British Journal of Pharmacology (1996) 117, 1127-1132

Characterization of the pharmacological profile of the potent LTB_4 antagonist CP-105,696 on murine LTB_4 receptors *in vitro*

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1 Binding of [^{3}H]-leukotriene B_4 ([^{3}H]-LTB_4) to murine spleen membranes (MSM) was determined.

2 Scatchard analyses of [^{3}H]-LTB_4 binding indicated the presence of high (K_{D1} = 1.7 nM) and low (K_{D2} = 7.5 nM) affinity receptors on MSM with B_{max} values of 151 fmol mg^{-1} protein (B_{max1}) and 354 fmol mg^{-1} protein (B_{max2}), respectively.

3 CP-105,696, a potent LTB_4 antagonist, inhibited [^{3}H]-LTB_4 (0.67 nM) binding to the high affinity receptor on MSM, IC_{50} = 30.2 nM, K_i = 17.7 nM with a Hill coefficient of 0.93.

4 Scatchard analyses of [^{3}H]-LTB_4 binding to MSM in the presence of CP-105,696 indicated that the high-affinity receptor was inhibited in a non-competitive manner and the low-affinity receptor in a competitive manner.

5 Isolated peripheral blood murine neutrophils (MN) responded chemotactically to LTB_4, EC_{50} = 2.5 nM. CP-105,696 blocked this response, IC_{50} = 2.3 nM. When examined over a full concentration-response range of LTB_4, CP-105,696 inhibited chemotaxis in a non-competitive manner.

6 Murine neutrophils in anticoagulated whole blood upregulated the integrin, complement receptor type 3 (CD11b/CD18, Mac-1) in response to LTB_4, EC_{50} = 20 nM and this was inhibited by CP-105,696 in a competitive manner.

7 These results provide evidence that MSM have specific binding sites for LTB_4, and as exemplified by CP-105,696, that these receptors may be useful for determining the potency and nature of antagonism of novel LTB_4 receptor antagonists.

Keywords: Leukotriene B_4 receptors; murine spleen membranes; neutrophils; chemotaxis; complement receptor 3; CD11b/CD18; Mac-1; CP-105,696

Introduction

Leukotriene B_4, a product of the 5-lipoxygenase pathway of arachidonic acid metabolism (Borgeat & Samuelsson, 1979) is a potent proinflammatory mediator thought to be involved in the pathogenesis of several inflammatory diseases (Ford-Hutchinson, 1990). Many reports over the past decade have provided evidence of specific high affinity receptors for LTB_4 on inflammatory cells such as neutrophils eosinophils and macrophages from several different species (Goldman & Goetzl, 1984; Lin *et al.*, 1984; Cristol *et al.*, 1988; Maghni *et al.*, 1991). In addition, membranes derived from guinea-pig and porcine spleen have also been shown to bind LTB_4 with high affinity (Cheng *et al.*, 1986; Miki *et al.*, 1990). Although there are a number of published reports demonstrating LTB_4-mediated leukocyte infiltration *in vivo* in mice (Suarez *et al.*, 1987; Thieroff *et al.*, 1988; Fretland *et al.*, 1990; Griswold *et al.*, 1991; Iwamoto *et al.*, 1993) to our knowledge there are no published reports showing direct evidence of LTB_4 receptors on murine cells or tissue. We therefore undertook the series of experiments described herein to determine if LTB_4 receptors could be detected on murine tissue (e.g. spleen) and to see if evidence could be provided that would link these receptors to functional responses of murine peripheral blood neutrophils. The ultimate goal of these experiments was to determine if the novel LTB_4 antagonist, CP-105,696 (Koch *et al.*, 1994) maintained potency on murine tissue/cells that would support its *in vivo* evaluation in murine inflammatory disease models.

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Methods

Membrane preparation

Spleens were dissected from 40 to 50 BALB/c mice and kept in 50 mM Tris-HCl buffer (pH = 7.3 at 25°C). After removal of connective tissue, the total weight of the spleens was determined. The pooled tissue was minced and homogenized twice by Polytron (Brinkmann Instruments, Westbury, NY, U.S.A.) PCU-2 at setting 7 for 10 s in 50 mM Tris at 3 ml g^{-1} tissue. The homogenate was centrifuged at 1000 *g* for 15 min and the resultant supernatant was centrifuged at 40,000 *g* for 20 min with washing of the intermediate pellet with 50 mM Tris. The final pellet was resuspended at a protein concentration of 5 mg ml^{-1} in 50 mM Tris pH = 7.3 with 10 mM $MgCl_2$ and stored at −70°C. The protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

