



Involvement of cysteinyl leukotrienes in airway smooth muscle cell DNA synthesis after repeated allergen exposure in sensitized Brown Norway rats

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1 Airway smooth muscle thickening is a characteristic feature of airway wall remodelling in chronic asthma. We have investigated the role of the leukotrienes in airway smooth muscle (ASM) and epithelial cell DNA synthesis and ASM thickening following repeated allergen exposure in Brown Norway rats sensitized to ovalbumin.

2 There was a 3 fold increase in ASM cell DNA synthesis, as measured by percentage bromodeoxyuridine (BrdU) incorporation, in repeatedly ovalbumin-exposed (4.1%, 3.6–4.6; mean, 95% c.i.) compared to chronically saline-exposed rats (1.3%, 0.6–2.1; $P < 0.001$).

3 Treatment with a 5-lipoxygenase enzyme inhibitor (SB 210661, 10 mg kg⁻¹, p.o.) and a specific cysteinyl leukotriene (CysLT₁) receptor antagonist, pranlukast (SB 205312, 30 mg kg⁻¹, p.o.), both attenuated ASM cell DNA synthesis. Treatment with a specific leukotriene B₄ (BLT) receptor antagonist (SB 201146, 15 mg kg⁻¹, p.o.) had no effect.

4 There was also a significant, 2 fold increase in the number of epithelial cells incorporating BrdU per unit length of basement membrane after repeated allergen exposure. This response was not inhibited by treatment with SB 210661, pranlukast or SB 201146.

5 A significant increase in ASM thickness was identified following repeated allergen exposure and this response was attenuated significantly by SB 210661, pranlukast and SB 201146.

6 Rats exposed to chronic allergen exhibited bronchial hyperresponsiveness to acetylcholine and had significant eosinophil recruitment into the lungs. Treatment with SB 210661, pranlukast or SB 201146 significantly attenuated eosinophil recruitment into the lungs, whilst having no significant effect on airway hyperresponsiveness.

7 These data indicate that the cysteinyl leukotrienes are important mediators in allergen-induced ASM cell DNA synthesis in rats, while both LTB₄ and cysteinyl leukotrienes contribute to ASM thickening and eosinophil recruitment following repeated allergen exposure.

Keywords: Allergic reactions; cysteinyl leukotrienes; airway smooth muscle; DNA synthesis; eosinophils; airway remodelling

Abbreviations: ASM, airway smooth muscle; BLT, leukotriene B₄ receptor; BrdU, 5-bromo-2'-deoxyuridine; CysLT, cysteinyl leukotrienes; CysLT₁, cysteinyl leukotriene receptor 1; CysLT₂, cysteinyl leukotriene receptor 2; DAPI, 4,6-diamidino-2-phenylindole; PC₂₀₀, concentration of acetylcholine required to increase baseline airways resistance 200%

Introduction

Asthma is a disease characterized by chronic inflammation, recruitment of pro-inflammatory cells into the airways and airway wall remodelling. Airway remodelling is distinguished by a number of processes including increased airway smooth muscle mass (Dunnill *et al.*, 1969), epithelial cell disruption (Laitinen *et al.*, 1985) and sub-epithelial fibrosis (Roche *et al.*, 1989). Increased airway smooth muscle mass consists of both smooth muscle cell hyperplasia and hypertrophy (Heard & Hossain 1973; Ebina *et al.*, 1993), and may contribute to bronchial narrowing and airway hyperresponsiveness.

Little is known about the mediators that induce changes in airway wall structure *in vivo*, but there is evidence to suggest that cysteinyl leukotrienes may be important in this process. The cysteinyl leukotrienes (CysLT: LTC₄, LTD₄ and LTE₄) are

lipid mediators that are present at elevated concentrations in the bronchoalveolar lavage fluid of asthmatics (Lam *et al.*, 1988; Wardlaw *et al.*, 1989). They also cause potent contraction of airway smooth muscle (Dahlén *et al.*, 1980; Barnes *et al.*, 1984), increase microvascular leakage (Woodward *et al.*, 1983; Hua *et al.*, 1985), stimulate mucus hypersecretion (Marom *et al.*, 1982; Johnson & McNee, 1983) but decrease mucociliary clearance (Bisgaard & Pedersen 1987), and also promote eosinophil accumulation (Laitinen *et al.*, 1993; Spada *et al.*, 1994). LTD₄ also potentiates human airway smooth muscle cell proliferation *in vitro* (Panettieri *et al.*, 1998b).

