ELSEVIER

International Immunopharmacology 3 (2003) 1829–1839

International Immunopharmacology

www.elsevier.com/locate/intimp

Differential inhibition of receptor activation by two mouse monoclonal antibodies specific for the human leukotriene B_4 receptor, BLT_1

A. Sabirsh*, A. Pettersson, Å. Boketoft, K. Kotarsky, C. Owman

Molecular Neurobiology, Lund University, BMC A12 Tornavägen 10, 224 81 Lund, Sweden

Received 13 July 2003; received in revised form 11 August 2003; accepted 22 August 2003

Abstract

The inflammatory mediator leukotriene B_4 (LTB_4) binds to and activates a G-protein-coupled receptor named BLT_1. We have previously produced two monoclonal antibodies, named 7B1 and 14F11, that bind specifically to this receptor. Using a HeLa cell line expressing human BLT_1, we find that both antibodies inhibit LTB_4-induced calcium release, and activation of a MAP-kinase-sensitive luciferase reporter system. The normal chemotactic movement of polymorphonuclear cells towards higher LTB_4 concentrations was also strongly inhibited by both antibodies. Neither antibody was found to activate BLT_1, and experiments using cyclic peptide fragments of the BLT_1 n-terminal and extracellular loops showed that these antibodies bind only to complex epitopes in the tertiary, membrane bound, conformation of the receptor protein. In ligand binding experiments, 7B1 was found to be a competitive antagonist, while 14F11 was a noncompetitive antagonist that inhibited receptor activation, but not agonist (LTB_4) binding. 14F11 will be a useful tool for studying the mechanisms of receptor activation.

Keywords: Receptor, cell surface; Epitope mapping; Binding; Enzyme-linked immunosorbent assay; Chemotaxis

1. Introduction

The eicosanoid lipid mediator leukotriene B_4 (LTB_4) binds to a G-protein-coupled receptor (GPCR) called BLT_1. BLT_1 is normally expressed primarily on immune cells (such as granulocytes) [1–3] where the receptors are important for steering these cells to sites of inflammation, and a variety of inflammatory conditions have been shown to involve BLT_1 [4–7]. The expression of BLT_1 may also be an important marker for tumourgenicity [8] and may be involved in tumour growth [9].

We have previously produced and characterised two mouse monoclonal antibodies that bind specifically to human BLT_1 receptors [1]. In order to further characterise these antibodies, we have investigated whether or not they interfere with agonist (LTB_4) binding to, and subsequent activation of, BLT_1. This question is interesting because the answer will provide information about how both LTB_4 and the antibodies bind to the receptor. Previously

* Corresponding author. Tel.: +46-46-222-0585; fax: +46-46-222-0568.

E-mail address: Alan.Sabirsh@mphy.lu.se (A. Sabirsh).

1567-5769/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.intimp.2003.08.009

reported BLT_1 antibodies were not compatible with this type of experiment because they recognised only latent receptor epitopes [10] or, were polyclonal antibodies that labelled multiple epitopes [2,11]. For use in biological systems, it is also advantageous to have an antibody that does not activate the receptor so that observing receptor expression will not affect that expression, or, alter the behaviour of the biological system the receptor is part of. Conversely, it is important to know if the use of the antibody blocks the normal function of the receptor ligand.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were purchased from Sigma, unless otherwise stated.

BLT_1 receptor fragments (Fig. 1) were synthesised and purified by Eurodiagnostica using Fmoc chemistry and a Milligen 9050 automated peptide synthesiser, followed by HPLC with a Kromasil C8 column and mobile phase consisting of a CH3CN/water/TFA gradient.

Fluorescence-labelled LTB_4 was produced using LTB_4-aminopropylamide (LTB_4-APA, Biomol) and the amine reactive succinimidyl ester of Alexa Fluor™ 568 (Molecular Probes). Alexa Fluor™ 568 was dissolved in methanol to a concentration of 1 mM. LTB_4-APA was dried in a nitrogen atmosphere and re-dissolved in methanol to a concentration of 1.28 mM.

LTB_4-APA and Alexa Fluor™ 568 solutions were mixed at a ratio of 1:2 and incubated for 60 min at room temperature with continuous stirring. The LTB_4-APA-Alexa Fluor™ product (LTB_4-FL) was isolated using reverse phase HPLC with a Nova Pak C_{18} column (Waters) and a mobile phase consisting of methanol/water/acetate at a ratio of 60:40:0.08 (pH set to 6.8 using NaOH). The unreacted Alexa Fluor™ dye passed through the column immediately followed by LTB_4-FL and unreacted LTB_4-APA. All three-reaction components could be detected using 270-nm absorption.

The fractions containing LTB_4-APA and LTB_4-FL were collected and the HPLC mobile phase was removed by diluting each sample 1:1 with water and then filtering through Supel Clean™ LC-18 columns (Supelco). Both LTB_4-APA and LTB_4-FL were retained in the columns and were washed once with water before elution in methanol. The purified substances were dried in a nitrogen atmosphere and re-dissolved in methanol. Concentrations were determined using adsorbance at 270 nm for LTB_4-APA and 365 or 578 nm for LTB_4-FL (the Alexa Fluor™ portion of the molecule absorbs light at 365 nm, whereas the unmodified LTB_4-APA does not; 578 nm is the absorption maximum for Alexa Fluor™ 568).

2.2. Cell culture and construction of HF1pBLT1 cell lines

Cultures of HeLa HF1pBLT1 luciferase reporter cells expressing BLT_1 were created and maintained according to Kotarsky et al. [12].

