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HUMAN NEUTROPHIL CHEMOTACTIC AND DEGRANULATING ACTIVITIES OF LEUKOTRIENE B_5 (LTB_5) DERIVED FROM EICOSAPENTAENOIC ACID

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Leukotriene B_4 exhibited 10- to 30-fold greater chemotactic potency for human neutrophils than eicosapentaenoic acid-derived leukotriene B_5, as assessed in modified Boyden micropore filter chambers. In contrast, leukotrienes B_4 and B_5 were equipotent stimuli of human neutrophil lysosomal degranulation in vitro, as quantified by the release of β-glucosaminidase. Analyses of competitive inhibition of the binding of [^{3}H] leukotriene B_4 to neutrophils indicated that leukotriene B_4 binds with a 500-fold greater association constant than leukotriene B_5 to a subclass of high-affinity receptors, which appears to transduce chemotactic responses efficiently, while leukotrienes B_4 and B_5 bind equally well to low-affinity receptors.

Mast cells, polymorphonuclear (PMN)[1] leukocytes, monocytes, macrophages, basophils, and some cells other than leukocytes 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 generated from 5-hydroperoxy-eicosatetraenoic acid (1-3). LTB_4 stimulates PMN leukocyte chemotaxis and other functions (4-6) and suppresses the proliferative and secretory activities of human T-lymphocytes (7). Receptors with a high degree of specificity for LTB_4 have been defined on human neutrophils (8).

Eicosapentaenoic acid (EPA) predominates over arachidonic acid in diets rich in cold-water fish and is absorbed, incorporated into membrane phospholipids, and metabolized by both cyclo-oxygenase and lipoxygenases (9,10). As the cyclo-oxygenase products of EPA differ in activity from those of arachi-

[1] Abbreviations used: PMN, polymorphonuclear; LTB_4, leukotriene B_4; EPA, eicosapentaenoic acid; HBSS, Hanks' balanced salt solution; LTB_5, leukotriene B_5; HPLC, high-performance liquid chromatography; HSA, human serum albumin; HEPES, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; and hpf, high power field.

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donic acid (10), which has functional consequences in vivo (9,10), the PMN leukocyte effects of the 5,12-di-hydroxy-eicosapentaenoic acid derivatives of EPA analogous to LTB_4 and the 6-trans isomers of LTB_4 now are characterized in terms of chemotaxis, release of lysosomal enzymes, and binding to LTB_4 receptors.

MATERIALS AND METHODS

Hanks' balanced salt solution (HBSS), Dulbecco's minimum essential medium (M.A. Bioproducts, Walkersville, Md.), recrystallized human serum albumin (HSA), eicosapentaenoic acid (EPA), N-formyl-methionyl-leucyl-phenylalanine (fMLP), 4-methyl-umbelliferyl-N-acetyl-β-D-glucosaminide (Sigma Chemical Co., St. Louis, Mo.), [^{3}H] leukotriene B_4 (180-221 Ci/mmole) (Amersham Corp., Arlington Heights, Ill.), Ficoll-Hypaque, 6 g % (w:v) dextran 70 in 0.15 M saline (Pharmacia Fine Chemicals, Piscataway, N.J.), cytochalasin B (Aldrich Chemical Co., Milwaukee, Wisc.), arachidonic acid (Supelco, Inc., Bellefonte, Pa.), and high-performance liquid chromatography (HPLC)-grade organic solvents that had been re-distilled from glass (Burdick and Jackson Laboratories, Inc., Muskegon, Mich.) were obtained as noted. Synthetic leukotriene B_4 was a gift from Dr. J. Rokach (Merck-Frosst Laboratories, Dorval, Canada).

