HETEROGENEITY OF HUMAN POLYMORPHONUCLEAR LEUKOCYTE RECEPTORS FOR LEUKOTRIENE B_4

Identification of a Subset of High Affinity Receptors that Transduce the Chemotactic Response

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Mast cells, polymorphonuclear (PMN)[1] leukocytes, monocytes, and macrophages convert arachidonic acid to 5(S),12(R)-dihydroxy-eicosa-6,14 cis-8,10 trans-tetraenoic acid or leukotriene B_4 (LTB_4) by 5-lipoxygenation and subsequent enzymatic hydration of the 5,6-epoxy-eicosatetraenoic acid derived from 5-hydroperoxy-eicosatetraenoic acid (1–5). When platelets are present with the leukocytes, platelet 12-lipoxygenation of 5-hydroxy-eicosatetraenoic acid (5-HETE) contributes significantly to the generation of LTB_4 (4, 6). LTB_4 stimulates PMN leukocyte chemotactic and chemokinetic migration (7–11), release of lysosomal enzymes in the presence of cytochalasin B (7, 12–14), adherence (15), aggregation (9, 16), expression of C3b receptors (17), and biochemical pathways that are specific prerequisities for the activation of PMN leukocytes (18, 19). Since the optimal expression of several activities of PMN leukocytes was elicited by different concentrations of LTB_4 (7, 13, 15–17), it was postulated that either the extent of saturation of a single class of receptors initiated different functions or that a distinct subset of receptors with a different affinity for LTB_4 was selectively coupled to each function.

Receptors for LTB_4 have been defined recently by quantifying the binding of [^{3}H]LTB_4 to human neutrophils (20, 21). Scatchard analysis of the binding data revealed $2.6–4.0 \times 10^4$ receptors per neutrophil with one apparent dissociation constant (K_d) of $1.1–1.4 \times 10^{-8}$ M. Other chemotactically active products of the 5-lipoxygenation of arachidonic acid inhibited by 50% the binding of [^{3}H]LTB_4 to the neutrophils at concentrations that evoked half-maximal chemotactic responses, whereas the chemotactically inactive leukotriene C_4 (LTC_4) did not compete with [^{3}H]LTB_4 for binding to the receptor. The chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and chemotactic fragments of C5 (C5fr) also failed to inhibit the binding of [^{3}H]LTB_4 to neutrophils, which

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[1] *Abbreviation used in this paper:* BSA, fatty acid-free bovine serum albumin; C5fr, chemotactic fragments of C5; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution with phenol red; 5-HETE, 5-hydroxy-eicosatetraenoic acid; hpf, high power field; HPLC, high performance liquid chromatography; LT, leukotriene; LTD_4, 5-hydroxy-6-S-cysteinyl-glycyl-eicosatetraenoic acid; OVA, ovalbumin; PMN, polymorphonuclear; 5(S),12(S)-6 trans-di-HETE, 5(S),12(S)-di-hydroxy-eicosa-6,8,10 trans-14 cis-tetraenoic acid.

established that the subset of chemotactic receptors for LTB_4 is distinct from those that mediate the responses to peptide chemotactic factors (20). The availability of purified [^{3}H]LTB_4 with a specific activity 7–10 times higher than that used in the initial characterization of the LTB_4 receptor has now permitted the demonstration of two classes of LTB_4 receptors on human PMN leukocytes that differ in affinity for [^{3}H]LTB_4 by ~150-fold. A critical functional role is suggested for the high affinity receptors, since they exhibit a K_d similar to the concentration of LTB_4 required to evoke a half-maximal PMN leukocyte chemotactic response and are eliminated selectively by chemotactic deactivation of PMN leukocytes to LTB_4.

Materials and Methods

Materials. Hanks' balanced salt solution with phenol red (HBSS) (M. A. Bioproducts, Walkersville, MD), *n*-butyl phthalate, fatty acid-free bovine serum albumin (BSA), fMLP, phenolphthalein glucuronic acid (Sigma Chemical Co., St. Louis, MO), ovalbumin (OVA) (Miles Laboratories, Inc., Elkhart, IN), [5,6,8,9,11,12,14,15(N)-^{3}H]LT B_4 (180–221 Ci/mmol; Amersham Corp., Arlington Heights, IL), Ficoll-Hypaque (6 g/100 ml dextran 70 in normal saline; Pharmacia Fine Chemicals, Piscataway, NJ), dinonyl phthalate (ICN Pharmaceuticals, Inc., Plainview, NY), cytochalasin B (Aldrich Chemical Co., Milwaukee, WI), and Hydrofluor (National Diagnostics, Inc., Somerville, NJ) were obtained from the suppliers noted. All organic solvents were redistilled from glass (HPLC grade; Burdick & Jackson Laboratories, Inc., Muskegon, MI). Partially purified chemotactic fragments of C5 (C5fr) were prepared from yeast-activated human serum as described (7, 10). Synthetic LTD_4 (5-hydroxy-6-S-cysteinyl-glycyl-eicosatetraenoic acid), LTB_4, and 12(S)-LTB_4 were kindly supplied by Dr. J. Rokach of Merck-Frosst Laboratories, Dorval, Canada. 5-HETE and the 5(S),12(S)-isomer of 6 trans-LTB_4 [5(S),12(S)-6 trans-di-HETE] were biosynthetically prepared and purified by reverse-phase high performance liquid chromatography (HPLC) as described (20).

Preparation of Human PMN Leukocytes. Human PMN leukocytes were prepared as described (22) from sodium citrate-anticoagulated venous blood of normal donors. Erythrocytes were removed by dextran sedimentation followed by a 20-s hypotonic lysis with 20 vol of ice-cold distilled water. Isotonicity was restored by adding 0.6 M KCl. PMN leukocytes of 96% or greater purity were obtained by centrifugation of mixed leukocytes on Ficoll-Hypaque cushions (23). The purified PMN leukocytes were suspended at concentrations of up to 5×10^7 per ml in HBSS containing 0.1 g/100 ml OVA and 10 mM Hepes (pH 7.3) (HBSS-OVA), stored at 4°C, and used within 1–2 h.