In Brown Norway rats, as few as three allergen exposures have been reported to increase airway smooth muscle mass (Sapienza *et al.*, 1991). LTD₄ has been implicated in allergen-induced airway smooth muscle thickening following repeated challenge (Wang *et al.*, 1993) and cysteinyl leukotriene

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breakdown products have been demonstrated to be increased after allergen exposure in Brown Norway rats (Martin *et al.*, 1993). Increased rates of DNA synthesis in both airway smooth muscle cells and epithelial cells have been observed in models of chronic allergen exposure using sensitized Brown Norway rats (Salmon *et al.*, 1997; Panettieri *et al.*, 1998a).

In the current study, we have investigated the influence of the leukotrienes on bronchial responsiveness to acetylcholine, DNA synthesis in airway smooth muscle and epithelial cells, smooth muscle area and eosinophil recruitment in a repeated allergen exposure model. We have used a 5-lipoxygenase (5-LO; E.C. 1.13.11.34) inhibitor, SB 210661 (McLoughlin *et al.*, 1998), and a cysteinyl leukotriene (CysLT₁) receptor antagonist, pranlukast (SB 205312, ONO-1078) (Obata *et al.*, 1992; Fujiwara *et al.*, 1993; Taki *et al.*, 1994), in order to evaluate the role of CysLT in bronchial responsiveness and airway wall remodelling. In addition, the potential contribution of another 5-LO product, leukotriene B₄, in these processes has been studied using a specific LTB₄ (BLT) receptor antagonist, SB 201146 (Daines *et al.*, 1993, 1996).

Methods

Sensitization and challenges

Pathogen-free, male Brown Norway rats weighing 220–250 g (Harlan, Bicester, U.K.) were sensitized on days 1, 2 and 3 using 1 mg kg⁻¹ intraperitoneal injections of ovalbumin in 0.9% sterile saline containing 100 mg Al(OH)₃ as adjuvant. On days 6, 9, 12, 15, 18 and 21, animals were exposed to 1% ovalbumin aerosol for 20 min. Challenges took place in a 0.8 m³ chamber, with free-breathing animals exposed to either saline or a 1% ovalbumin aerosol mist produced by a DeVilbiss PulmoSonic nebulizer (DeVilbiss Health Care Ltd., Feltham, U.K.). The aerosol mist was pumped at a rate of 0.6 l min⁻¹ by a small animal ventilator. At all other times, rats were housed in a caging system receiving clean filtered air (Maximiser, Thorens Caging System Inc., Hazleton, PA, U.S.A.). Five groups of animals were studied in total (*n* = 6 in each group):

- group A: ovalbumin-sensitized, saline-challenged, vehicle-treated;
- group B: ovalbumin-sensitized, ovalbumin-challenged, vehicle-treated;
- group C: ovalbumin-sensitized, ovalbumin-challenged, SB 210661-treated;
- group D: ovalbumin-sensitized, ovalbumin-challenged, SB 201146-treated;
- group E: ovalbumin-sensitized, ovalbumin-challenged, pranlukast-treated.