[^{3}H]-LTB_4 receptor binding assays

The [^{3}H]-LTB_4 mouse spleen binding assay was performed according to a method adapted from Cheng *et al.* (1986). The [^{3}H]-LTB_4 binding assays were carried out in a volume of 150 µl containing 50 µg membrane preparation, 0.67 nM [^{3}H]-LTB_4, 50 mM Tris pH = 7.3, 10 mM $MgCl_2$ and 10% methanol in the presence or absence of various concentrations of unlabelled LTB_4 or CP-105,696 for 30 min at 4°C. The assay was performed in triplicate in microtitre plates (Costar, Cambridge, MA, U.S.A.) and filtered through Whatman GF/B glass fibre filters using a beta plate apparatus (Pharmacia LKB, Piscataway, NJ, U.S.A.) to separate free from bound [^{3}H]-LTB_4. Specific [^{3}H]-LTB_4 binding represents the value obtained when nonspecific binding (value obtained in the

presence of 5 μM unlabelled LTB_4) is subtracted from total binding. For saturation binding experiments, concentrations of [^{3}H]-LTB_4 ranging from 0.25 to 20 nM were used. [^{3}H]-LTB_4 binding to the spleen membranes was unchanged for up to 6 months.

Murine neutrophil isolation and chemotaxis assay

Neutrophils were isolated from pooled anticoagulated blood obtained from BALB/c mice by intracardiac puncture according to the procedure described by Rot (1991). Isolated cells (usually ~30% neutrophils) were adjusted to a density of 2.5×10^6 neutrophils ml^{-1} in HBSS containing 1.6 mM Ca^{2+} and 0.7 mM Mg^{2+}, 10 mM HEPES and 1% BSA (pH 7.2). Chemotaxis was performed using a 48-well chamber apparatus (Neuroprobe, Cabin John, MD, U.S.A.) with cellulose nitrate filters (pore size, 3.0 μm) as previously described (Harvath *et al.*, 1987). Under these assay conditions, neutrophils are the only cells that migrate into the filters. The total number of cells (observed at ×400 magnification in 3–5 fields) migrating from 20 μm beneath the surface monolayer to the leading front (usually 40–60 μm 60 min^{-1} at optimal chemotactic factor concentrations) were summed and provided an index of the chemotactic response. Each experiment was performed on at least three separate occasions. In experiments where CP-105,696 was examined for antagonist properties, it was present in both compartments of the chemotaxis chamber.

Upregulation of Mac-1 on neutrophils in whole blood

Whole blood obtained by intracardiac puncture from BALB/c mice was anticoagulated with EDTA (0.37%); 100 μl of blood was then added to 12 × 75 mm borosilicate test tubes containing 10 μl of 10 × stock concentration of CP-105,696 for 5 min at 37°C. LTB_4 (20 nM final concentration) was added to the samples and incubated for 10 min at 37°C. Tubes were then removed and placed in an ice water bath and ~1 ml of cold PBS containing 10 mM EDTA, pH 7.25, 0.2% sodium azide and 0.2% heat-inactivated foetal bovine serum (PBS-wash). The cells were then pelleted at 200 *g* and incubated with either FITC-conjugated rat anti-mouse Mac-1 mAb or rat IgG-2b isotype control Ab for the mouse cells. The RBC were lysed with FACS Lysing Solution (Becton-Dickinson, San Jose, CA, U.S.A.) and the remaining cells washed with PBS-wash at 4°C. Fluorescent labelling was quantified immediately with a B.D. FACScan flow cytometer with a live gate on the neutrophils. Data from 5000 neutrophils per sample were recorded and expressed as the percentage maximal mean channel fluorescence.

Data analysis

Results are expressed as the means ± s.e.mean from at least three independent experiments unless otherwise indicated. IC_{50} values were calculated by plotting the mean of the percent control of each compound-treated group on a logscale, fitting the linear portion of the graphs to a linear regression curve and calculating the x value at y = 50. Binding site densities and respective affinities for [^{3}H]-LTB_4 were calculated according to the published method (Scatchard, 1949) using London Software Receptor Fit Saturation Two-site Program. K_i was calculated according to the method of Cheng & Prusoff, 1973.

Materials

[^{3}H]-LTB_4 (specific activity = 200 Ci $mmol^{-1}$) was purchased from DuPont/NEN (Boston, MA, U.S.A.); LTB_4 from Biomol, Plymouth Meeting, PA, U.S.A., BALB/c mice from Jackson Laboratories, Bar Harbor, ME, U.S.A. CP-105,696, (+)-1-(3S,4R)-[3-(4-phenyl-benzyl)-4-hydroxy-chroman-7-yl]-cyclopentane carboxylic acid was synthesized according to Koch *et al.* (1994) and its structure is shown in Figure 1.

Results

Evidence for specific [^{3}H]-LTB_4 binding sites on MSM

Figure 2 shows a representative saturation isotherm and Scatchard plot for the binding of [^{3}H]-LTB_4 to murine spleen membranes. Analysis of this data indicated the presence of two distinct binding sites with the higher affinity site exhibiting a dissociation constant (K_{D1}) (mean ± s.e.) of 1.7 ± 0.4 nM (n = 3) and a total binding capacity (B_{max1}) of 151 ± 17 fmol mg^{-1} protein, and a lower affinity binding site exhibiting a dissociation constant (K_{D2}) of 7.5 ± 2.1 nM (n = 3) and a total binding capacity (B_{max2}) of 354 ± 59 fmol mg^{-1} protein. [^{3}H]-LTB_4 binding was linear over a protein concentration range of 5–50 μg/well and half-maximal binding occurred at ~10 min reaching equilibrium at 30 min (results not shown).