Measurement of the Binding of [^{3}H]LTB_4 to PMN Leukocytes. Methanol solutions of [^{3}H]-LTB_4 and nonradiocative fatty acids were reduced nearly to dryness in separate glass tubes under nitrogen and taken up in HBSS-OVA at 4°C, immediately before use as described (20). In each experiment, $5 \pm 3 \times 10^{-14}$ mol (mean ± range) of [^{3}H]LTB_4 and different concentrations of each nonradioactive fatty acid were incubated with replicate suspensions of 2×10^6 or 1×10^7 PMN leukocytes in a final volume of 0.5 ml for 40 min in an ice water bath. The amount of radioactivity bound to the PMN leukocytes was determined by layering each suspension on 0.3 ml of a mixture of *n*-butyl phthalate and dinonyl phthalate (7:2, vol/vol) in a 1.5-ml polypropylene tube and centrifuging for 30 s at 8,000 *g* in a Beckman microfuge B (Beckman Instruments, Inc., Fullerton, CA). The tip of each polypropylene tube containing the PMN leukocyte pellet was cut off with a razor blade, the contents of the tip were resuspended with a Pasteur pipette in 4 ml of Hydrofluor, and the amount of radioactivity in the pellet and 0.1 ml of the upper aqueous layer was determined separately.

In a few experiments, [^{3}H]LTB_4 was incubated with 2×10^7 PMN leukocytes in 1 ml of HBSS-OVA for 60 min on ice. The suspensions then were acidified to pH 3.5 with glacial acetic acid, 1 vol of isopropanol was added, and the suspension was extracted twice with 1 vol of ethyl ether and once with 1 vol of chloroform. The organic layers were

pooled and dried under a stream of nitrogen, and the extracted lipids were redissolved in chromatography solvent for analysis.

HPLC. LTB_4, other 5,12-di-HETE isomers, and 5-HETE were resolved by reverse-phase HPLC on a 4.6-mm × 25-cm column of 10 μm octadecyl(C18)-silane (Ultrasil; Altex Scientific Inc., Berkeley, CA) that was developed isocratically at a flow rate of 1.7 ml/min with methanol/water/acetic acid (70:30:0.03, vol/vol, titrated to pH 4.5 with ammonium hydroxide) (3). Analysis of the stock solutions of [^{3}H]LTB_4 revealed that 99% of the radioactivity cochromatographed with a synthetic LTB_4 standard and the remaining 1% eluted at several earlier times. The purified and synthetic 5,12-di-HETE isomers and 5-HETE contained <3% optically determined impurities. The [^{3}H]LTB_4 also was converted to the methyl ester and subjected to standard-phase HPLC on a μPorasil column (Waters Associates, Milford, MA) that was developed isocratically with hexane/2-propanol (91:9, vol/vol) at 1.3 ml/min and again was shown to contain <1% radiochemical contamination. In three separate studies, 97.2 ± 2.4% (mean ± 2 SD) of the [^{3}H]LTB_4 was bound by specific rabbit anti-LTB_4 serum and >98% was displaced by synthetic LTB_4 (3).

Analysis of Binding Data. The specific activity of the [^{3}H]LTB_4 in each incubation tube was determined by dividing the total cpm recovered from the cell pellet and the aqueous layer by the total number of moles of LTB_4 present in the incubation tube. The cpm bound to the PMN leukocytes divided by the specific activity and the total volume of the incubation suspension equaled the concentration of bound LTB_4. The concentration of free LTB_4 was determined by dividing the cpm in the aqueous layer by the specific activity. The binding data were fit by a method of weighted nonlinear least squares to the ligand-binding model developed by Feldman (24) using a Wang 2200 series computer (Wang Laboratories, Inc., North Chelmsford, MA). The curve-fitting program is based on the LIGAND program described by Munson and Rodbard (25) and uses the Newton-Gauss-Marquardt-Levenberg algorithm as described by Fletcher and Schrager (26) to find those values for each parameter which minimized the weighted sum of the squares. The variance for the bound LTB_4 was determined to be 5% of the concentration of bound LTB_4 across the range of free LTB_4 concentrations tested. The "extra sum of squares" test (F statistic) (25) was used to compare the aptness of fit to models of one and two classes of receptors. Nonspecific binding was estimated by the curve-fitting program and was subtracted from the total binding data to yield the specific binding data. Analysis of the data collected for inhibition of [^{3}H]LTB_4 binding by LTB_4 analogs was performed using the curve-fitting program by setting the association constants for LTB_4 binding equal to the values previously determined in the absence of a competitive ligand and then calculating nonspecific binding, the total concentration of the high and low affinity receptors, and the association constants of the competing ligand for each of the receptors.

Deactivation of Human PMN Leukocyte Chemotaxis. Replicate suspensions of 1×10^7 PMN leukocytes/ml of HBSS-OVA were incubated with a range of concentrations of LTB_4 or buffer alone (control PMN leukocytes) for 10 min at 37°C. At the end of the incubation, 5 vol of ice-cold HBSS containing 0.2 g/100 ml fatty acid-free BSA (HBSS-BSA) were added to each suspension of PMN leukocytes. The PMN leukocytes were washed twice with HBSS-BSA and three times with HBSS at 4°C. The LTB_4-treated PMN leukocytes were suspended at 2×10^7 per ml of HBSS-OVA on ice. The total and nonspecific binding of [^{3}H]LTB_4 was determined by incubating 4×10^6 PMN leukocytes with 1×10^{-10} M [^{3}H]LTB_4 in 0.5 ml HBSS-OVA in the absence and presence of 1.5 μM LTB_4, which displaced 88.4 ± 3.6% (mean ± SD, $n = 7$) of the total binding. Subtracting the nonspecifically bound radioactivity from the total bound radioactivity gave the specific binding component. The amount of radioactivity specifically bound to control PMN leukocytes pretreated at 37°C in HBSS-OVA alone was defined as 100%.