Drug pre-treatments

All oral drug pre-treatments were given at a time prior to allergen challenges such that blood plasma levels were maximal or near-maximal at the time of the challenge. A second dose was then administered so that drug plasma levels were near-maximal approximately 7 h after the allergen challenge. The 5-LO inhibitor, SB 210661, was administered 1 h before allergen challenge at a dose of 10 mg kg⁻¹ (in 1 ml of a 1 : 1 solution of 10% polyethyleneglycol 200 and 1% methylcellulose). The BLT receptor antagonist, SB 201146, was administered 30 min before allergen challenge at a dose of 15 mg kg⁻¹ (in 1 ml of 0.9% saline). The CysLT₁ receptor antagonist, pranlukast, was administered 2 h before allergen challenge at a dose of

30 mg kg⁻¹ (in 1 ml of a 1 : 1 solution of 0.5 M NaOH and polyethyleneglycol 400). Vehicle-treated rats received 1 ml of the same vehicle at the same time points as those rats receiving drugs.

Bromodeoxyuridine dosing

5-Bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co., Poole, U.K.) was dissolved in dimethylsulphoxide (DMSO) and diluted with sterile water, giving a final concentration of DMSO <7%. Rats were injected intraperitoneally with 50 mg kg⁻¹ BrdU in 1 ml of solution immediately following each allergen challenge and received a second dose 8 h later (total of 12 injections).

Measurement of bronchial responsiveness to acetylcholine

Bronchial responsiveness was measured 18–24 h after the final allergen challenge as previously described (Elwood *et al.*, 1991). Briefly, rats were anaesthetized, tracheostomy performed and lung resistance calculated using a computer program (LabVIEW 2, National Instruments, Austin, TX, U.S.A.) by the method of von Neergard & Wirz (1927). Increasing half-log₁₀ concentrations of acetylcholine were administered by inhalation for 45 breaths and lung resistance was measured. Challenges were halted when lung resistance exceeded 200% of initial baseline values. The concentration of acetylcholine required to increase baseline resistance by 200% (PC₂₀₀) was determined by linear interpolation of log concentration-lung resistance curves.

Tissue collection

Lung resistance was allowed to return to baseline levels and then rats were killed using an overdose of sodium pentobarbitone (500 mg kg⁻¹; i.p.). The lungs were rapidly removed and insufflated with O.C.T. Tissue Tek[®] mounting medium (Raymond A. Lamb, London, U.K.) diluted 1 : 1 with phosphate-buffered saline. Regions of the left and right lung lobes were mounted on cork blocks with the main bronchi uppermost, snap-frozen in melting isopentane and stored at –25°C.

Bromodeoxyuridine and α -smooth muscle actin immunohistochemistry

Cryostat sections 5 μ m thick were cut and thaw-mounted onto glass slides treated with Vectabond (Vector Laboratories, Peterborough, U.K.). Left lung blocks were cut between the first and second division of the main bronchi, at an angle perpendicular and lateral to the conducting airways. Tissue sections were fixed in a 1 : 1 mixture of acetone and methanol for 10 min at 12°C and endogenous peroxidase was blocked by immersing sections in methanol containing 0.3% (v v⁻¹) hydrogen peroxide for 20 min. A primary anti-BrdU monoclonal antibody (mAb) solution (clone BU-1; Amersham International, Buckinghamshire, U.K.) was applied at 37°C for 75 min. A secondary biotinylated rat adsorbed antiserum to mouse IgG (Vector Laboratories) was then applied followed by a 45-min incubation with a peroxidase-linked avidin-biotin complex solution (ABC-Elite kit, Vector Laboratories). BrdU-positive cells were visualized using 3,3'-diaminobenzidine-tetrachloride solution (Sigma) with glucose oxidase-nickel enhancement to give a black end-product (Shu *et al.*, 1988). Sections were then rinsed and a primary anti- α -smooth muscle

actin mAb (clone 1A4; Sigma) was applied for 1 h at room temperature. A secondary biotinylated rat adsorbed antiserum to mouse IgG (Vector Laboratories) was applied to the sections, followed by an avidin-biotin complex reagent conjugated to alkaline phosphatase (Vector Laboratories).

The α -smooth muscle actin staining was visualized using Sigma *FAST* (4chloro-2-methylbenzenediazonium/3-hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate (α -naphthol AS-MX) and Fast Red TR) in Tris buffer (Sigma) to give a red end-product. Nuclei that were not immunoreactive for BrdU were counterstained by application of the fluorescent DNA ligand 4,6-diamidino-2-phenylindole hydrochloride (DAPI; Sigma) at a concentration of 0.00001% and mounted under glass coverslips.