LTB_4, leukotriene B_5 (LTB_5), and the respective 6-trans isomers of each were generated, purified, and characterized as described (11). Replicate suspensions of 3 x 10^7 guinea pig peritoneal PMN leukocytes from sodium caseinate exudates were incubated for 8 min at 37°C in 1 ml of Dulbecco's medium with 50 mM Tris-HCl (pH 7.4), 20 μM calcium ionophore A23187 (Calbiochem, Inc., LaJolla, Calif.), and either 100 μm arachidonic acid or eicosapentaenoic acid, that had been purified to over 95% prior to use. The reactions were terminated with 1 ml of methanol at 0°C and the products were extracted into chloroform and purified by sequential silicic acid chromatography and HPLC on a semi-preparative C18 5 μm particle reverse-phase column (IBM Instruments, Inc., Danbury, Conn.) developed at 1 ml/min with methanol:water:glacial acetic acid (60:40:0.01, v:v). The individual products in each series were purified to over 97% by re-chromatography on an analytical C18 column (IBM Instruments, Inc.) developed at 1 ml/min with methanol:water:glacial acetic acid (65:35:0.01, w:v) and shown to be identical to compounds authenticated by gas chromatography-mass spectrometry analyses in terms of UV spectrum, and the times of elution from reverse-phase HPLC and standard-phase HPLC as methyl esters (11). Double lipoxygenation products were not found, as expected from the absence of 12-HETE in the extracts.

Human neutrophils were obtained from sodium citrate-anticoagulated venous blood of normal subjects, purified to over 98% by sedimentation and lysis of erythrocytes and by centrifugation through Ficoll-Hypaque cushions (4), and suspended in HBSS containing 0.1 g % HSA and 0.001 M N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.2) (HBSS-HSA). Chemotaxis was assessed in 0.2 ml blind-well modified Boyden chambers (Neuroprobe, Inc., Bethesda, Md.) fitted with 3 μm pore diameter filters (Schleicher and Schuell, Inc., Keene, N.H.) and containing 2 x 10^6 neutrophils in 0.5 ml. Neutrophils were enumerated microscopically in 10 high power fields (hpfs), five from each of duplicate filters, at a depth of 80-100 μm from the source of neutrophils in order to achieve a background count of 3-6 neutrophils per hpf. Responses are expressed as net neutrophils per hpf, after subtraction of background migration in control chambers lacking a stimulus. The stimulation of release of β-glucosaminidase by chemotactic factors was quantified by incubating replicate 0.2 ml suspensions of 1 x 10^6 neutrophils in HBSS-HSA containing 5 μg/ml of cytochalasin B for 20 min at 37°C. After centrifugation of the

suspensions, the β-glucosaminidase activity in supernates and sonicates of neutrophil pellets was determined by the cleavage of 4-methyl-umbelliferyl-N-acetyl-β-glucosaminide using a standard fluorimetric assay (12). The net percentage release of β-glucosaminidase was calculated by subtracting the percentage release in the absence of a stimulus. The binding of [^{3}H]LTB_4 to 6 x 10^6 neutrophils in 0.35 ml of HBSS-HSA without or with unlabeled LTB_4, LTB_5, or their 6-trans isomers was quantified by incubating replicate suspensions for 40 min at 0°C, separating the neutrophil-bound from unbound radioactivity by centrifugation on a mixture of phthalate oils, and determining the radioactivity in the neutrophil pellets and aqueous supernates as described (8). After calculation of the concentration of free and bound [^{3}H]LTB_4 in each sample (8), the data were fit by a weighted non-linear method of least-squares to one- and two-receptor models with a technique modified from the LIGAND program (13-15) using an HP-86A computer (Hewlett-Packard, Inc., Mountainview, Calif.). Specific binding was calculated by subtracting the non-specific binding of [^{3}H]LTB_4, in the presence of a 1500-fold higher concentration of unlabeled LTB_4, from the total binding. The value for 100% specific binding of [^{3}H]LTB_4 was used to assess the competitive inhibition of binding by LTB_4, LTB_5, and their 6-trans isomers.