2.3. BLT_1 antibodies

The two antibodies specific for BLT_1 (called 7B1 and 14F11, IgG2a and IgG1 isotypes, respectively) were produced and characterised according to Pettersson et al. [1], following immunisation of mice with cells expressing BLT_1. Molar antibody concentrations were calculated using the molecular weight of the appropriate (mouse) immunoglobulin isotype obtained from crystal structures [13,14]. The concentration of protein in each antibody solution was determined using a protein assay according to the manufacturer's instructions (BCA Protein Assay™, Pierce, Rockford, IL, USA). Antibody concentrations should be regarded as approximate due uncertainties regarding their actual weight and the concentration of *active* antibody in antibody solutions.

2.4. Membrane preparation

Stably transfected monoclonal HF1pBLT1 cells were grown in tissue culture plates until confluent and then chilled to 4 °C. The cell growth medium was removed and the cells were rinsed once with ice-cold PBS, before ice-cold Tris–HCl buffer (50 mM Tris base, 5 mM $MgCl_2$, 1 mM EGTA, pH 7.5) was added to each plate. The cells were then scraped off and

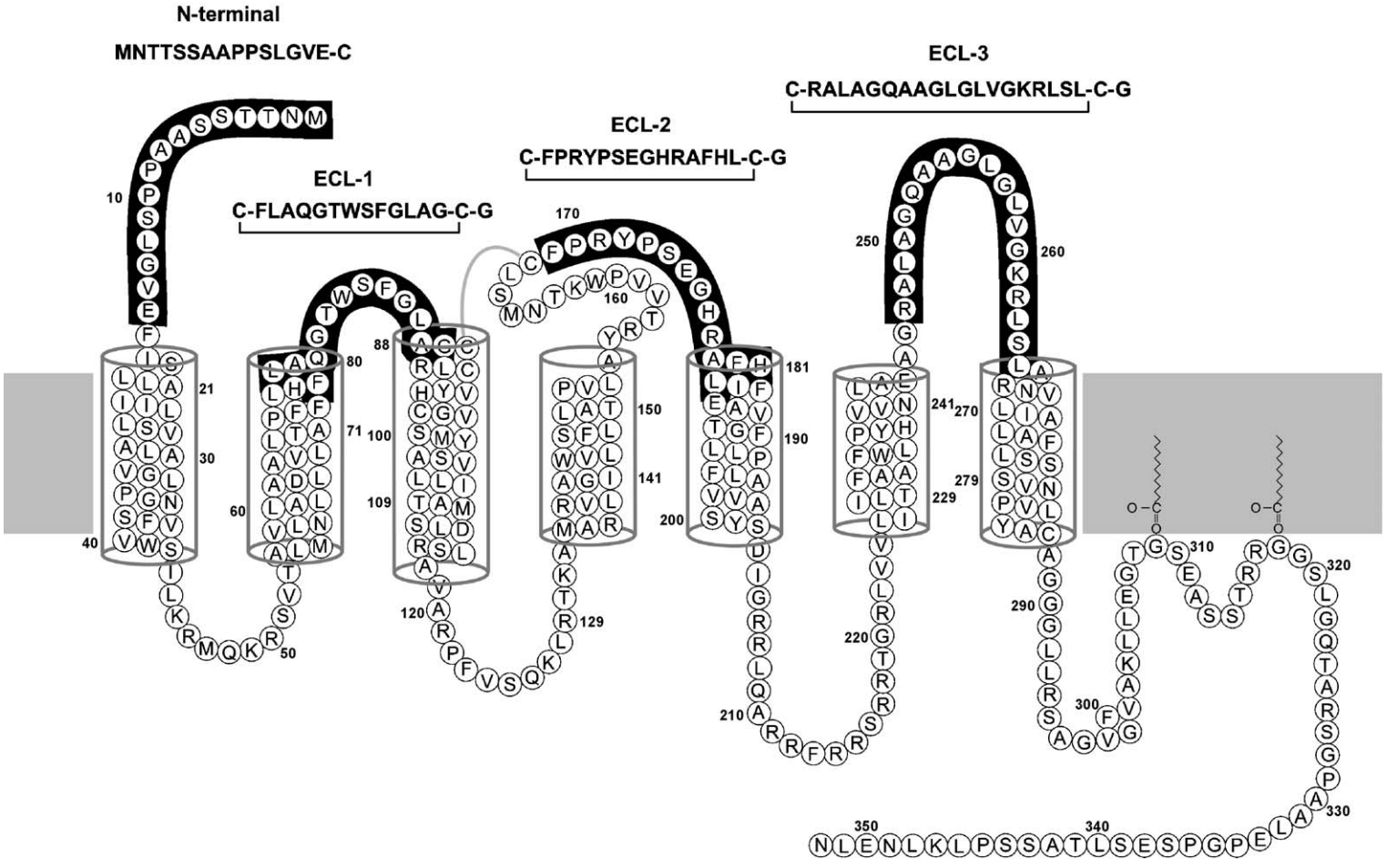

Fig. 1. This two-dimensional representation of BLT_1 shows the locations of the four BLT_1 fragments (highlighted in black) along with the approximate locations of the transmembrane helices (obtained from the rhodopsin crystal structure [26] following alignment with rhodopsin). The cyclic peptide sequences used experimentally, including additional cysteins and glycines, are shown above each loop.

transferred to 50 ml centrifuge tubes on ice and homogenised using an Ultra Turrax™ T25 polytron.

The cell homogenates were centrifuged at 1000 × *g* for 10 min at 4 °C. The pellet was discarded, additional buffer was added, and the supernatant was re-homogenised. The resulting homogenate was centrifuged at 100,000 × *g* for 30 min at 4 °C, and the supernatant was discarded, additional buffer was added, and the pellet was re-homogenised. The membrane protein concentration was determined (BCA Protein Assay™, Pierce), and the membrane isolates were portioned into aliquots before they were frozen at − 80 °C until use.