Tissue sections of spleen, colon and thymus were used to confirm positive BrdU immunoreactivity. Negative controls were performed on sections of lung for BrdU and α -smooth muscle actin with antibodies of the same immunoglobulin class or in the absence of primary antibody.

Eosinophil major basic protein immunohistochemistry and counting

Sections were fixed in acetone and then incubated with a mAb against human major basic protein (MBP; clone BMK-13; Monosan, Uden, The Netherlands) at a concentration of 1:80 for 1 h at room temperature for detection of rat eosinophils. After labelling with a biotinylated horse anti-mouse mAb, positive cells were visualized by using an avidin-biotin complex reagent conjugated to alkaline phosphatase (Vector Laboratories). Eosinophils were visualized using Sigma *FAST* in Tris buffer, with positive cells appearing red. Sections were counterstained with haematoxylin (BDH, Lutterworth, U.K.) and mounted under glass coverslips. Eosinophil recruitment around the airways and in parenchyma was evaluated. Eosinophil influx around the five largest airways in each lung section was assessed as the number of positive cells in the airway wall (consisting of epithelium, submucosa, smooth muscle and lamina propria), and expressed per millimetre of basement membrane length. Counts in parenchyma were assessed as the number of positive cells in five randomly selected low power fields.

Quantification of DNA synthesis and airway smooth muscle area using computer-assisted image analysis

Quantification of images was performed using a Zeiss microscope fitted for both transmitted light and fluorescence imaging, with images captured using a monochrome camera at maximum sensitivity, and analysed using an image analysis system (Sonata model, Seescan, Cambridge, U.K.).

The field of interest containing the whole airway was visualized with a $\times 10$ objective using DAPI fluorescence and converted to a monochrome image. The airway of interest was identified and internal perimeter, cross-sectional area and breadth (feret diameter) measured. The airway was then visualized using a $\times 20$ objective, the transmitted light image containing BrdU-positive cells captured and converted to a monochrome image. Without moving the section, a red fluorescence image of the alkaline phosphatase-Fast Red labelled α -smooth muscle actin immunoreactivity was captured. A blue fluorescence image for DAPI-positive nuclei was then captured and all three images were converted to stored monochrome images. The area of α -smooth muscle actin immunoreactivity was measured and then a mask created of this region which was overlaid onto the transmitted light image

of the same area and the number of BrdU-positively stained nuclei was counted. The mask was then overlaid onto the DAPI fluorescent image and the number of nuclei counted. DNA synthesis in epithelium was measured in a similar way by creation of an epithelial mask by interactive delineation using the DAPI-fluorescence image, and then counting the number of BrdU-positive nuclei within the mask and measuring basement membrane length.

DNA synthesis in airway smooth muscle cells was measured as the number of BrdU-immunoreactive nuclei divided by the total number of nuclei (BrdU plus DAPI nuclei) within the α -smooth muscle actin-defined immunoreactive area. Epithelial DNA synthesis was measured as the number of BrdU-positive cells per unit length of basement membrane within the DAPI-defined epithelial mask. Airway smooth muscle thickness was measured as the total α -smooth muscle actin immunoreactive area around each airway per unit length of basement membrane.

Airway number and section variability

BrdU indices were measured from the five largest conducting airways in a section of lung tissue cut perpendicular and lateral to the plane of the airways between the first and second division of the main bronchi. In preliminary experiments, the airway smooth muscle BrdU index was measured in the five largest airways from five consecutive lung tissue sections from a sensitized and ovalbumin-challenged rat. The coefficient of variation for BrdU index decreased with increasing section number, but the differences were small, with a standard error of less than $\pm 15\%$ of the mean index when comparing one against five consecutive sections. BrdU indices in this study were therefore calculated from the five largest airways from a single lung section for each rat in all treatment groups. All counts in this study were performed with the investigator blinded to treatment group.