The chemotaxis and lysosomal enzyme release of PMN leukocytes preincubated with LTB_4 or in buffer alone (controls) were assessed in parallel with the binding of [^{3}H]LTB_4. Chemotaxis was performed in modified Boyden chambers (Adaps, Inc., Dedham, MA) assembled with micropore filters of 3 μm pore diameter (Sartorius, Bottingen, Federal Republic of Germany) (22, 27). PMN leukocytes were enumerated microscopically in 10

high power fields (hpf), 5 from each of duplicate filters, at a depth of 80–100 μm from the cell source. The depth for counting was selected to achieve a background count of three to six PMN leukocytes per hpf. The response is expressed as net PMN leukocytes per hpf, after subtraction of background migration in control chambers lacking a stimulus. The altered chemotactic migration of PMN leukocytes pretreated with LTB_4 is expressed as a percentage of the migration of replicate portions of PMN leukocytes preincubated in HBSS-OVA alone.

The stimulation of release of β-glucuronidase from lysosomal granules was determined by incubating replicate suspensions of 2×10^6 PMN leukocytes in 0.4 ml of HBSS-OVA containing the stimulus or buffer alone and 5 μg/ml of cytochalasin B at 37°C for 30 min. Each supernate was assayed colorimetrically for β-glucuronidase activity as described (7). The amount of release of β-glucuronidase is expressed as a percentage of the total amount of activity present in replicate suspensions of PMN leukocytes that had been disrupted by sonication on ice. The percentage of β-glucuronidase released in the absence of a stimulus was subtracted to determine the net percentage release. The altered release of β-glucuronidase by PMN leukocytes pretreated with LTB_4 is denoted as a percentage of the release by PMN leukocytes preincubated in HBSS-OVA alone.

The statistical significance of binding parameters for deactivated PMN leukocytes relative to the binding parameters for untreated PMN leukocytes was determined by a standard *t* test for unpaired populations of samples with unequal variance. The statistical significance of the results of studies of the function of deactivated PMN leukocytes relative to the data for controls was determined by a standard two-sample *t* test for paired samples.

Results

Characteristics of the Binding of [^{3}H]LTB_4 by Human PMN Leukocytes. PMN leukocytes were incubated with 1×10^{-10} M [^{3}H]LTB_4 for 40 min at 0°C in the presence and absence of nonradioactive LTB_4 to quantify specific binding. The binding of [^{3}H]LTB_4 in the absence of unlabeled nonradioactive LTB_4 was rapid, reaching a plateau level within 10 min (Fig. 1). The nonspecific binding of [^{3}H]-

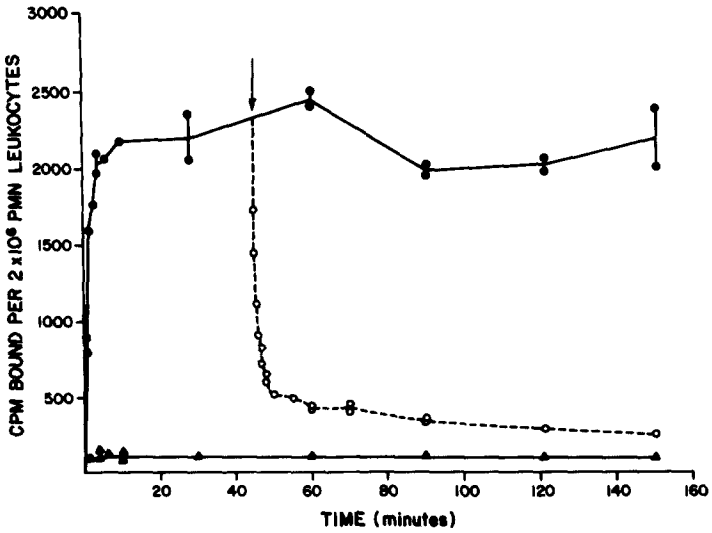

FIGURE 1. Time course of the human PMN leukocyte binding and release of [^{3}H]LTB_4. Binding was quantified in the absence (●) and presence (▲) of 1.5 μM nonradioactive LTB_4. The dissociation of [^{3}H]LTB_4 was assessed by incubating PMN leukocytes with [^{3}H]LTB_4 for 45 min and then adding nonradioactive LTB_4 (as indicated by the arrow) to a final concentration of 1.5 μM to block rebinding of dissociated [^{3}H]LTB_4 (○).

LTB_4 to PMN leukocytes was defined as that detected in the presence of 1.5 μM nonradioactive LTB_4. In seven experiments where the effect of 1.5 μM nonradioactive LTB_4 was assessed, the extent of inhibiton of the total binding of [^{3}H]-LTB_4 to PMN leukocytes was 88.4 ± 3.6% (mean ± SD) after 40 min at 0°C. Under the same binding conditions, the rate of degradation of [^{3}H]LTB_4 was slow compared with the rate of binding. When [^{3}H]LTB_4 was incubated with 2 × 10^7 PMN leukocytes for 60 min at 0°C and recovered by extraction, >95% of the radioactivity cochromatographed with synthetic LTB_4 on reverse-phase HPLC, as compared with [^{3}H]LTB_4 from the stock solution where 99% of the radioactivity cochromatographed with the synthetic standard.

The specific binding of [^{3}H]LTB_4 by PMN leukocytes reached a plateau level by 10 min, which remained constant for up to 150 min at 0°C (Fig. 1). When 1.5 μM nonradioactive LTB_4 was added to PMN leukocytes that had been incubated with [^{3}H]LTB_4 for 45 min at 0°C, 77.7 ± 4.8% (mean ± SD, n = 3) of the specifically bound [^{3}H]LTB_4 was dissociated within 10 min after the addition of the LTB_4 and the remainder was dissociated at a slower rate (Fig. 1). By 105 min after the addition of 1.5 μM LTB_4, 89.6 ± 2.0% of the specifically bound [^{3}H]LTB_4 had been released from the PMN leukocytes.