2.5. Enzyme-linked immunosorbant assays (ELISA)

ELISA was performed using four different synthetic peptides representing the extracellular loops (ECLs) and N-terminal of BLT_1 (Fig. 1). Flanking cysteins (C) where added to BLT_1 sequences to create cyclic peptides and additional cystein (N-terminal peptide) or glycine (ECLs) residues were added for conjugation to Keyhole Limpet Hemocyanin (KLH), for immunization purposes.

Maxisorp™ ELISA plates (Nunc) were coated with peptides by adding 0.5 μg/well of the appropriate peptide or peptide mixture (0.2 μg/well for KLH conjugates) dissolved in 50 μl PBS and incubated overnight at 4 °C. The plates where blocked using 150 μl/well PBS containing 0.2% fat-free milk powder and 0.1% Tween 20 (this blocking buffer also used for wash steps and antibody solutions).

Anti-BLT_1 antibodies were added at concentrations of 20 μg/ml in a volume of 50 μl/well and incubated for 1 h at RT (serum diluted 1:200 from a mouse immunized with peptide–KLH conjugate as a positive control). Secondary goat anti-mouse immunoglobulins coupled to horseradish peroxidase were added after washing, and incubated for 1 h at room temperature. A final wash with PBS was performed before the addition of tetramethylbenzidine (TMB, Zymed) substrate. The reaction was stopped by the addition of 1 M HCl, and evaluated using absorption at 450 nm.

2.6. Flow cytometry

Flow cytometric analysis of HF1pBLT1 cells stained with the anti-BLT_1 antibodies at 4 μg/ml was performed using a FACSCalibur™ (BD Biosciences). The cells were stained either indirectly using an RPE conjugated F(ab′)2 goat anti-mouse immunoglobulin (DAKO) as secondary reagent, or directly using FITC conjugated 7B1 (Serotec) or FITC or RPE conjugated 14F11 (R&D Systems). Anti-BLT_1 antibodies and the cells were incubated together, with or without 0.5 mg/ml of individual BLT_1-peptide fragments (Fig. 1) or a mixture of all four at a concentration of 0.125 mg/ml each. An unrelated peptide fragment from the $P2Y_{12}$ receptor was used as a control.

2.7. Fluorescence polarisation assays of antibody binding

Fluorescence polarisation assays were performed in a total volume of 50 μl of binding buffer (0.02 M HEPES, 10 mM $CaCl_2$, 10 mM $MgCl_2 \cdot 6\ H_2O$, pH 7.5) in black, low-volume plates from Molecular Devices. Membrane preparations were added to each well (at a concentration of 10 μg protein/well) together with the appropriate antibody or LTB_4, and 10 nM LTB_4-FL. The membranes were incubated at room temperature for 1 h before fluorescent ligand binding was analysed using a fluorometer (Polarstar™, BMG) equipped to measure fluorescence polarization.

For each antibody or LTB_4, the concentration that inhibited half of the specific binding (IC_{50}) of LTB_4-FL was determined when possible from competitive binding curves using nonlinear regression (Graph Pad, Prism) of specific binding data, and this in turn was used to calculate K_i according to Cheng and Prusoff [15]. There was a linear correlation between the amount of specific binding (LTB_4-FL polarization) and the amount of membrane protein added to each well. No specific binding to sham-transfected HF1 cells was observed. Specific binding is reported as millipolars (mP).

2.8. Intracellular calcium concentration assay of BLT_1 activity

Confluent cultures of monoclonal HF1pBLTR1 cells were dissociated using EDTA, diluted and seeded into black, clear bottomed 96-well plates (Corning Costar). The plates were incubated at 37 °C, 7% CO_2 for at least 2 days, until the cells were 80–90%

confluent. The growth medium was then exchanged for growth medium containing 4 μM FURA-2AM, 2.5 mM Probenecid and antibodies at a concentration of 100 nM or buffer as appropriate, and the cells were incubated for 1 h at 37 °C and 7% CO_2. The cells were then washed four times with 50 μl of a buffer solution (135 mM NaCl, 4.6 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 11 mM glucose, 11 mM Hepes, 2.5 mM Probenecid, pH 7.4) before a final 45 μl volume of buffer was added to each well. An additional 5 μl of buffer solution, containing antibodies at 10 × the final concentration, was then re-

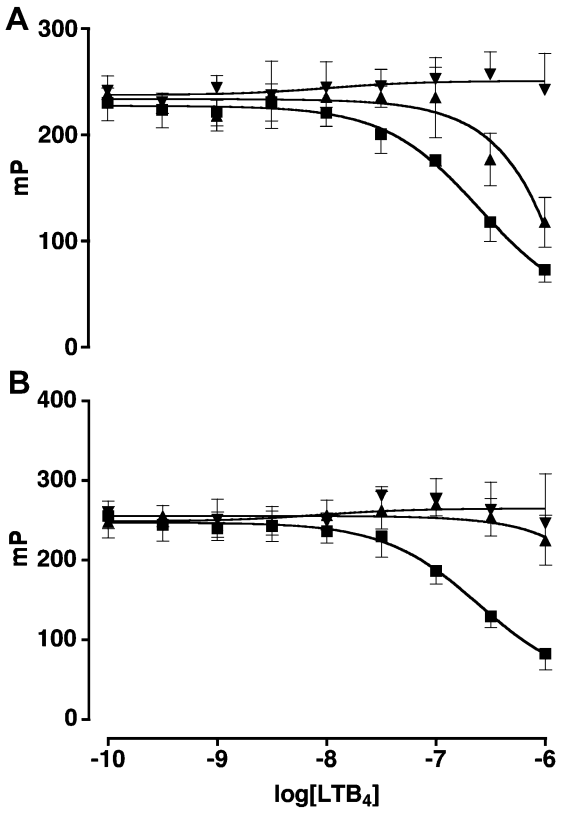

Fig. 2. Shown are BLT_1 antibodies (7B1 in A, 14F11 in B) in competition for LTB_4-FL binding, compared to their respective isotype controls and LTB_4. The anti-BLT_1 antibodies, their isotype controls or unlabelled LTB_4 were incubated with 0.2 μg/μl (10 μg) of membrane protein and 10 nM LTB_4-FL for 1 h before fluorescence polarisation was analysed. Results are shown as mean ± 95% CI from duplicate wells in three experiments. ■: LTB_4, ▲: anti-BLT_1 antibody, ▼: isotype control.