Effect of CP-105,696 on [^{3}H]-LTB_4 binding to high affinity receptors on MSM

Figure 3 demonstrates the ability of CP-105,696 to compete for [^{3}H]-LTB_4 binding to the high-affinity binding site on MSM. An IC_{50} value of 30.2 ± 1.2 nM (K_i = 17.7 ± 0.7 nM) (n = 3) was obtained for CP-105,696; this is ~10 fold higher than the IC_{50} value obtained for LTB_4, 3.1 ± 0.5 nM (n = 3). Scatchard analysis of [^{3}H]-LTB_4 binding to high-affinity receptors on MSM in the presence and absence of fixed concentrations of CP-105,696 showed clear evidence of noncompetitive interactions of CP-105,696 on [^{3}H]-LTB_4 binding to this high affinity site (Figure 4). The K_D values of [^{3}H]-LTB_4 obtained in the presence of CP-105,696 at 1 nM and 5 nM were 1.9 nM and 1.9 nM

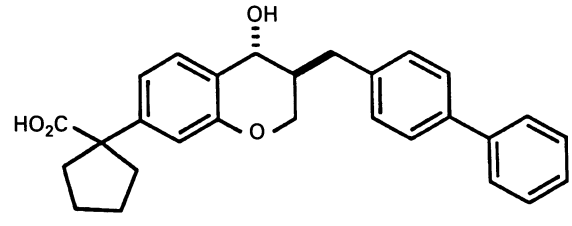

Figure 1 Structure of CP-105,696.

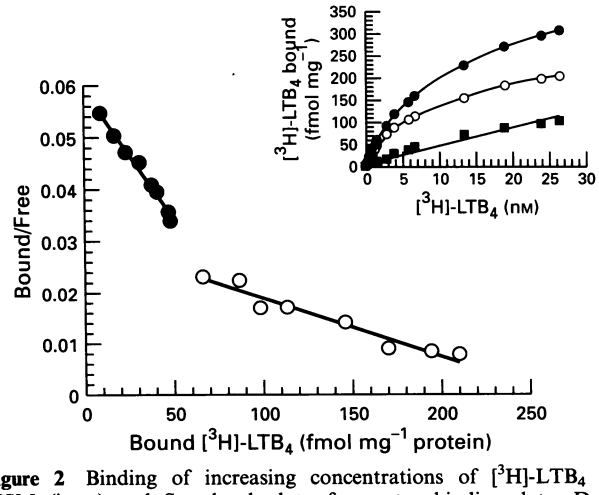

Figure 2 Binding of increasing concentrations of [^{3}H]-LTB_4 to MSM (inset) and Scatchard plot of receptor binding data. Data shown are representative of three independent experiments. Data were fitted to a two-site model using the Lundon Software Receptor Fit Saturation Binding Programme. Symbols in inset represent (●) total, (■) nonspecific and (○) specific [^{3}H]-LTB_4 binding. Calculated K_{D1} = 1.98 nM and B_{max1} = 118 fmol mg^{-1} (●) and K_{D2} = 8.98 nM and B_{max2} = 270 fmol mg^{-1} (○).

respectively compared to the control value of 2.0 nM. The corresponding densities of high-affinity [^{3}H]-LTB_4 binding sites per cell in the presence of CP-105,696 at 1.0 nM and 5.0 nM were 105 fmol mg^{-1} and 76 fmol mg^{-1} respectively, compared with the control value of 118 fmol mg^{-1}.

Effect of CP-105,696 on [^{3}H]-LTB_4 binding to low-affinity receptors on MSM

Figure 5 shows the Scatchard analysis of [^{3}H]-LTB_4 binding to low-affinity receptors on MSM in the presence and absence of fixed concentrations of CP-105,696. Evidence of competitive interactions at this low-affinity binding site were obtained. The K_D values of [^{3}H]-LTB_4 obtained in the presence of CP-105,696 at 1 nM and 5 nM were 16 nM and 22 nM respectively compared to the control value of 10.0 nM. The corresponding densities of low affinity [^{3}H]-LTB_4 binding sites per cell in the presence of CP-105,696 at 1.0 nM and 5.0 nM were 456 fmol mg^{-1} and 466 fmol mg^{-1} respectively, compared with the control value of 468 fmol mg^{-1}.