The rapid association and dissociation of the bulk of the [^{3}H]LTB_4 specifically bound to PMN leukocytes suggested that the system was at equilibrium in the standard binding studies and that equilibrium binding parameters could be derived accurately. The total amount of LTB_4 bound to PMN leukocytes was a function of the free concentration of LTB_4 (Fig. 2*A*). Whereas total and nonspecific binding increased with the concentration of LTB_4 up to 1,600 nM, specific binding was saturated at LTB_4 concentrations of 800 nM and greater. The specific binding of LTB_4 at 1,600 nM was 108 ± 23% (mean ± SD, n = 5) of the specific binding at 800 nM. Computer analysis of the concentration dependence of total binding (Fig. 2*A*) revealed that the data were best fit ($P < 0.01$) by a model consisting of two different classes of binding sites for LTB_4 and a nonspecific binding component. The biphasic nature of the Scatchard plot of the values for specific binding of LTB_4 (Fig. 2*B*) supported the suitability of a model of two classes of receptors for the binding of LTB_4 to PMN leukocytes. The high affinity and low affinity binding sites had mean K_d values of 3.9 × 10^{-10} M and 6.1 × 10^{-8} M, respectively, for LTB_4 (Table I). There were 4.4 ± 1.2 × 10^3 high affinity receptors (mean ± SD) and 2.7 ± 1.8 × 10^5 low affinity receptors per PMN leukocyte.

Structural Determinants of the Binding of [^{3}H]LTB_4 to PMN Leukocytes. The inhibition of binding of [^{3}H]LTB_4 to PMN leukocytes by chemotactically less potent 5,12 di-HETE isomers was assessed in parallel with studies of chemotactic activity (Fig. 3 and Table I). Both of the other 5,12 di-HETE isomers inhibited by up to 100% the specific binding of [^{3}H]LTB_4 to PMN leukocytes. Further, the concentrations at which the isomers inhibited by 50% the binding of [^{3}H]-LTB_4 (IC_{50} values) were in the same relative rank order as the concentrations required to achieve 50% of the maximal chemotactic response (EC_{50}) (Fig. 3 and Table I). Hill coefficients of 0.61 ± 0.09, 0.78 ± 0.04, and 0.94 ± 0.13 (mean ± SD, n = 3) were derived from the inhibition of [^{3}H]LTB_4 binding by LTB_4, 12(S)-LTB_4, and 5(S),12(S)-6 trans-di-HETE, respectively. The Hill coefficients

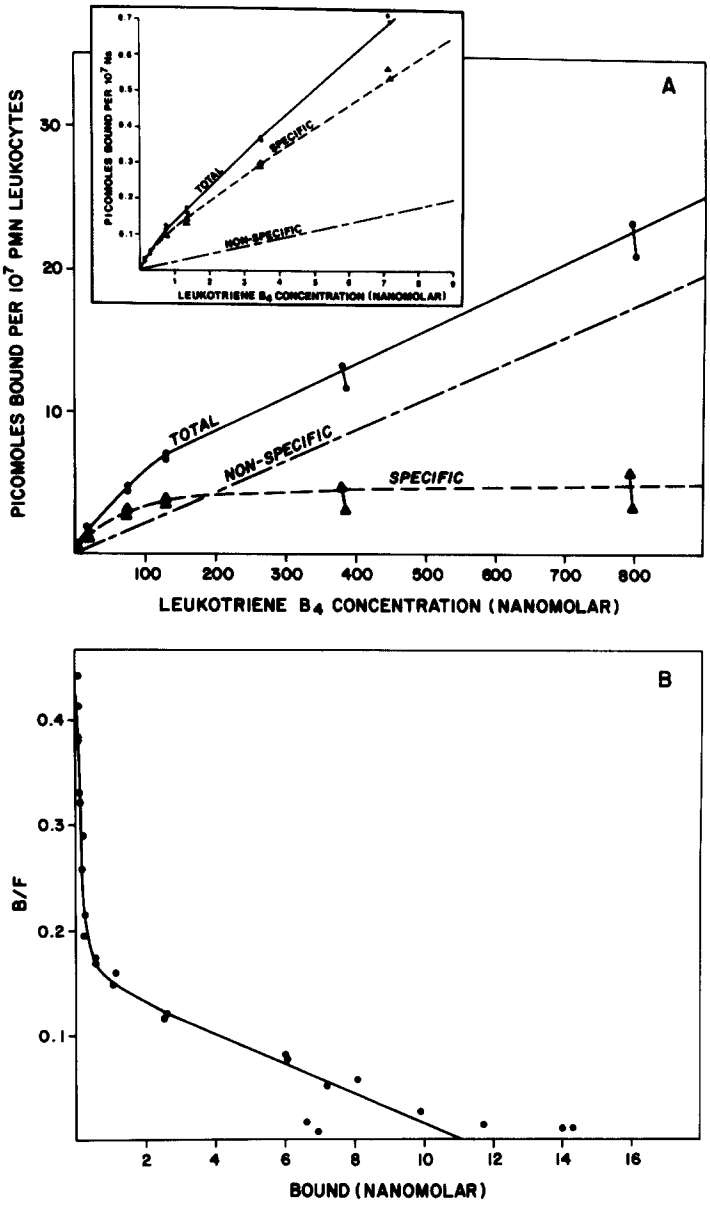

FIGURE 2. Concentration dependence of the binding of [^{3}H]LTB$_4$ to human PMN leukocytes. (*A*) Each data point represents a single determination at each LTB$_4$ concentration. We used binding affinities of $K_{d1} = 3.57 \times 10^{-10}$ M and $K_{d2} = 7.55 \times 10^{-8}$ M and receptor densities of 3,670 and 334,000 receptors per PMN leukocyte, respectively, to construct the solid line that represents the best fit of the total binding data by the nonlinear least squares curve-fitting program. Specific binding (▲) was determined by subtracting nonspecific binding (——) of 2.22×10^{-5} pmol per 10^7 PMN leukocytes $\times$ M^{-1} from the total binding (●). (*B*) The Scatchard plot was derived from the specific binding data in *A*. The solid line was constructed from the characteristics of binding determined in *A*, the units for which were expressed in terms of the molar concentration of bound LTB$_4$. B/F, bound/free concentration of LTB$_4$.