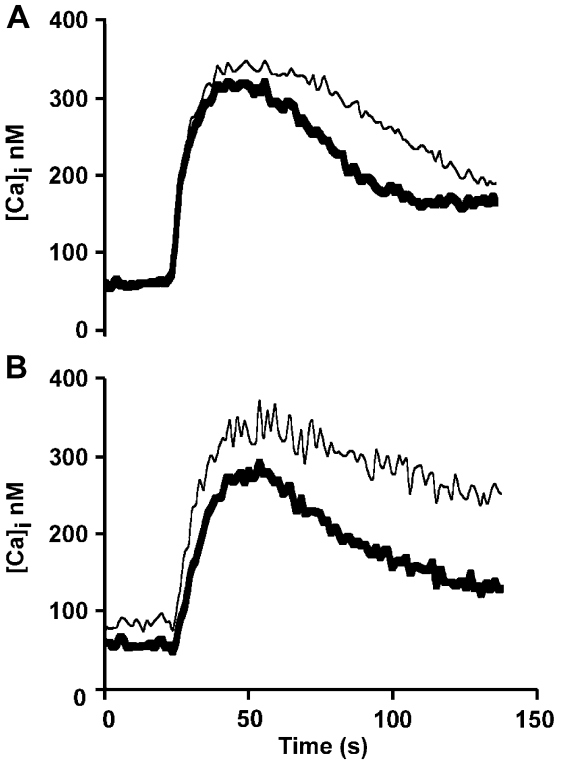

Fig. 3. Representative curves from four experiments in quadruplicate showing calcium release induced by challenge with 100 nM LTB_4 (thin lines) at 20 s following a 60-min pre-treatment with (A) 100 nM 7B1, (B) 100 nM 14F11 (thick lines).

added to every second well (results are reported as final concentrations). The remaining wells received 5 μl of control solutions, and the plates were incubated for another 5 min. Following pre-treatment, the plates were transferred to a fluorometer (Fluostar™, BMG Technologies), and 50 μl of buffer containing 200 nM LTB_4 or the appropriate antibody solution was injected into individual wells and the cells were monitored for the next 120 s. Control wells containing cells that had not been exposed to FURA-2 were used to subtract background auto-fluorescence. The ratio of emitted fluorescence at 520 nm (following stimulation by 340- and 380-nm light) was calculated following background subtraction at each wavelength.

The ratiometric data was converted to calcium concentrations according to Grynkiewicz et al. [16], following a calibration based on Williams and Fay [17] using a kit designed for this purpose from

Molecular Probes. Cytoplasmic loading of HF1pBLTR1 cells with FURA-2AM was optimised using fluorescent microscopic analysis of loaded cells [18]. Results are shown as representative curves from each experiment. Sample group sizes are given as n which refers to the number of pairs of control and treatment wells (e.g. $n=2$ is four wells).

Calcium concentrations were recorded for 20 s before the cells were exposed to 100 nM LTB_4 (final concentration). Responses from cells incubated with control solutions are shown as thin lines.

2.9. Luciferase assay of BLT_1 activity

The assay for agonist-induced luciferase production was performed according to Kotarsky et al. [12]. Briefly, HF1pBLTR1 cells were seeded as above into white, clear bottomed 96-well plates (Corning Costar) and grown until they were 80–90% confluent. PBS solutions containing LTB_4 with or without antibodies were then added (in a volume not exceeding 10 μl) to the wells (results are reported as final ligand or antibody concentrations). Following a further 16 h of incubation, the cells were washed once with PBS and lysed. Luciferase activity was measured using a 96-well luminometer (Lumistar™, BMG). The half-maximum effective ligand concentration (EC_{50}) was determined from the luciferase assay concentration–response curves using nonlinear regression (Graph Pad, Prism). Results are shown as amplification of luciferase activity (ALA) which is calculated by dividing the response from treated