Analysis of data

All data are expressed as the arithmetic mean index derived from the five largest airways in one section of lung from each rat. Mean indices were statistically analysed after logarithmic transformation by one-way analysis of variance (ANOVA), followed by *t*-tests with Bonferroni correction used to evaluate significant differences between groups. Values are expressed as means with 95% confidence intervals, with *P* values of < 0.05 considered significant.

Results

Bronchial responsiveness to acetylcholine

There was no significant difference in the baseline lung resistance (R_L) values following saline challenge among the five groups of rats. There was a significant increase in bronchial responsiveness to acetylcholine in the sensitized and repeatedly allergen-exposed rats (PC_{200} : 5.62 mM, 3.89–8.13) (mean, 95% c.i.) compared to sensitized and repeated-saline exposed rats (PC_{200} : 17.3 mM, 10.2–29.5; $P < 0.01$). There was no significant attenuation of this increased bronchial responsiveness in rats treated with either SB 210661 or SB 201146. There was a trend towards inhibition of bronchial responsiveness in the pranlukast-treated rats compared to the repeatedly allergen- and vehicle-treated group, but this was not significant (Figure 1).

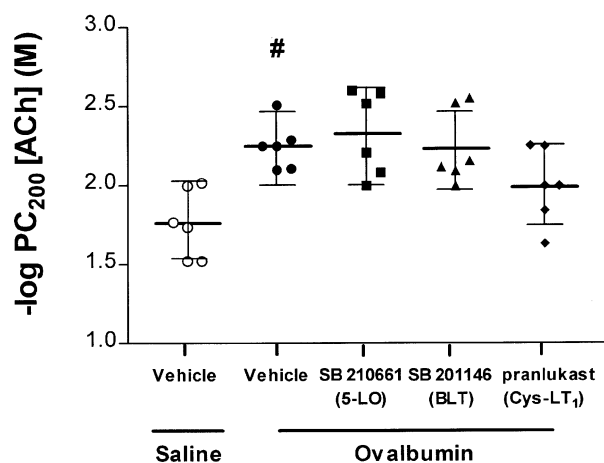


Figure 1 Bronchial responsiveness to acetylcholine in sensitized Brown Norway rats exposed repeatedly to saline or ovalbumin. There was a half-log order decrease in the mean $-\log PC_{200}$ value in repeatedly allergen-exposed rats compared to the saline-exposed group ($\#P < 0.05$). Following repeated allergen exposure, treatment with SB 210661, SB 201146 or pranlukast caused no significant attenuation of mean $-\log PC_{200}$ values compared to vehicle-treated rats. Data are expressed as mean $-\log PC_{200}$ values with error bars representing 95% confidence intervals.

Airway smooth muscle and epithelial cell DNA synthesis

The BrdU index of airway smooth muscle cells in the sensitized and repeatedly saline-challenged rats was 1.3% (0.6–2.1) and this was increased to 4.1% (3.6–4.6) in sensitized and repeatedly allergen-exposed rats ($P < 0.001$). Treatment with the 5-LO inhibitor SB 210661 (2.0%, 1.3–2.7; $P < 0.01$) or the CysLT₁ receptor antagonist pranlukast (2.1%, 1.5–2.7; $P \geq 0.05$) attenuated airway smooth muscle cell DNA synthesis. The BLT receptor antagonist SB 201146 did not, however, significantly inhibit airway smooth muscle cell DNA synthesis (Figure 2A).

There was a greater than 2 fold increase in epithelial cell DNA synthesis in sensitized and repeatedly allergen-exposed rats, with 13.5 BrdU-positive cells per mm basement membrane (9.3–17.7) compared to 6.6 (2.5–10.6) in sensitized and repeatedly saline-exposed rats ($P < 0.05$). Treatment with any of the three drugs did not cause significant inhibition of epithelial cell DNA synthesis (Figure 2B).