Effect of CP-105,696 on LTB_4-mediated murine neutrophil chemotaxis

Using the 48-microwell chemotaxis chamber and cellulose nitrate filters of 3 μm pore size we observed half-maximal effects of LTB_4 on neutrophil migration at 2.5 nM and maximal effects at 100 nM (see inset Figure 6). With 5 nM as the fixed concentration of LTB_4 in the lower chamber, half maximal inhibition of chemotaxis occurred at a concentration of 2.3±0.7 nM (n=5) of CP-105,696. When chemotaxis was assessed over a full range of concentrations of LTB_4, the effect of

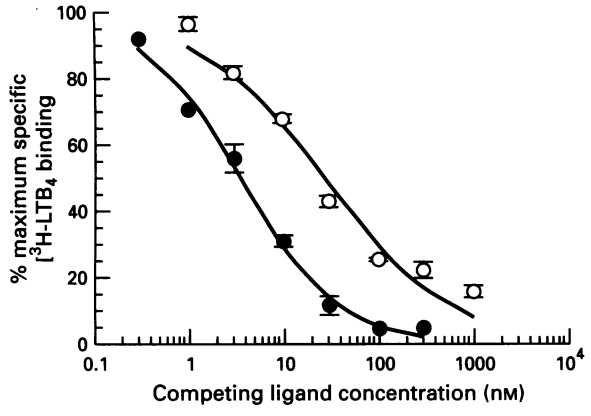

Figure 3 Inhibition of [^{3}H]-LTB_4 binding to MSM by CP-105,696 (○) and LTB_4 (●). IC_{50} for LTB_4=3.14±0.48 nM and for CP-105,696=30.2±1.2 nM. MSM were incubated with 0.67 nM [^{3}H]-LTB_4 for 30 min at 4°C in the presence and absence of various concentrations of CP-105,696 or LTB_4 and specific binding was determined. Data are means±s.e. from three experiments each performed in triplicate. Zero percent specific binding refers to the level of [^{3}H]-LTB_4 binding obtained in the presence of 1.0 μM unlabelled LTB_4.

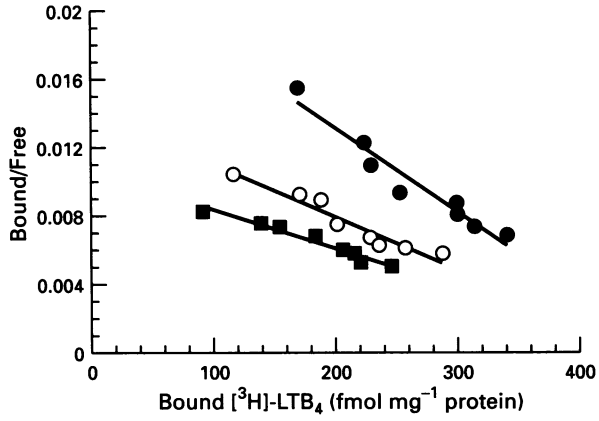

Figure 5 Scatchard plots of [^{3}H]-LTB_4 binding to low-affinity receptors in MSM in the absence (●) and presence of (○) 1 nM and (■) 5 nM CP-105,696. MSM were incubated with concentrations of [^{3}H]-LTB_4 (4.0 nM–20.0 nM) for 30 min at 4°C in the absence and presence of CP-105,696. Control, K_D=10.2 nM, B_{max}=468 fmol mg^{-1}; 1 nM CP-105,596, K_D=16.0 nM, B_{max}=456 fmol mg^{-1}; 5 nM CP-105,596, K_D=21.8 nM, B_{max}=466 fmol mg^{-1}. Data shown are representative of three independent experiments. Data were fitted to a one-site model using the Lundon Software Receptor Fit Saturation Binding Programme.

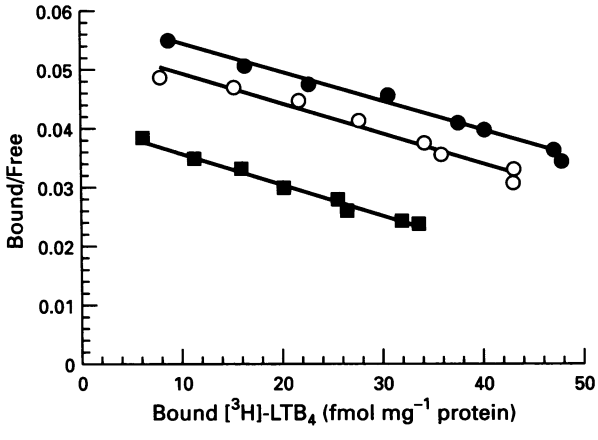

Figure 4 Scatchard plots of [^{3}H]-LTB_4 binding to high-affinity receptors on MSM in the absence (●) and presence of (○) 1 nM and (■) 5 nM CP-105,696. MSM were incubated with concentrations of [^{3}H]-LTB_4 (0.25 nM–2.0 nM) for 30 min at 4°C in the absence and presence of CP-105,696. Control K_D=1.98 nM, B_{max}=118 fmol mg^{-1}; 1 nM CP-105,596, K_D=1.93 nM, B_{max}=105 fmol mg^{-1}; 5 nM CP-105,596, K_D=1.85 nM, B_{max}=76.3 fmol mg^{-1}. Data shown are representative of three independent experiments. Data were fitted to a one-site model using the Lundon Software Receptor Fit Saturation Binding Programme.