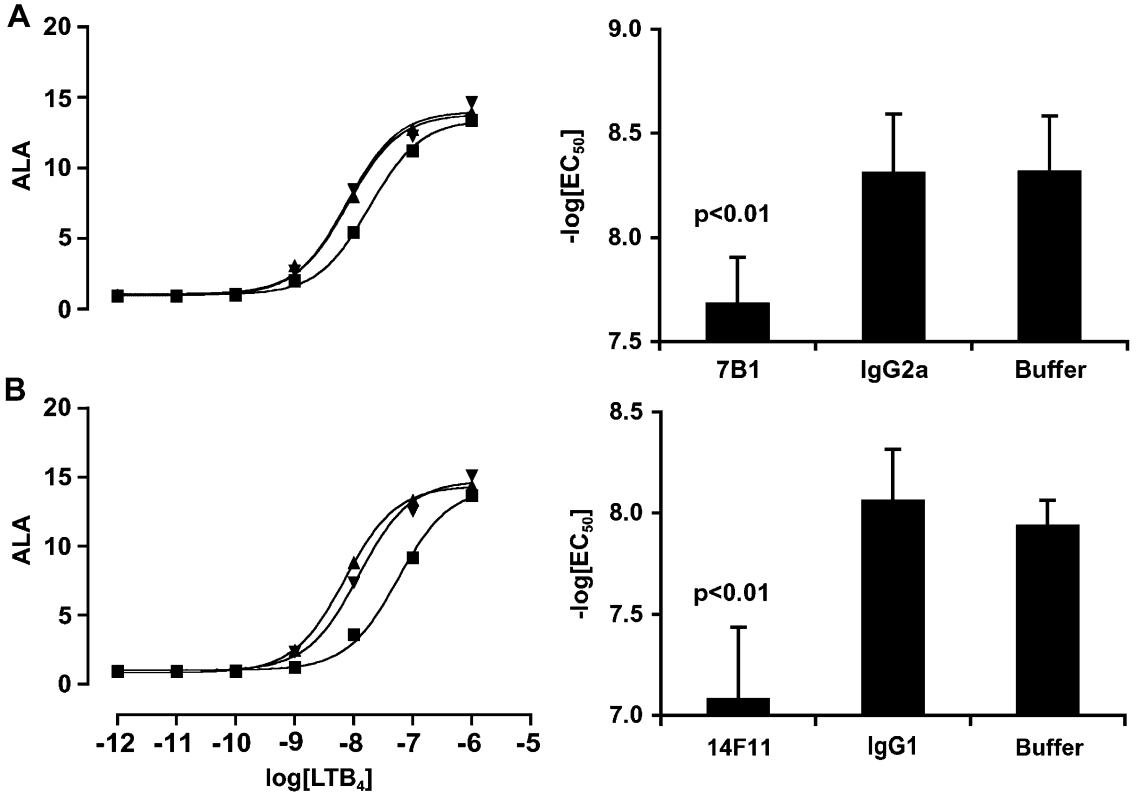

Fig. 4. Representative concentration curves showing luciferase activity induced by challenge with 100 nM LTB_4 following a 60-min pretreatment with (A) 100 nM 7B1, $n=12$, (B) 100 nM 14F11, $n=9$. ▼: LTB_4 only, ▲: LTB_4 and appropriate isotype control antibody, ■: LTB_4 and 7B1 or 14F11. The entire data set is depicted on the right as mean EC_{50} values for LTB_4, with and without (buffer control) antibodies. Variation is shown as 95% CI.

wells by the background, obtained from wells receiving control solutions.

2.10. Chemotaxis using blood polymorphonuclear cells (PMNs)

The chemotactic procedure followed Frevert et al. [19] with minor modifications. Samples of venous blood taken from healthy donors were drawn into EDTA tubes and mixed with dextran to a final concentration of 0.6%. Following a 30-min room temperature incubation the leukocyte-rich fraction was removed, and the PMNs were pelleted by layering this fraction onto a lymphoprep density gradient (Nycomed) and centrifuging at 1000 rpm for 30 min. Remaining red blood cells were removed from the pellet using a short hypotonic shock before the cells were washed and re-suspended in RPMI (Gibco). Calcein-AM (Molecular Probes) and appropriate solutions of antibodies were then added to final concentrations of 1 μM and 100 nM, respectively, and the cells were incubated for and additional 60 min at room temperature. The cells were then washed twice in PBS-Dulbecco's (Gibco) and resuspended to a final concentration of 4.0×10^6 cells/ml. The cells were then added to the upper surface of a polycarbonate filter with 8 μm pores (ChemoTX™, Neuroprobe), and the wells below the filter were filled with appropriate doses of LTB_4 and, antibodies at a final concentration of 100 nM. Following a 1-h incubation at 37 °C, the fluorescence intensity of the wells below the filters was analysed using a fluorometer (Fluorostar™, BMG). Cell migration was defined as a fraction of the maximum possible fluorescence (cells added directly to bottom well) following correction for nonspecific cell migration and autofluorescence. EC_{50} values were calculated from the LTB_4 concentration–response curves, as above.

Calcein loading and cell viability was greater than 99% as confirmed by fluorescence microscopy and trypan blue exclusion respectively. Following each experiment, the isolated cells were fixed and subjected to histologic examination after staining with May-Grünwald–Giemsa. Cell populations averaged 93% neutrophils, 5.5% eosinophils and 1.5% other cell types.

3. Results

We have examined how two monoclonal mouse anti-human BLT_1 antibodies previously raised in our laboratory affect leukotriene B_4 (LTB_4) binding to BLT_1 and subsequent receptor activation. We found that only one of the antibodies, 7B1, inhibited ligand binding to the receptor. BLT_1 receptor peptide fragments did not inhibit antibody labelling of cell surface BLT_1 receptors, and the antibodies did not recognise the synthetic fragments. However, both antibodies inhibited the LTB_4-induced activation of second messenger systems by BLT_1, such as intracellular calcium mobilisation and MAP kinase activation. Finally, the chemotactic movement of human polymorphonucleocytes ex vivo was strongly inhibited following pretreatment with anti-BLT_1 antibodies.

We used a fluorescent variant of LTB_4 (LTB_4-FL) to study antibody binding to BLT_1 by allowing the