Airway smooth muscle area and cell number

In sensitized rats, repeated allergen challenge significantly increased airway smooth muscle area compared to repeated saline challenge. The saline-challenged group had a mean airway smooth muscle thickness (defined as ASM area/basement membrane length) of 19.3 μm per mm basement membrane (17.5–21.2), compared to 25.9 μm (19.7–32.1) in the allergen-challenged group ($P < 0.05$). There was a significant attenuation of the increased ASM thickness with SB 210661 (17.9, 15.0–20.8; $P < 0.05$), SB 201146 (16.6, 13.9–19.3; $P < 0.01$) and pranlukast (19.0, 13.1–24.9; $P < 0.05$) compared to vehicle-treated and repeatedly allergen-exposed rats (Figure 3A). The total number of airway smooth muscle cells was counted to determine if increases in ASM area were due to an increase in the overall number of cells (i.e. ASM cell hyperplasia). Repeatedly allergen-exposed rats had more smooth muscle nuclei per mm basement membrane, with 82.6 (57.9–107.3) compared to 64.9 (47.9–82.0) in the saline-

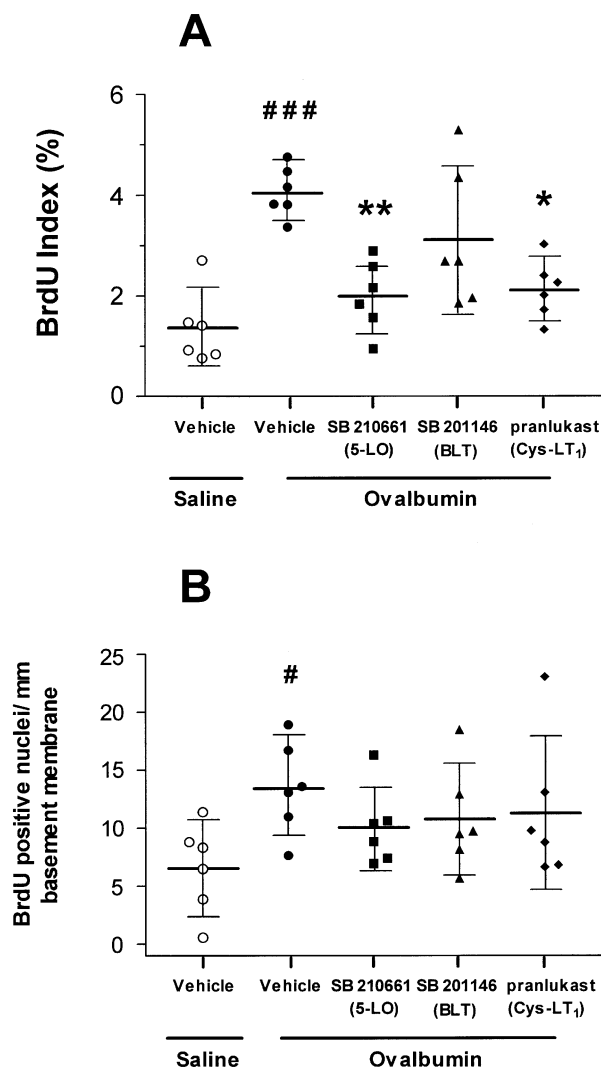


Figure 2 Effects of SB 210661, SB 201146 and pranlukast on airway smooth muscle cell (A) and epithelial cell (B) bromodeoxyuridine (BrdU) incorporation following repeated allergen challenge. (A) There was a greater than 3 fold increase in mean BrdU index of the airway smooth muscle cells of repeated allergen compared to repeated saline-exposed rats (### $P < 0.001$). Treatment with SB 210661 and pranlukast but not SB 201146 caused significant attenuation of airway smooth muscle BrdU index compared to vehicle-treated rats ($*P < 0.05$, $**P < 0.01$). (B) There was a greater than 2 fold increase in epithelial cell BrdU incorporation of repeated allergen compared to repeated saline-exposed rats ($\#P < 0.05$). Treatment with any of the three drugs did not cause an attenuation in epithelial cell BrdU incorporation. Horizontal bars are the mean values for each group of rats with error bars representing the 95% confidence intervals.

exposed group, but this increase was not significant (Figure 3B).