TABLE I

Correlation of the Binding Affinity of LTB_4 and Other Isomers of 5,12 di-HETE with Chemotactic Potency

Ligand	Dissociation constants*		Chemotactic potency EC_{50}‡
	High affinity (K_{d1})	Low affinity (K_{d2})	
	M		*M*
LTB_4	$3.9 \pm 2.4 \times 10^{-10}$	$6.1 \pm 2.9 \times 10^{-8}$	3.0×10^{-9}
12(S)-LTB_4	$2.2 \pm 0.4 \times 10^{-9}$	$2.0 \pm 1.6 \times 10^{-7}$	6.3×10^{-8}
5(S),12(S)-6 trans-di-HETE	$5.8 \pm 2.7 \times 10^{-8}$	—	$0.8–1.0 \times 10^{-6}$

* The dissociation constants were determined by fitting the binding data to one- and two-site binding models using a nonlinear least squares curve-fitting program. The binding data were best fit by a two-site model for LTB_4 and 12(S)-LTB_4 ($P < 0.001$) and by a one-site model for the 5(S),12(S)-6 trans-di-HETE, which yielded a single K_d.

‡ The EC_{50} values for the stimulation of human PMN leukocyte chemotaxis by LTB_4 and 5(S),12(S)-6 trans-di-HETE had been previously determined (10, 11), while that for 12(S)-LTB_4 was derived from results of three current studies, where the mean ± SD was $6.3 \pm 2.9 \times 10^{-8}$ M.

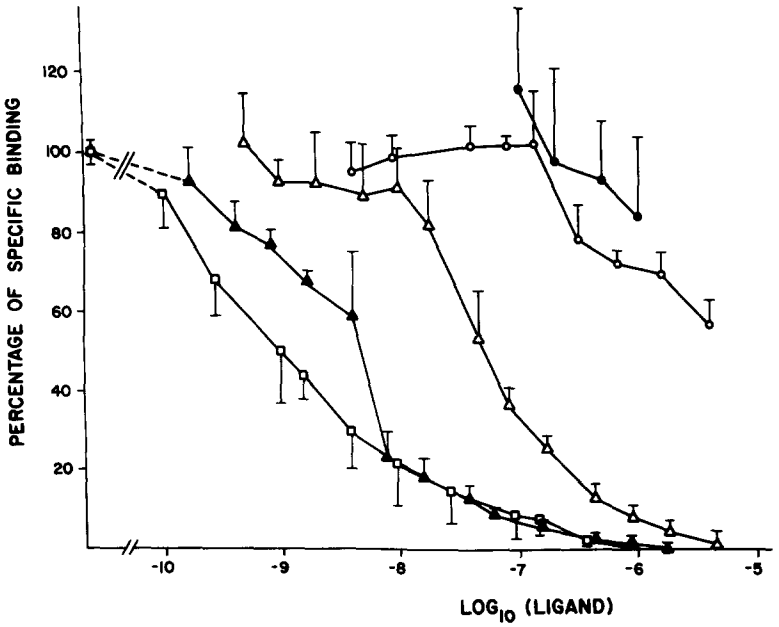

FIGURE 3. Inhibition of the specific binding of [^{3}H]LTB_4 by unlabeled LTB_4 (□), 12(S)-LTB_4 (▲), 5(S),12(S)-6 trans-di-HETE (△), LTD_4 (●), and 5-HETE (○). Each data point represents the mean ± SD of duplicate measurements from three experiments. The inhibitory dissociation constants for the three isomers of 5,12-di-HETE are summarized in Table I.

suggest that LTB_4 and 12(S)-LTB_4 each bind with markedly different affinities to the two classes of sites, whereas 5(S),12(S)-6 trans-di-HETE exhibited approximately the same affinity for the two types of LTB_4 binding sites. These conclusions were confirmed by a nonlinear least squares analysis of the data (Fig. 3), which yielded characteristic inhibitory dissociation constants for LTB_4 and the other isomers of 5,12-di-HETE (Table I). The IC_{50} values for the binding of the 5,12 di-HETE isomers to the high affinity receptor correlate closely with the

EC_{50} values for chemotactic potency, suggesting that the high affinity receptors are involved in mediating the chemotactic response of PMN leukocytes to all of the related fatty acids.

The chemotactically inactive lipid LTD_4, as well as 10^{-6} M fMLP and a maximally chemotactic concentration of C5fr, did not inhibit significantly the binding of [^{3}H]LTB_4 to PMN leukocytes, suggesting that both sets of receptors are specific for LTB_4 and structurally related fatty acids and are distinct from the receptors previously defined for the chemotactic peptides. The quantitatively predominant 5-lipoxygenase product of human PMN leukocytes, 5-HETE, also inhibited the specific binding of [^{3}H]LTB_4 to PMN leukocytes, but at sufficiently high concentrations that 100% inhibition of the binding of [^{3}H]LTB_4 could not be achieved.