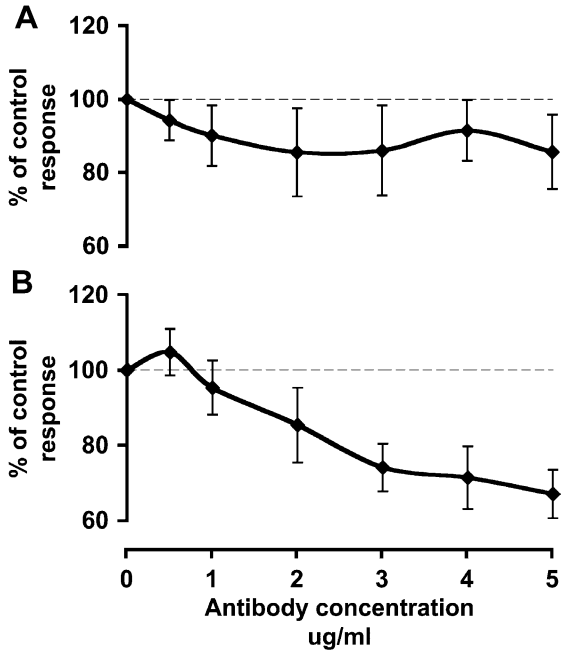

Fig. 5. Increasing antibody concentration inhibits HF1pBLTR1 luciferase production stimulated by 100 nM LTB_4. (A) 7B1, (B) 14F11. Mean responses from three experiments in quadruplicate ($n = 12$) are shown, relative to wells receiving appropriate isotype control antibodies. Errors are shown as 95% CI. Concentrations of 1 to 5 μg/ml correspond to approximately 7 to 35 nM. Antibodies were incubated with the cells for 60 min prior to LTB_4 stimulation.

antibodies to compete for LTB_4-FL binding sites and measuring subsequent changes in LTB_4-FL polarisation. Only antibody 7B1 could inhibit LTB_4-FL binding to membranes prepared from HF1pBLTR1 cells (Fig. 2) with a K_i of 6.6 (0.3–140) nM (mean and 95% CI from six samples). LTB_4-FL binding was not inhibited by antibody 14F11 or appropriate isotype controls, but native LTB_4 could displace LTB_4-FL with a K_i of 0.8 (0.6–1.2) nM.

In an effort to define which portions of the BLT_1 receptor protein form the epitopes bound by the antibodies, we used two strategies involving cyclic fragments of the receptor peptide (Fig. 1). Cyclic fragments were used in an effort to simulate the conformation the fragments would normally have as part of the receptor protein [20]. In repeated experiments, neither 7B1 nor 14F11 bound to peptide fragments immobilised on plastic either individually, or as a mixture. Control experiments using the same technique with the peptide fragments and sera from immunised mice were successful however. Furthermore, the peptide fragments did not inhibit antibody labelling of cell surface BLT_1 receptors, measured using flow cytometry, indicating that the cyclic peptides could not compete with BLT_1-receptors for antibody binding sites (results not shown).

Both of the antibodies tested reduced the activity of second messenger systems following BLT_1 activation by LTB_4. BLT_1-mediated calcium release was inhibited following exposure to these antibodies (Fig. 3), but not by isotype controls. Using a luciferase reporter system as an indicator of MAP kinase activity, we found that the EC_{50} for LTB_4 increased 4-fold in the presence of 7B1 and 10-fold in the presence of

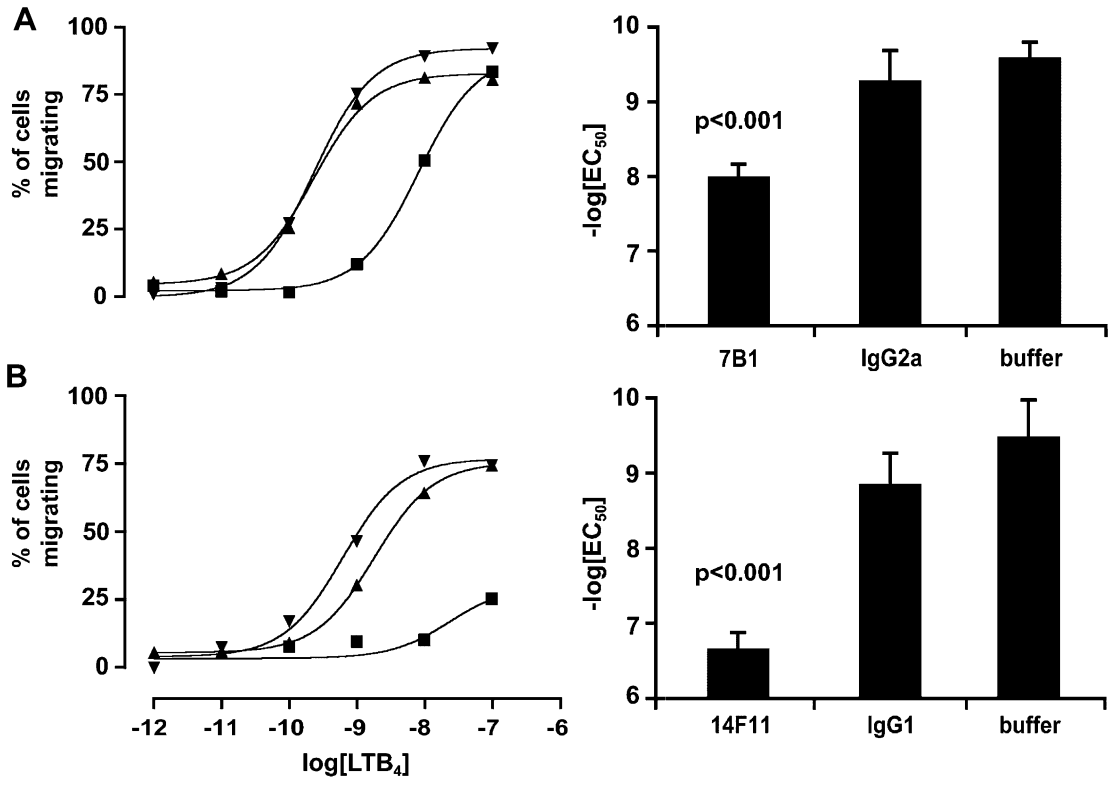

Fig. 6. Representative concentration curves showing neutrophil chemotactic activity induced by challenge with 100 nM LTB_4 following a 60-min pre-treatment with (A) 100 nM 7B1, $n=9$, (B) 100 nM 14F11, $n=12$. ▼: LTB_4 only, ▲: LTB_4 and appropriate isotype control antibody, ■: LTB_4 and 7B1 or 14F11. The entire data set is depicted on the right as mean EC_{50} values for LTB_4, with and without (buffer control) antibodies. Variation is shown as 95% CI.