Eosinophils in the airway wall and parenchyma

Sensitized and repeatedly allergen-exposed rats had significantly more eosinophils in the airways, with 26.9 (19.7–34.2) MBP-positive cells per mm basement membrane compared to 4.5 (1.6–7.4) in the sensitized and repeatedly saline-exposed ($P < 0.001$). There was a significant reduction in eosinophil numbers around the airways following drug treatment with SB 210661 (14.9, 12.2–17.6; $P < 0.001$), SB 201146 (17.4, 14.2–20.7; $P < 0.01$) or pranlukast (12.4, 7.0–17.8; $P < 0.001$) compared to vehicle-treated rats (Figure 4A). The trend in

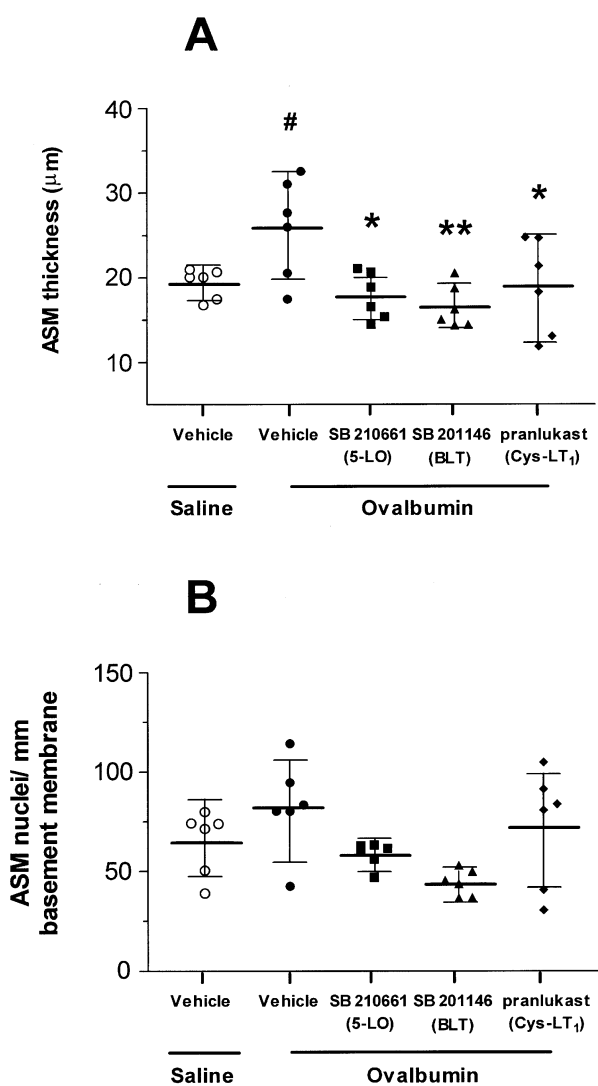


Figure 3 Effect of SB 210661, SB 201146 and pranlukast on airway smooth muscle thickness (A) and cell number (B). (A) Following repeated allergen exposure there was a significant increase in airway smooth muscle thickness per unit length of basement membrane compared to the repeated saline-exposed group ($\#P < 0.05$). Treatment with SB 210661, SB 201146 or pranlukast caused a significant reduction in ASM thickness compared to the vehicle-treated group ($*P < 0.05$, $**P < 0.01$). (B) There was no significant increase in the total number of ASM nuclei per mm of basement membrane in the repeated-allergen compared to the repeated saline-exposed group of rats.

eosinophil migration in parenchyma was similar to that observed around the airways. The sensitized and chronically saline-exposed rats had a mean of 23.2 MBP-positive cells (10.1–36.3) in five low-power microscope fields, compared to 74.0 (44.1–103.9) in the chronically allergen-exposed group ($P < 0.01$). Again, significant attenuation of eosinophil recruitment was observed following treatment with all three drugs (Figure 4B).

