₄ binding was greater with [¹⁴C]LTB₄ than with the stereoisomers 6-trans-LTB₄ and 12-epi-6-trans-LTB₄, 5-HETE, arachidonic acid itself, or 12-hydroxystearate, another hydroxylated long chain fatty acid. The discrimination between LTB₄ and its two 6-trans isomers indicates that there is stereospecificity of binding. The relative activities of the three 5,12-diHETE in inhibiting LTB₄ binding is consistent with their relative potencies in inducing chemokinesis (21). The binding of LTB₄ was not due to "contamination" by the 5S,12S-trans,8-cis,10-trans,14-cis,18-ETE isomer, 5S,12S-DHETE (30, 42) which is minimally represented in our LTB₄ preparations due to our use of TLC system, which largely separates this isomer from LTB₄ in our routine preparative procedure. Moreover, if anything, this isomer blocks [³H]LTB₄ binding less well than LTB₄. (d) At equivalent cell concentrations both total and specific binding were greater in PMN than lymphocytes. Both the molecular and cellular specificities of LTB₄ binding demonstrated in this study are consistent with the existence of a specific receptor for LTB₄ on PMN.

Precise quantitation of LTB₄ binding with increasing ligand concentrations may be complicated by the ability of this agent to aggregate PMN and increase their adherence to foreign surfaces. Both of these properties increase with increasing concentrations of LTB₄, incubation time, and incubation temperature. Quantitation of protein in the cell pellets, however, showed no loss of cell recovery under any of the conditions tested. Moreover, the ability of PMN to bind [³H]FMLP was not affected by [¹⁴C]LTB₄ under conditions which maximally inhibited [³H]LTB₄ binding. Thus, little if any of the inhibition seen by blocking concentrations of [¹⁴C]LTB₄ could be attributed to these effects.

The binding of our [³H]LTB₄ appeared to be irreversible at 4°C, but not at 37°C. The most likely explanation is a very slow off rate (kinetic dissociation constant). While one could speculate as to whether the LTB₄ is sequestered in some manner (by incorporation into phospholipids), or internalization in endocytotic vesicles, studies with LTB₄ have failed to demonstrate any esterification into phospholipids or triglycerides in neutrophils (W. Stenson and C. W. Parker, unpublished data) or monocytes (37). The availability of LTB₄ for metabolism and transport back out of the cell at 37°C strongly argues against any irreversible sequestration into a non-reactive lipid pool. Based on the data at 37°C alone, it might be argued that LTB₄ is stimulating endocytosis, facilitating its own uptake and metabolism by cells. However, the blocking of [³H]LTB₄ binding by [¹⁴C]LTB₄ is hard to explain on this basis unless the endocytosis itself is initiated through a specific receptor. The stereospecific uptake of LTB₄ at 4°C, where increased endocytosis in response to an exogenous stimulus would not be expected, would also appear to require a specific receptor.

The physiological relevance of this specific LTB₄ binding is unknown. However, the relative abilities of [¹⁴C]LTB₄, 6-trans-LTB₄, 12-epi-6-trans-LTB₄, 5-HETE, and arachidonic acid to inhibit the binding of LTB₄ do correspond to their relative potencies in inducing chemotaxis. Further attempts to correlate function with specific binding of LTB₄ will require that the cellular constituents involved in LTB₄ binding be characterized both in terms of location and molecular structure.

Summary

In this paper we have described the binding of nanomolar concentrations of [³H]leukotriene B₄ (LTB₄) to human polymorphonuclear leukocytes. Because up to
80% of the total \[^{3}\text{H}]\text{LTB}_4\) binding was blocked by excess (>100 times) \[^{14}\text{C}]\text{LTB}_4\), the majority of binding is specific. Stereospecificity of the \(\text{LTB}_4\) binding is demonstrated by the diminished relative abilities of the 6-trans- and 12-epi-6-trans- isomers of \(\text{LTB}_4\) to block \[^{3}\text{H}]\text{LTB}_4\) binding. With these two isomers 3–10-fold higher than \[^{14}\text{C}]\text{LTB}_4\) concentrations were needed for equivalent inhibition of \[^{3}\text{H}]\text{LTB}_4\) binding. This difference is quantitatively less dramatic than the differences between these isomers in many in vitro functional assays such as chemokinesis, chemotaxis, and degranulation. Binding of \[^{3}\text{H}]\text{FMLP}\) is not blocked at >100-fold excess of \(\text{LTB}_4\). The binding of \[^{3}\text{H}]\text{LTB}_4\) to cells appears to be essentially irreversible at 4°C, but not at 37°C where initially bound \(\text{LTB}_4\) is rapidly converted to metabolites which then enter the medium. These results suggest the presence of a saturable, stereospecific site for \(\text{LTB}_4\) on PMN. The association of \(\text{LTB}_4\) binding and the initiation of pharmacological responses to \(\text{LTB}_4\) will require further studies.

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