14F11 (Fig. 4), but no increases were observed in the presence of isotype controls. Using a range of antibody concentrations commonly employed to label surface receptors, we also observed that for 14F11, the level of inhibition was also dependent on the antibody concentration (Fig. 5), although this relationship was less clear for 7B1. To ensure that the BLT_1-binding antibodies did not activate BLT_1, we added each antibody (or their isotype controls), without LTB_4, to HF1pBLT1 cell cultures and observed no increases in intracellular calcium levels or reporter activity. Control studies using ATP to activate endogenously expressed P2Xn receptors on HeLa HF1 cells showed that neither anti-BLT_1 antibody could inhibit functional responses (calcium release and MAP kinase activation) to other ligands (results not shown).

The antagonistic effects of the 7B1 and 14F11 were also evaluated using granulocytes that were first treated with the antibodies before they were exposed to an LTB_4 gradient and allowed to migrate across a membrane in a modified Boyden chamber. The efficacy of these antibodies in this more physiological setting was even greater than when using transfected cells or sensitive reporter assay systems. We found that the mean EC_{50} for LTB_4 measured using cell migration increased from an average of 0.5 nM (with control antibody) to 10 nM (a 20-fold change) and from 1.4 to 222 nM (a 150-fold change), for 7B1 and 14F11, respectively (Fig. 6). The BLT_1 antibodies did not induce cell migration in absence of LTB_4, and their isotype controls did not inhibit responses to LTB_4. Neutrophil chemotaxis towards *N*-formylpeptide (fMLP) was unaffected (results not shown).

4. Discussion

We have examined how two different monoclonal antibodies (7B1 and 14F11) binding specifically to the LTB_4-receptor BLT_1 [1] can affect agonist binding and receptor activation. Using a transgenic HeLa cell line expressing BLT_1, we found that only one antibody (7B1) could specifically inhibit ligand binding, while both could inhibit receptor activation, and chemotactic movement towards LTB_4. Unlike previous work with anti-BLT_1 receptor antibodies that were found to both activate BLT_1 and inhibit LTB_4 binding [11], our antibodies are pure antagonists and bind to BLT_1 without activating it.

Our two BLT_1 antibodies were both produced by immunizing mice with whole cells expressing BLT_1 before producing hybridoma clones using spleen cells taken from the mice. These hybridomas were in turn screened for reactivity against BLT_1 [1]. The two antibodies are different isotypes and presumably recognise different portions of the receptor protein exposed on the cell surface. This is reflected by differences in their ability to prevent LTB_4 binding and receptor activation. While only 7B1 inhibited both ligand binding and receptor activation, 14F11 prevented receptor activation more potently than 7B1 without affecting LTB_4 binding.

Our attempts to determine which epitopes on the receptor protein are important for antibody binding were unsuccessful. The antibodies probably recognise complex but specific structures in the folded, membrane bound, receptor protein that cannot be emulated using protein fragments. In support of this hypothesis, neither of these antibodies was found to be compatible with Western blotting techniques, which involve denatured BLT_1 proteins.

7B1 interferes with ligand binding, and both allosteric and orthosteric interactions may be important. Even simple steric hindrance may be involved. 14F11 does not interact with the ligand binding site however. This antibody probably binds to the extracellular portions of the receptor in a way that impedes the conformational changes necessary for receptor activation, but not ligand binding. 14F11 will therefore be a valuable tool for studying the mechanics of BLT_1 activation because it functions as a noncompetitive antagonist. This complements currently available BLT_1-specific ligands, such as the partial agonist U75302, and, the antagonist CP-105696, which was originally reported to be noncompetitive, but appears to be competitive in preparations expressing BLT_1 exclusively [21].

The antibodies were found to be more potent antagonists when they were studied using the endogenous BLT_1 receptors expressed on granulocytes. A 9-fold increase in LTB_4 concentration was necessary to overcome 14F11 antagonism in a HeLa cell reporter assay for MAP kinase activity, but a 150-fold increase was necessary when using a granulocyte chemotactic assay. The chemotaxis assay is more sensitive, but this

does not explain the increased potency of the antibodies as antagonists. There may be several ways to explain these observations. The antibodies recognise specific antigenic epitopes and if the receptor protein conformation changes so as to alter those epitopes, the antibodies will no longer bind to the receptor, even if the receptor is accessible on the cell surface. Receptor modifications, such as phosphorylation, could conceivably affect the receptor protein conformation, even if the intracellular portion of the receptor was modified, but this does not appear to be the case for BLT_1 [22]. Partitioning of the receptor into different membrane microenvironments could affect antibody-binding epitopes [23]. Different rates of receptor protein circulation between the cellular membrane and internal organelles could also be important, although there is evidence that this is not the case, at least in granulocytes [24]. Differential expression of G proteins can affect ligand binding to BLT_1, and this may also offer an explanation for the differences observed [25]. It is possible that when LTB_4 and one of the antibodies binds to BLT_1, receptor conformations are induced which are more or less compatible with the various signalling pathways involved in the different functional experiments presented in the present study. This hypothesis is not directly supported by our data however, and experiments designed to address this question will have to be performed before any conclusions can be drawn.

The two antibodies (7B1 and 14F11) will be important for investigating BLT_1 pharmacology because although they both function as antagonists, only 7B1 interferes with LTB_4 binding. Further investigation of how 14F11 binds to BLT_1 will provide important information regarding the conformational changes that follow agonist binding because this antibody inhibits only receptor activation, not ligand docking.