RESULTS AND DISCUSSION

LTB_4, the 6-trans isomers of LTB_4, and fMLP elicited neutrophil chemotactic responses which reached a similar maximum level (Fig. 1). The potency of LTB_4 was 100- to 300-fold greater than that of the 6-trans isomers of LTB_4 over the range of concentrations assessed, which is consistent with the results of previous studies (4). LTB_5 also evoked a maximum neutrophil chemotactic response similar in magnitude to that of LTB_4, but was 10- to 30-fold less potent than LTB_4 (Fig. 1). The 6-trans isomers of LTB_5 exhibited approximately 30%-100%, 3%-10%, and 0.1%-0.3% of the neutrophil chemotactic potency of the 6-trans isomers of LTB_4, LTB_5, and LTB_4, respectively, but failed to elicit a maximum chemotactic response similar in magnitude to those of the other factors at concentrations compatible with complete solubility in aqueous buffers.

The differences in potency between the isomers of LTB_4 and LTB_5 were substantially less when evaluated in terms of the release of β-glucosaminidase from neutrophil lysosomal granules (Fig. 2). LTB_4 and LTB_5 evoked a similar maximum release reaction over the range of concentrations examined, but the mean maximum level of release of β-glucosaminidase for each was only approximately 40% of that evoked by 10^{-6}M fMLP. Although the mean potency of LTB_4 appeared to be 3- to 10-fold higher than that of LTB_5, the differences were