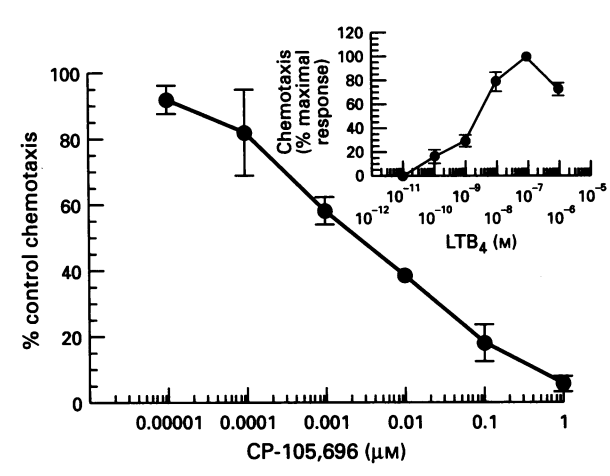

Figure 6 Inhibition of LTB_4-mediated murine neutrophil chemotaxis by CP-105,696. LTB_4 was used at a fixed concentration of 5 nM in the lower chamber and CP-105,696 was present at various concentrations (10 pM–1 μM) in both the upper and the lower chamber. Chambers were incubated for 60 min at 37°C and then the filters were removed, processed and counted as described in Methods. Data are the means±s.e. from 5 independent experiments. Inset shows LTB_4 concentration-response curve.

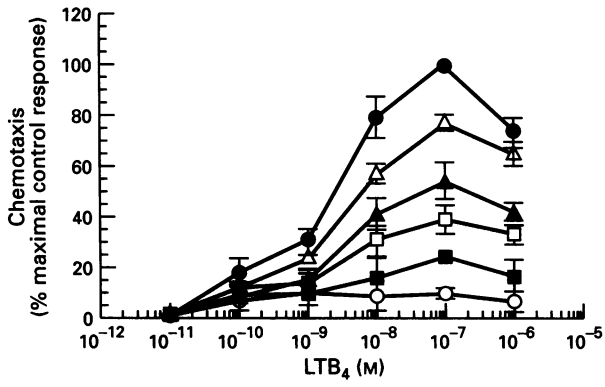

Figure 7 Effect of CP-105,696 on the concentration-dependent curve of LTB_4-mediated chemotaxis. Chemotaxis was induced by a range of concentrations of LTB_4 (10 pM – 1 μM) in the absence (●) and presence of (△) 10^{-9}M; (▲) 10^{-8}M; (□) 10^{-7}M; (■) 10^{-6}M; (○) 10^{-5}M CP-105,696. Data are expressed as the percentage of maximal chemotaxis occurring to LTB_4 in the absence of CP-105,696 and are means ± s.e. from three independent experiments.

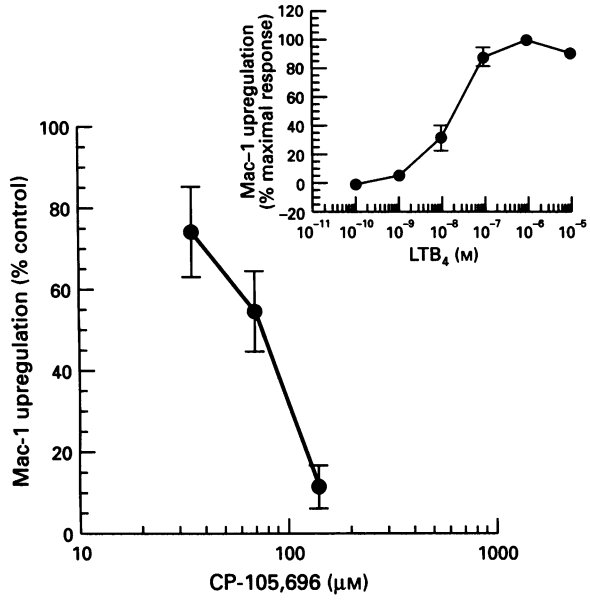

Figure 8 Inhibition of LTB_4-mediated Mac-1 upregulation in murine neutrophils in whole blood. LTB_4 was used at a fixed concentration of 100 nM. CP-105,696 was present at various concentrations (35 – 140 μM). Data are expressed as the percentage of the response occurring in the absence of CP-105,696 and are the means ± s.e. from 4 independent experiments. Inset shows LTB_4 concentration-response curve.

the addition of CP-105,696 could not be overcome by increasing concentrations of LTB_4, which offers evidence that a noncompetitive form of inhibition is occurring at the LTB_4 receptor mediating neutrophil chemotaxis (Figure 7).