Correlation of the Reduction in [^{3}H]LTB_4 Binding with the Decrease in Chemotactic Response of Deactivated PMN Leukocytes. Preincubation of PMN leukocytes with LTB_4, followed by washing, resulted in a decreased chemotactic response to LTB_4, whereas the chemotactic response to other stimuli was only minimally decreased (27). A similarly selective decrease in the chemotactic responsiveness of PMN leukocytes pretreated with *N*-formyl-methionyl peptides has been termed deactivation and shown to correlate with a decrease in the number of PMN leukocyte receptors for *N*-formyl-methionyl peptides (28, 29). PMN leukocytes pretreated at 37°C for 10 min with different concentrations of LTB_4 exhibited significantly diminished chemotactic responses to subsequent stimulation with LTB_4 (Fig. 4*A*). The chemotactic responses to 3×10^{-9} M and 3×10^{-8} M LTB_4 were suppressed significantly by preincubation of PMN leukocytes with concentrations of LTB_4 as low as 0.1 and 0.3 nM, respectively. The amount of [^{3}H]-LTB_4 bound to the PMN leukocytes decreased concomitantly with the chemotactic response. The chemotactic response to C5fr also decreased in a conentration-dependent manner after pretreatment of the PMN leukocytes with LTB_4, but a 30–100-fold higher LTB_4 concentration was required to achieve the same extent of deactivation to C5fr as to 3×10^{-8} M LTB_4. With preincubation concentrations of 3×10^{-10} M LTB_4 or higher, the chemotactic response to C5fr was significantly greater ($P < 0.05$, paired Student's *t* test) than that to 3×10^{-8} M LTB_4.

Scatchard analysis of the binding of [^{3}H]LTB_4 to PMN leukocytes pretreated with 1×10^{-8} M LTB_4 revealed a complete loss of the high affinity receptors along with a significant change in the number and affinity of the low affinity receptor for LTB_4 (Fig. 5). The PMN leukocytes pretreated with LTB_4 exhibited a single class of LTB_4 receptors with a K_d of $3.1 \pm 2.8 \times 10^{-7}$ M (mean ± SD, $n = 3$) and a receptor density of $1.1 \pm 1.0 \times 10^6$ per PMN leukocyte. The receptors expressed on the LTB_4-deactivated PMN leukocytes exhibited a significantly lower affinity ($P = 0.04$) and higher density ($P = 0.05$) than those observed on untreated PMN leukocytes using a Student's *t* test for unpaired populations of samples. Preincubation of PMN leukocytes with HBSS-OVA alone at 37°C did not alter the binding of [^{3}H]LTB_4 (Fig. 5).

The selective loss of the high affinity receptors for LTB_4 and the parallel reduction in chemotactic responses to LTB_4 after deactivation suggested that the high affinity receptors may mediate the chemotactic responses of PMN

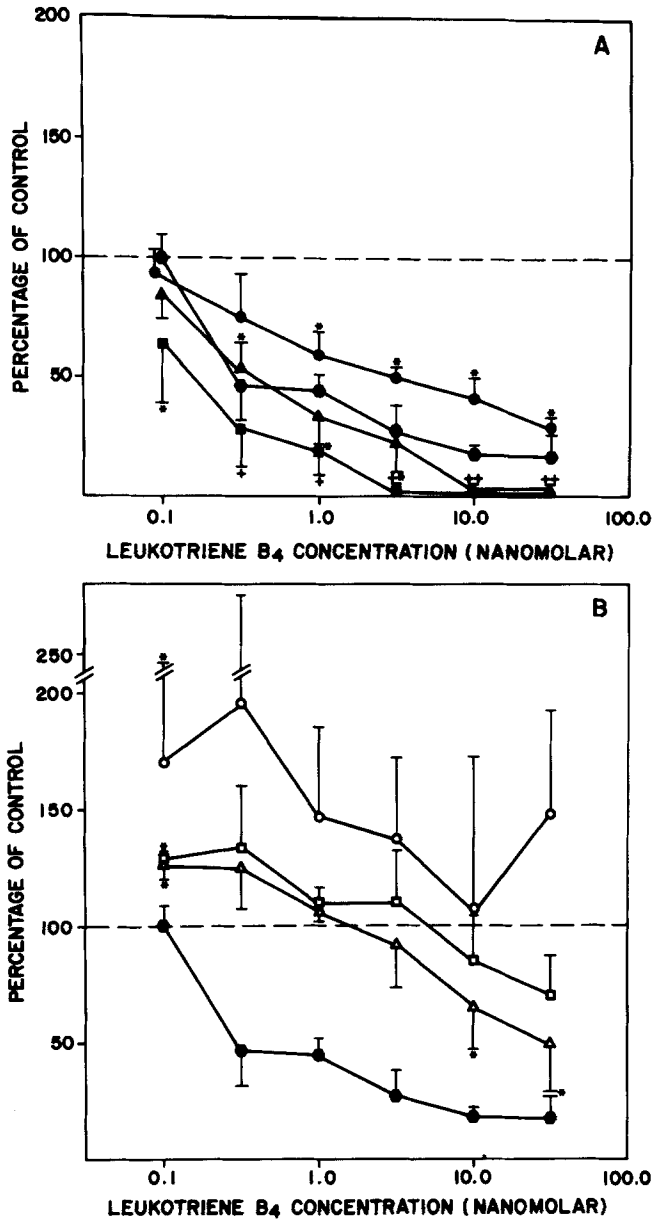

FIGURE 4. [^{3}H]LTB_4-induced chemotaxis and β-glucuronidase release, and binding of [^{3}H]-LTB_4 by PMN leukocytes pretreated with LTB_4. After preincubation with different concentrations of LTB_4 or buffer alone for 10 min at 37°C, the PMN leukocytes were washed and used to assess binding of [^{3}H]LTB_4 and functions: (*A*) Chemotactic responses to a 1:30 dilution of C5fr (●), 3×10^{-9} M LTB_4 (■), and 3×10^{-8} M LTB_4 (▲). Control PMN leukocytes incubated in HBSS-OVA alone gave (100%) migration values of 25.1 ± 2.4, 10.3 ± 3.0, and 21.8 ± 2.6 net PMN leukocytes per hpf (mean ± SD, $n = 3$) in response to C5fr, 3×10^{-9} M LTB_4, and 3×10^{-8} M LTB_4, respectively. (*B*) β-glucuronidase release. Control PMN leukocytes incubated in HBSS-OVA alone released 5.0 ± 1.6, 18.9 ± 3.7, and 23.3 ± 3.8 net percentage of the total β-glucuronidase (mean ± SD, $n = 3$) in response to a 1:20 dilution of C5fr (○), 3×10^{-8} M LTB_4 (△), and 3×10^{-7} M LTB_4 (□), respectively. Specific binding of 1×10^{-10} M [^{3}H]LTB_4 (●) is shown in both *A* and *B*. Each data point represents the mean ± SD for three experiments. A paired Student's *t* test was used to assess the statistical significance of differences between control leukocytes and LTB_4-treated leukocytes; levels of significance are as follows: *, $P < 0.05$; +, $P < 0.01$; ++, $P < 0.005$.