References

[1] Pettersson A, Boketoft A, Sabirsh A, Nilsson NE, Kotarsky K, Olde B, et al. First-generation monoclonal antibodies identifying the human leukotriene B(4) receptor-1. Biochem Biophys Res Commun 2000;279(2):520–5.

[2] Dasari VR, Jin J, Kunapuli SP. Distribution of leukotriene B4 receptors in human hematopoietic cells. Immunopharmacology 2000;48(2):157–63.

[3] Yokomizo T, Izumi T, Shimizu T. Co-expression of two LTB4 receptors in human mononuclear cells. Life Sci 2001; 68(19–20):2207–12.

[4] Fretland DJ, Gokhale R, Mathur L, Baron DA, Paulson SK, Stolzenbach J. Dermal inflammation in primates, mice, and guinea pigs: attenuation by second-generation leukotriene B4 receptor antagonist, SC-53228. Inflammation 1995;19(3): 333–46.

[5] Griffiths RJ, Pettipher ER, Koch K, Farrell CA, Breslow R, Conklyn MJ, et al. Leukotriene B4 plays a critical role in the progression of collagen-induced arthritis. Proc Natl Acad Sci U S A 1995;92(2):517–21.

[6] Tager AM, Dufour JH, Goodarzi K, Bercury SD, von Andrian UH, Luster AD. BLTR mediates leukotriene B(4)-induced chemotaxis and adhesion and plays a dominant role in eosinophil accumulation in a murine model of peritonitis. J Exp Med 2000;192(3):439–46.

[7] Aiello RJ, Bourassa PA, Lindsey S, Weng W, Freeman A, Showell HJ. Leukotriene B4 receptor antagonism reduces monocytic foam cells in mice. Arterioscler Thromb Vasc Biol 2002;22(3):443–9.

[8] Hennig R, Ding XZ, Tong WG, Schneider MB, Standop J, Friess H, et al. 5-Lipoxygenase and leukotriene B(4) receptor are expressed in human pancreatic cancers but not in pancreatic ducts in normal tissue. Am J Pathol 2002;161(2): 421–8.

[9] Tong WG, Ding XZ, Hennig R, Witt RC, Standop J, Pour PM, et al. Leukotriene B4 receptor antagonist LY293111 inhibits proliferation and induces apoptosis in human pancreatic cancer cells. Clin Cancer Res 2002;8(10):3232–42.

[10] Harvey JP, Koo CH, Boggs JM, Young RN, Goetzl EJ. Mouse monoclonal antibody to a latent epitope of leucocyte receptors for leukotriene B4. Immunology 1992;76(1): 122–8.

[11] Gifford LA, Chernov-Rogan T, Harvey JP, Koo CH, Goldman DW, Goetzl EJ. Recognition of human polymorphonuclear leukocyte receptors for leukotriene B4 by rabbit anti-idiotypic antibodies to a mouse monoclonal antileukotriene B4. J Immunol 1987;138(4):1184–9.

[12] Kotarsky K, Owman C, Olde B. A chimeric reporter gene allowing for clone selection and high-throughput screening of reporter cell lines expressing G-protein-coupled receptors. Anal Biochem 2001;288(2):209–15.

[13] Harris LJ, Skaletsky E, McPherson A. Crystallographic structure of an intact IgG1 monoclonal antibody. J Mol Biol 1998; 275(5):861–72.

[14] Harris LJ, Larson SB, Hasel KW, McPherson A. Refined structure of an intact IgG2a monoclonal antibody. Biochemistry 1997;36(7):1581–97.

[15] Cheng Y, Prusoff WH. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 1973;22(23):3099–108.

[16] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985;260(6):3440–50.

[17] Williams DA, Fay FS. Intracellular calibration of the fluores-

cent calcium indicator Fura-2. Cell Calcium 1990;11(2–3): 75–83.
[18] Roe MW, Lemasters JJ, Herman B. Assessment of Fura-2 for measurements of cytosolic free calcium. Cell Calcium 1990; 11(2–3):63–73.
[19] Frevert CW, Wong VA, Goodman RB, Goodwin R, Martin TR. Rapid fluorescence-based measurement of neutrophil migration in vitro. J Immunol Methods 1998;213(1):41–52.
[20] Benjamin DC, Berzofsky JA, East IJ, Gurd FR, Hannum C, Leach SJ, et al. The antigenic structure of proteins: a reappraisal. Annu Rev Immunol 1984;2:67–101.
[21] Yokomizo T, Kato K, Hagiya H, Izumi T, Shimizu T. Hydroxyeicosanoids bind to and activate the low affinity leukotriene B4 receptor, BLT2. J Biol Chem 2001;276(15):12454–9.
[22] Chung-a-on KO, Soyombo O, Spur BW, Lee TH. Stimulation of protein kinase C redistribution and inhibition of leukotriene B4-induced inositol 1,4,5-trisphosphate generation in human neutrophils by lipoxin A4. Br J Pharmacol 1996;117(6): 1334–40.
[23] Nguyen DH, Taub D. Cholesterol is essential for macrophage inflammatory protein 1 beta binding and conformational integrity of CC chemokine receptor 5. Blood 2002;99(12): 4298–306.
[24] Boggs JM, Koo CH, Goetzl EJ. Down-regulation of receptor antigen in leukotriene B4-induced chemotactic deactivation of human polymorphonuclear leucocytes. Immunology 1991; 73(2):212–6.
[25] Masuda K, Itoh H, Sakihama T, Akiyama C, Takahashi K, Fukuda R, et al. A combinatorial G protein-coupled receptor reconstitution system on budded baculovirus: evidence for Gαi and Gαo coupling to a human leukotriene B4 receptor. J Biol Chem 2003;278(27):24552–62.
[26] Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, et al. Crystal structure of rhodopsin: a G protein-coupled receptor [see comments]. Science 2000;289(5480): 739–45.