Effect of CP-105,696 on LTB_4-mediated upregulation of the adhesion molecule Mac-1 on murine neutrophils in whole blood

Half-maximal effects of LTB_4 on Mac-1 upregulation on murine neutrophils in whole blood occurred at ~20 nM with maximal effects at ~1.0 μM (see inset Figure 8). Using 100 nM as the fixed concentration of LTB_4, we observed half maximal inhibition of Mac-1 upregulation by CP-105,696 at 66.5 ± 10.7 μM (n=4). When Mac-1 upregulation was examined over a full range of concentrations of LTB_4, the addition of increasing concentrations of CP-105,696 caused parallel rightward shifts in the LTB_4-concentration curves, which indicates that competitive interactions at the LTB_4 receptor mediating CD11b upregulation are occurring (Figure 9).

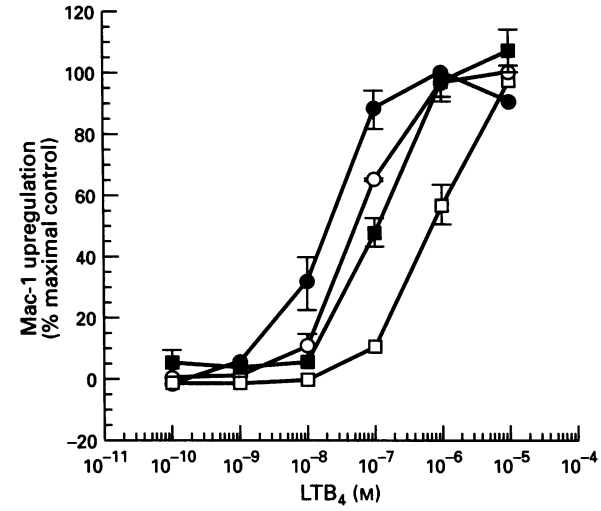

Figure 9 Effect of CP-105,696 on the concentration-dependent curves of LTB_4-mediated upregulation of Mac-1 in neutrophils in whole blood. Mac-1 upregulation was induced by a range of concentrations of LTB_4 in the absence (●) and presence of (○) 35 μM; (■) 70 μM; (□) 140 μM CP-105,696. Data are expressed as the percentage of maximal response occurring to LTB_4 in the absence of CP-105,696 and are from four experiments.

Discussion

Several published reports have provided evidence of specific LTB_4 receptors on inflammatory cells and that these receptors were probably linked to functional activation of cells such as human neutrophils (Goldman & Goetzl, 1984; Lin *et al.*, 1984). More recently through the use of the specific LTB_4 receptor antagonist, CP-105,696, we have presented pharmacological evidence that the high affinity LTB_4 receptor (K_D = 0.65 nM) appears to mediate chemotaxis, and the low affinity LTB_4 receptor (K_D = 5.8 nM) is linked to secretory events in human neutrophils (Showell *et al.*, 1995). Two other reports have demonstrated the presence of high affinity binding sites for LTB_4 on membranes derived from guinea-pig and porcine spleen (Cheng *et al.*, 1986; Miki *et al.*, 1990). Although the specific cell source(s) contributing these binding sites for [^{3}H]-LTB_4 were not defined it was felt that these receptors were physiologically relevant, and for guinea-pigs, possibly unrelated to the LTB_4 binding site present on granulocytes from this species (Cheng *et al.*, 1986). As it is impractical to obtain sufficient numbers of circulating murine neutrophils suitable for [^{3}H]-LTB_4 receptor binding assays and as mouse spleen is known to serve a myelopoietic function (Bannerman, 1983) we reasoned that membranes derived from this tissue may be a useful surrogate for murine neutrophils and thus possess LTB_4 receptors reflective of this cell population. Using [^{3}H]-LTB_4 as a specific probe we show here evidence for the presence of specific binding sites for LTB_4 on membranes derived from murine spleen with affinities in the nanomolar range. Scatchard analysis of the [^{3}H]-LTB_4 binding isotherms revealed the presence of two distinct receptors with affinities of 1.7 nM (K_{D1}) and 7.5 nM (K_{D2}) respectively. Interestingly, guinea-pig (Cheng *et al.*, 1986) and porcine (Miki *et al.*, 1990) membranes yielded single affinity [^{3}H]-LTB_4 binding sites with K_Ds of 1.7 nM and 1.0 nM respectively and receptor capacities of 274 fmol mg^{-1} protein and 120 fmol mg^{-1} protein. For mouse spleen membranes the high and low affinity [^{3}H]-LTB_4 receptors exhibited binding capacities of 151 fmol mg^{-1} protein and 354 fmol mg^{-1} respectively. From our own work and