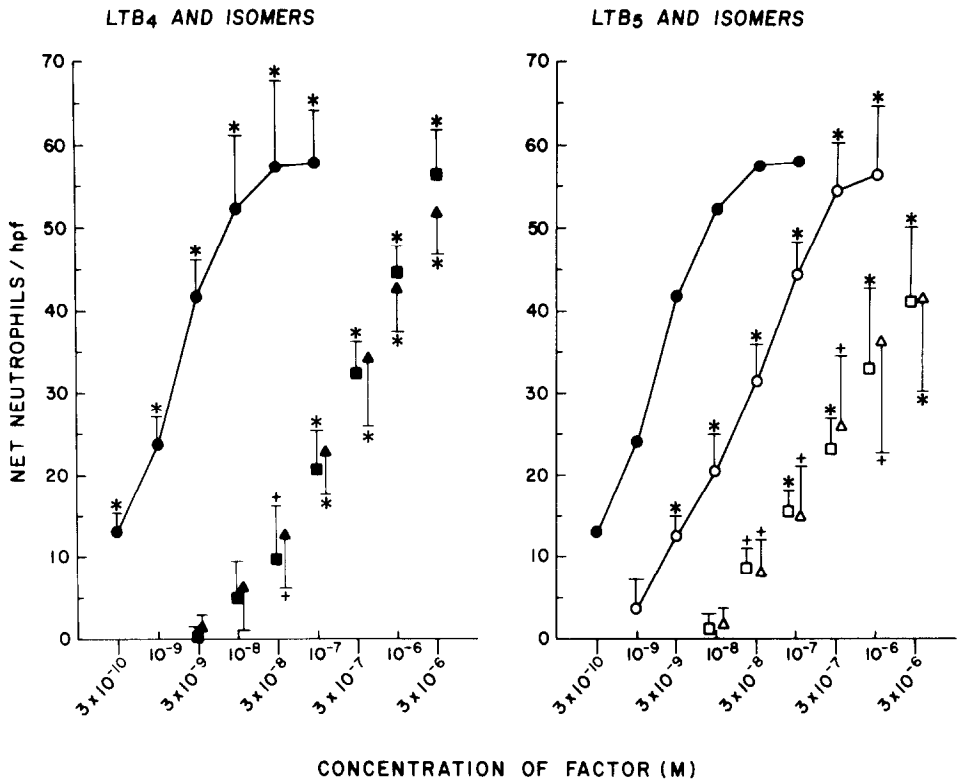

Figure 1 - Human neutrophil chemotactic activity of LTB_4 (●), 5(S),12(S)-6 trans-LTB_4 (■), 5(S),12(R)-6 trans-LTB_4 (▲) (left-hand frame), LTB_5 (○), 5(S),12(S)-6 trans-LTB_5 (□), and 5(S),12(R)-6 trans-LTB_5 (△) (right-hand frame). Each point and bracket are the mean and S.D. of the results of four (LTB_4 and isomers) or three (LTB_5 and isomers) experiments with different neutrophils. The mean data for LTB_4 are presented again in the right-hand frame for comparison. The chemotactic responses to 3×10^{-9}M synthetic LTB_4 and 10^{-7}M and 10^{-6}M fMLP were 46.4 ± 5.9 (mean ± S.D.), 36.9 ± 9.0, and 56.7 ± 10.0 net neutrophils/hpf, respectively, above a background of 4.5 ± 0.8 neutrophils/hpf. The statistical significance of responses (standard t-test) is indicated by a + = $p < 0.05$ or a * = $p < 0.01$.

not statistically significant. The 6-trans isomers of LTB_4 and LTB_5 exhibited 1%-10% of the potency of LTB_4 and LTB_5, respectively (Fig. 2).

LTB_4 displaced the binding of [^{3}H]LTB_4 from human neutrophils in a concentration-dependent relationship from 10^{-10}M - 10^{-6}M LTB_4 (Fig. 3), which is consistent with the presence of a mean of 5.4×10^3 high-affinity receptors (Kd = $4.9 \pm 0.8 \times 10^{-10}$M, mean ± S.D.) and 1.0×10^6 low-affinity receptors (Kd = $2.9 \pm 2.6 \times 10^{-7}$M). In contrast, the displacement of [^{3}H]LTB_4 by LTB_5 (Fig. 3) and the 6-trans isomers of LTB_4 and LTB_5 demonstrated a similar concentration-dependence for each, that was most compatible with binding of these factors equally to both classes of receptors. The affinity (Kd) of the