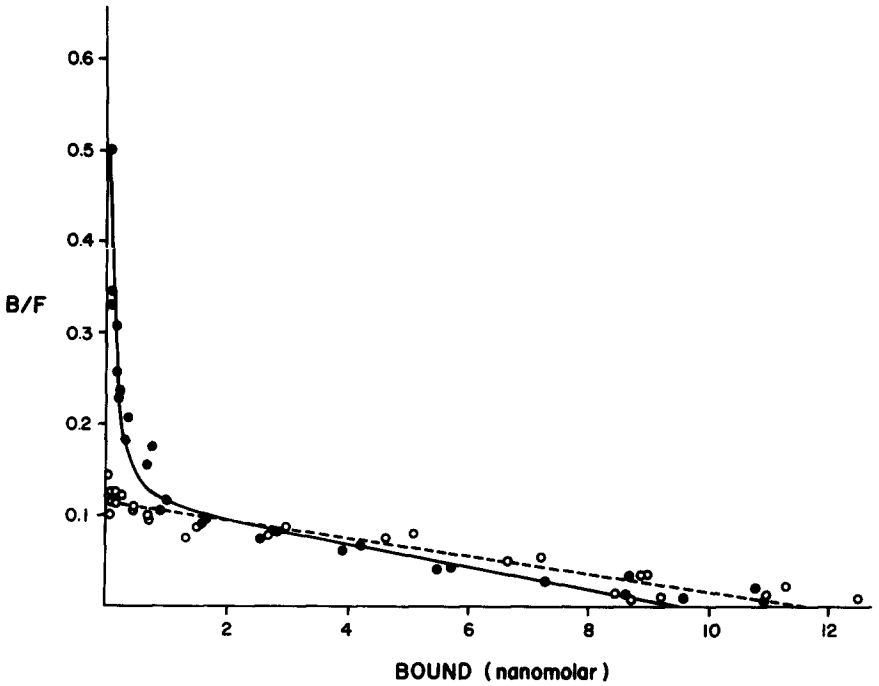

FIGURE 5. Scatchard analysis of the specific binding of LTB_4 to PMN leukocytes deactivated chemotactically by pretreatment with LTB_4. B/F, bound/free concentration of LTB_4. PMN leukocytes were preincubated in the absence (●) and presence (○) of 1×10^{-8} M LTB_4 for 10 min at 37°C and washed. The solid and dashed lines were constructed from the binding parameters determined with the nonlinear least squares curve-fitting program. The binding data for the control PMN leukocytes preincubated in HBSS-OVA alone was best fit by a two-site binding model with a K_{d1} of 3.6×10^{-10} M and K_{d2} of 8.8×10^{-8} M, and a receptor density of 4,600 and 284,000 receptors per PMN leukocyte for the high and low affinity sites, respectively. The binding data for the PMN leukocytes preincubated with 1×10^{-8} M LTB_4 was best fit by a one-site binding model with a single K_d of 1.0×10^{-7} M and a receptor density of 353,000 per PMN leukocyte.

leukocytes to LTB_4. The elicitation of release of granule enzymes in the presence of cytochalasin B requires significantly higher concentrations of LTB_4 than chemotaxis and thus was considered to be mediated by the low affinity receptors for LTB_4. Pretreatment of PMN leukocytes with 1×10^{-8} M LTB_4 at concentrations that inhibited completely the chemotactic response to LTB_4 did not significantly inhibit the release of β-glucuronidase evoked by 3×10^{-7} M LTB_4 and only minimally suppressed that elicited by 3×10^{-8} M LTB_4 (Fig. 4*B*). That none of the high affinity receptors were expressed on chemotactically deactivated PMN leukocytes implies that LTB_4 stimulates enzyme release principally by binding to the low affinity PMN leukocyte receptors for LTB_4.

Discussion

The availability of [^{3}H]LTB_4 of high specific activity and purity and the application of computer-based least squares fitting of the results of binding to PMN leukocytes permitted the definition of two classes of receptors of different affinities. The specific binding of [^{3}H]LTB_4 to PMN leukocytes accounted for

>88% of the total binding at equilibrium, as assessed by the extent of inhibition of binding by a 15,000-fold higher concentration of nonradioactive LTB_4. Maximum specific binding of [^{3}H]LTB_4 was attained rapidly, reaching a plateau level within 10 min, and was rapidly dissociated by the addition of a 15,000-fold higher concentration of nonradioactive LTB_4 (Fig. 1). The specific binding of LTB_4 was concentration dependent and reached saturation at 800 nM LTB_4. Analysis of the curvilinear Scatchard plot of the LTB_4 binding data indicated that the best fit was achieved by a model of two classes of receptors of different affinities (Fig. 2). The two classes of LTB_4 receptors consisted of a mean of 4.4×10^3 and 2.7×10^5 sites per PMN leukocyte with respective mean affinities of 3.9×10^{-10} M (K_{d1}) and 6.1×10^{-8} M (K_{d2}) (Table I).