that of others using human neutrophils we believe the high affinity receptor for LTB_4 to be coupled functionally to chemotaxis and the low affinity receptor for LTB_4 to secretion. Thus the results obtained with mouse spleen membranes suggest that the high affinity [^{3}H]-LTB_4 binding site is most probably reflective of a receptor involved in murine neutrophil chemotaxis and the low affinity [^{3}H]-LTB_4 receptor involved in secretory phenomena. Previously we have shown the LTB_4 receptor antagonist, CP-105,696 to inhibit [^{3}H]-LTB_4 binding to human neutrophil high affinity receptor non-competitively and that was seen for CP-105,696 inhibition of the LTB_4-mediated chemotactic response (Showell *et al.*, 1995). As demonstrated here, CP-105,696 is also a non-competitive inhibitor of both [^{3}H]-LTB_4 binding to the high affinity receptor on murine spleen membranes and LTB_4-mediated murine neutrophil chemotaxis. Taken together we believe that the findings with CP-105,696 and the above relationship of ligand binding and functional overlap for neutrophil chemotaxis strengthens the argument that the high affinity receptor for LTB_4 is the receptor mediating chemotaxis. With regard to the low affinity receptor for LTB_4 on murine spleen membranes, it appears that this receptor, as was previously shown for human neutrophils (Showell *et al.*, 1995), is coupled to secretory events of murine neutrophils. Again pharmacological evidence using CP-105,696 supports this view. Using murine neutrophils in whole blood, competitive inhibition of LTB_4-mediated Mac-1 upregulation was found and occurs at similar concentrations to that for human neutrophil CD11b upregulation in whole blood (Showell *et al.*, 1995). The potency of CP-105,696 in this whole blood assay is underestimated by $\sim$2.5 log because of the very high level of protein binding that obtains for this compound (Liston *et al.*, unpublished). In addition [^{3}H]-LTB_4 binding to the low affinity LTB_4 receptor on MSM is competitively inhibited by CP-105,696 as was previously seen for human neutrophils.

Together the data presented here suggest that murine spleen membranes are a relatively easily obtained and useful surrogate for predicting the inhibitory potency and nature of antagonism of prototype LTB_4 antagonists on murine neutrophil functional responses. Furthermore murine and human neutrophils appear to be similarly sensitive to LTB_4 with respect to induction of biological responses and sensitivity to a variety of chemically distinct LTB_4 antagonists (Showell *et al.*, unpublished). The value of first being able to demonstrate a concordance between results obtained with human cells and cells from an animal species used for preclinical efficacy testing is considerable. Here we believe we have shown that mice are such a species and evaluation of the LTB_4 antagonist, CP-105,696, in murine models such as collagen-induced arthritis (Griffiths *et al.*, 1995) and experimental allergic encephalomyelitis (Gladue *et al.*, 1995) have provided some compelling data supportive of clinical evaluation of LTB_4 antagonists in a variety of human inflammatory diseases.

We are indebted to Deborah A. Baker and Eben D. Salter for their assistance in collecting mouse spleens and blood. Niall Doherty, Ron Gladue, Richard Griffiths and E. Roy Pettipher are thanked for their critical reading of the manuscript. Kathryn L. Smith and Deborah H. Sanford are thanked for their help in manuscript preparation.

References

BANNERMAN, P.M. (1983). Hematology. In *The Mouse in Biomedical Research*. Volume III *Normative Biology, Immunology and Husbandry*, ed. Foster, H.L., Small, J.D. & Fox, J.G. pp. 293–312. New York: Academic Press.

BORGEAT, P. & SAMUELSSON, B. (1979). Transformation of arachidonic acid by rabbit polymorphonuclear leukocytes. Formation of a novel dihydroxyeicosatetraenoic acid. *J. Biol. Chem.*, **254**, 2643–2650.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

CHENG, J.B., CHENG, E.I-P., KOHI, F. & TOWNLEY, R.G. (1986). [^{3}H]Leukotriene B_4 binding to the guinea pig spleen membrane preparation: A rich tissue source for a high-affinity leukotriene B_4 receptor site. *J. Pharmacol. Exp. Ther.*, **236**, 126–132.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibitory constant (K_i) and the concentrations of an inhibitor which causes 50 percent inhibition (IC_{50}) of an enzyme reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.

CRISTOL, J.P., PROVENCAL, B., BORGEAT, P. & SIROIS, P. (1988). Characterization of leukotriene B_4 binding sites on guinea pig lung macrophages. *J. Pharmacol. Exp. Ther.*, **247**, 1199–1203.

FORD-HUTCHINSON, A.W. (1990). Leukotriene B_4 in inflammation. *Crit. Rev. Immunol.*, **10**, 1–12.

FRETLAND, D., WIDOMSKI, D., ANGLIN, C. & GAGINELLA, T. (1990). CD18 monoclonal antibody inhibits neutrophil diapedesis in the murine dermis induced by leukotriene B_4 and 12(R)-hydroxyeicosatetraenoic acid. *Eicosanoids*, **3**, 171–174.

GLADUE, R.P.,. CARROLL, L.A., SALTER, E.D., PETTIPHER, E.R., MILICI, A.J., KOCH, K. & SHOWELL, H.J. (1995). Protective effects of LTB_4 receptor antagonist CP-105,696 in a murine model of passively induced EAE. *Inflamm. Res.*, **44**, Suppl. 3, S232.