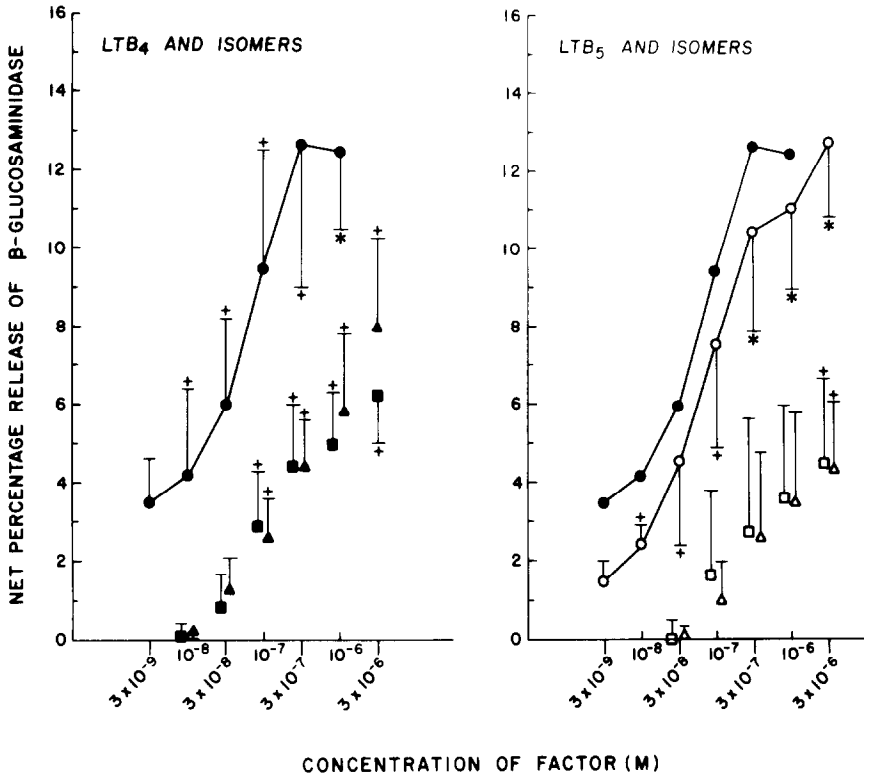

Figure 2 - Release of β-glucosaminidase from human neutrophils by LTB_4 (●), 5(S),12(S)-6 trans-LTB_4 (■), 5(S),12(R)-6 trans-LTB_4 (▲) (left-hand frame), LTB_5 (○), 5(S),12(S)-6 trans-LTB_5 (□), and 5(S),12(R)-6 trans-LTB_5 (△) (right-hand frame). The number of experiments, designation of statistical significance, and repetition of LTB_4 mean results in the right-hand frame are as in Fig. 1. The net release of β-glucosaminidase by 3 x 10^{-8}M synthetic LTB_4 and 10^{-6}M fMLP were 6.4 ± 1.2% (mean ± S.D.) and 32.2 ± 5.7%, above a background release of 4.5 ± 0.9% in buffer alone.

neutrophil receptors for LTB_5 was 2.8 ± 0.5 x 10^{-7}M (mean ± S.D.). 5(S),12(S)-6-trans-LTB_4, 5(S),12(R)-6-trans-LTB_4, 5(S),12(S)-6-trans-LTB_5, and 12(S),12(R)-6-trans-LTB_5 displaced a mean of 50% of the [^{3}H]LTB_4 from neutrophils at respective concentrations of 3 x 10^{-7}M, 1 x 10^{-8}M, 3 x 10^{-7}M, and 1 x 10^{-6}M.

The reduced chemotactic potency of LTB_5 relative to LTB_4 (Fig. 1) would not be predicted by the functional relationship of the C6-peptide leukotrienes of the pentaene and tetraene series, as the smooth muscle contractile potencies of LTC_5 and LTD_5 are similar to those of LTC_4 and LTD_4, respectively (16). The association constants of LTB_5 and the 6-trans isomers of LTB_4 and LTB_5 for the neutrophil high-affinity receptors were approximately 20- to 2000-fold lower than for LTB_4 (Fig. 3). The concomitantly diminished chemotactic potency

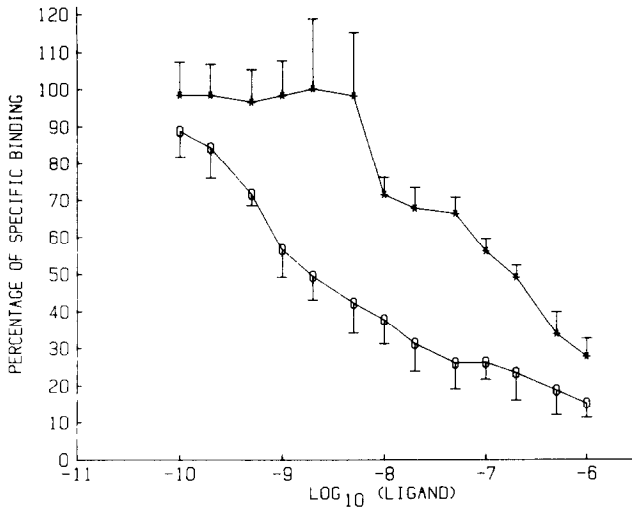

Figure 3 - Inhibition of the specific binding of [^{3}H]LTB_4 to human neutrophils by LTB_4 (o) and LTB_5 (*). Each point and bracket are the mean and S.D. of the results of four (LTB_4) or three (LTB_5) studies. Kd values are presented in the text.

and binding affinity of LTB_5 and the two series of 6-trans isomers support the contention that the neutrophil chemotactic response to LTB_4 is transduced most efficiently by high-affinity receptors. The similarity of potency of LTB_5 and LTB_4 with respect to the stimulation of release of β-glucosaminidase (Fig. 2) suggests that low-affinity receptors have a more central role in lysosomal degranulation than in chemotaxis. Although the cellular mechanisms of the effects of LTB_4 and LTB_5 on neutrophils will only be defined by further studies, it can be predicted that diets enriched in EPA will modify the contributions of leukotriene B to inflammatory and immunological reactions.

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