The coupling of the high affinity receptors for LTB_4 to the stimulation of PMN leukocyte chemotaxis was suggested initially by the similarity of the K_{d1} for LTB_4 and the concentration of LTB_4 required to elicit a chemotactic response that was 50% of maximum (EC_{50}) (Table I). This relationship was confirmed by the results of studies of chemotactic structural isomers of LTB_4 (Fig. 3, Table I). 12(S)-LTB_4 is a chemotactic factor for PMN leukocytes that possessed only 5% of the potency of LTB_4 and exhibited a K_{d1} ~5.5-fold greater than that of LTB_4. The 5(S),12(S)-6 trans-di-HETE had only 1/100th the chemotactic potency of LTB_4 and had a K_{d1} ~150-fold greater than that of LTB_4. The K_{d1} for 5(S),12(S)-6 trans-di-HETE was indistinguishable from the K_{d2}, indicating that unlike LTB_4 and 12(S)-LTB_4, it binds with equal affinity to the two classes of LTB_4 receptors.

The results of binding and functional studies of PMN leukocytes deactivated chemotactically by preincubation with LTB_4 and washing confirmed the importance of high affinity receptors in the transduction of chemotaxis. A fully deactivating exposure to LTB_4 reduced by >80% the specific binding of [^{3}H]-LTB_4 (Fig. 4), which was attributable predominantly to loss of binding by the high affinity receptors (Fig. 5). The extent of loss of high affinity binding of [^{3}H]-LTB_4 with prior exposure to different concentrations of LTB_4 correlated with the degree of reduction in chemotactic response to LTB_4, whereas the lysosomal degranulating effect of a low concentration of LTB_4 was diminished only minimally and that of a high concentration of LTB_4 was unchanged (Fig. 4).

Several theoretical models have been proposed to account for the upwardly concave curvilinear Scatchard plots seen in studies of other receptors, including fixed heterogeneity of combining sites, negatively cooperative site-site interactions, multi-step binding reactions to distinct components of a single class of receptors, and competition of an unlabeled ligand for the nonspecific binding component. The latter possibility is unlikely because three structurally distinct chemotactic isomers of 5,12-di-HETE all suppressed the binding of [^{3}H]LTB_4 to the same level of nonspecific binding. The remaining models for curvilinear Scatchard plots cannot be differentiated on the basis of equilibrium binding data alone, but require further information on the kinetics of ligand binding, on the structure of the binding sites, and on the possible association of the binding sites with other putative effector molecules of the PMN leukocytes.

We have chosen to interpret the binding of LTB_4 to PMN leukocytes in terms of two independent classes of binding sites because of the evidence relating high affinity binding sites to the chemotactic responses of PMN leukocytes to LTB_4.

The possible relationship between the low affinity binding site and the stimulation by LTB_4 of lysosomal enzyme release is not proven, but is suggested both by the similarity of K_{d2} and the concentrations of LTB_4 required to achieve β-glucuronidase release and by the failure of chemotactic deactivation to suppress either the expression of the low affinity binding sites or PMN leukocyte enzyme release in response to LTB_4.

Other support for the functional role of high affinity LTB_4 receptors is derived from the results of studies of the expression of PMN leukocyte receptors for fMLP. Chemotaxis is elicited by low concentrations and lysosomal enzyme secretion by higher concentrations of fMLP (31, 32). Human PMN leukocyte receptors for fMLP in isolated membrane preparations exist in both high and low affinity states, that are in part interconvertible through a mechanism regulated by guanine nucleotides (33). Aliphatic alcohols (34) and polyene antibiotics (35) influence the affinity state of the fMLP receptors on intact PMN leukocytes and the effects of such agents have supported a relationship of high affinity receptors to chemotaxis and of low affinity receptors to lysosomal enzyme secretion. For example, the shift from low to high affinity sites induced by the aliphatic alcohols enhances chemotaxis and suppresses enzyme secretion, while the opposite effect of polyene antibiotics on receptor affinity diminishes chemotaxis and enhances enzyme secretion. While such studies have not been performed for LTB_4 receptors, intact human PMN leukocytes before any manipulation have two clearly definable classes of receptors for LTB_4 (Fig. 2, Table I), but not for fMLP (32). Nonetheless the net effective expression of the high affinity receptors for LTB_4 may be attributable to complex cellular mechanisms, as chemotactic deactivation alters rapidly and strikingly the affinity and/or number of such receptors (Fig. 5). The relationship of alterations in the high affinity receptors for LTB_4 to chemotactic defects induced in vitro and acquired in some human diseases is now susceptible to analysis and may provide new approaches for correcting such functional defects.

Summary

Human polymorphonuclear (PMN) leukocytes bound [^{3}H]leukotriene B_4 ([^{3}H]-LTB_4) specifically, as assessed by the displacement of 88% or more of the bound radioactivity by a 15,000-fold higher concentration of nonradioactive LTB_4 or by micromolar concentrations of structural isomers of LTB_4. The specific binding of [^{3}H]LTB_4 by PMN leukocytes was characterized by rapid association and dissociation, and was saturable at 800 nM LTB_4. The results of computer analyses of the concentration dependence of binding of [^{3}H]LTB_4 were consistent with the expression of two classes of receptors having respective mean affinities of 3.9×10^{-10} M and 6.1×10^{-8} M and mean densities of 4.4×10^5 and 2.7×10^5 per PMN leukocyte. Structural isomers of LTB_4 inhibited the binding of [^{3}H]LTB_4 to PMN leukocytes at concentrations similar to those required to elicit chemotaxis, while chemotactic peptides did not inhibit binding. PMN leukocytes that were deactivated by prior exposure to LTB_4 lost high affinity binding sites selectively and concurrently with a reduction in the chemotactic response to LTB_4. Chemotactic deactivation altered, but did not eliminate, the low affinity receptors for LTB_4 and reduced only minimally the lysosomal degranulation

elicited by LTB_4. The high affinity receptors for LTB_4 on normal human PMN leukocytes appear to transduce the chemotaxis evoked by LTB_4 without substantially modifying lysosomal degranulation.

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