GOLDMAN, D.W. & GOETZL, E.J. (1984). Heterogeneity of human polymorphonuclear leukocyte receptors for leukotriene B4. Identification of a subset of high affinity receptors that transduce the chemotactic response. *J. Exp. Med.*, **159**, 1027–1041.

GRIFFITHS, R.J., PETTIPHER, E.R., KOCH, K., FARRELL, C.A., BRESLOW, R., CONKLYN, M.J., SMITH, M.A., HACKMAN, B.C., WIMBERLY, D.J., MILICI, A.J., SCAMPOLI, D.N., CHENG, J.B., PILLAR, J.S., PAZOLES, C.J., DOHERTY, N.S., MELVIN, L.S., REITER, L.A., BIGGERS, M.S., FALKNER, F.C., MITCHELL, B.Y., LISTON, T.E. & SHOWELL, H.J. (1995). Leukotriene B_4 plays a critical role in the progression of collagen-induced arthritis. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 517–521.

GRISWOLD, D.E., WEBB, E.F. & HILLEGARS, L.M. (1991). Induction of plasma exudation and inflammatory cell infiltration by leukotriene C_4 and leukotriene B_4 in mouse peritonitis. *Inflamm.*, **15**, 251–258.

HARVATH, L., MCCALL, C.E., BASS, D.A. & MCPHAIL, L.C. (1987). Inhibition of human neutrophil chemotaxis by the protein kinase inhibitor, 1-(5-isoquinolinesulfonyl)piperazine. *J. Immunol.*, **139**, 3055–3061.

IWAMOTO, I., TOMOE, S., TOMIOKA, H. & YOSHIDA, S. (1993). Leukotriene B_4 mediates substance P-induced granulocyte infiltration into mouse skin. *J. Immunol.*, **151**, 2116–2123.

KOCH, K., MELVIN, JR. L.S., REITER, L.A., BIGGERS, M.S., SHOWELL, H.J., GRIFFITHS, R.J., PETTIPHER, E.R., CHENG, J.B., MILICI, A.J., BRESLOW, R., CONKLYN, M.J., SMITH, M.A., HACKMAN, B.C., DOHERTY, N.S., SALTER, E., FARRELL, C.A. & SCHULTE, G. (1994). (+)-2-(3S,4R)-[3-(Phenylbenzyl)-4-hydroxychroman-7-y]cyclopentane carboxylic acid, CP-105,696. A highly potent selective leukotriene B_4 antagonist with oral activity in the murine collage induced arthritis model. *J. Med. Chem.*, **37**, 1397–1399.

LIN, A.H., RUPPEL, P.L. & GORMAN, R.R. (1984). Leukotriene B_4 binding to human neutrophils. *Prostaglandins*, **28**, 837–849.

MAGHNI, K., DEBRUM-FERNANDES, A.J., FOLDES-FILET, M., GAUDRY, M., BORGEAT, P. & SIROIS, P. (1991). Leukotriene receptors on guinea pig alveolar eosinophils. *J. Pharmacol. Exp. Ther.*, **258**, 784–789.

MIKI, I., WANTANABE, T., NAKAMURA, M., SEYAMA, Y., UI, M., SATO, F. & SHIMIZU, T. (1990). Solubilisation and characterization of leukotriene B_4 receptor-GTP binding protein complex from porcine spleen. *Biochem. Biophys. Res. Commun.*, **166,** 342–348.

ROT, A. (1991). Chemotactic potency of recombinant human neutrophil attractant/activating protein-1 (Interleukin-8) for polymorphonuclear leukocytes of different species. *Cytokine*, **3,** 21–27.

SCATCHARD, G. (1949). The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51,** 660–672.

SHOWELL, H.J., PETTIPHER, E.R., CHENG, J.B., BRESLOW, R., CONKLYN, M.J., FARRELL, C.A., HINGORANI, G.P., SALTER, E.D., HACKMAN, B.C., WIMBERLY, D.J., DOHERTY, N.S., MELVIN, JR. L.S., REITER, L.A., BIGGERS, M.S. & KOCH, K. (1995). The *in vitro* and *in vivo* pharmacologic activity of the potent and selective leukotriene B_4 receptor antagonist CP-105,696. *J. Pharmacol. Exp. Ther.*, **273,** 170–184.

SUAREZ, C.R., PICKETT, W.C., BELL, D.H., MCCLINTOCK, D.K., ORONSKY, A.L. & KERWAR, S.S. (1987). Effect of low dose methotrexate on neutrophil chemotaxis induced by leukotriene B_4 and complement C5a. *J. Rheumatol.*, **14,** 9–11.

THIEROFF, R., INHLICH, W. & EKERDT, R. (1988). Chemotactic activity of LTB_4 in the skin of different laboratory animals *in vivo*. *J. Invest. Dermatol.*, **90,** 254.

(Received August 17, 1995
Revised November 15, 1995
Accepted November 23, 1995)