Discussion

Following repeated allergen exposure of sensitized Brown Norway rats we detected significant increases in both airway smooth muscle and epithelial cell DNA synthesis, as measured by BrdU incorporation, compared to saline-exposed controls. Treatment of sensitized and repeatedly allergen-exposed rats

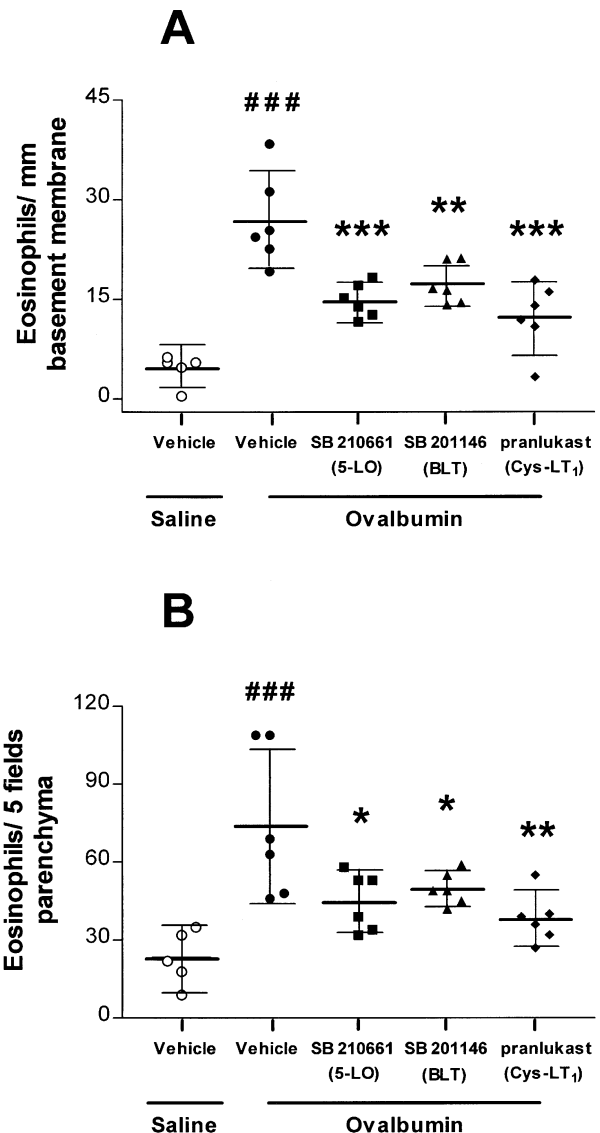


Figure 4 Effect of SB 210661, SB 201146 and pranlukast on eosinophil recruitment to the airways (A) and parenchyma (B). (A) There was a significant number of MBP-positive eosinophils around the airways following repeated allergen exposure of rats compared to repeated saline exposure ($###P < 0.001$). Following treatment with SB 210661, SB 201146 or pranlukast there was a significant attenuation of eosinophil recruitment to the airways compared to the vehicle-treated group ($**P < 0.01$, $***P < 0.001$). (B) There was also a significant increase in MBP-positive eosinophil recruitment into the parenchyma of repeatedly allergen-exposed compared to repeatedly saline-exposed rats ($###P < 0.001$). Treatment with SB 210661, SB 201146 or SB 205312 caused a significant attenuation of eosinophil recruitment compared to vehicle-treated rats ($*P < 0.05$, $**P < 0.01$).

with the 5-LO inhibitor SB 210661 and the CysLT₁ receptor antagonist SB 205312 (pranlukast) significantly attenuated airway smooth muscle cell DNA synthesis whereas the BLT receptor antagonist SB 201146 was without effect. These data indicate that airway smooth muscle cell DNA synthesis was attenuated by blocking the production or action of cysteinyl leukotrienes. In contrast, increased epithelial cell DNA synthesis following chronic allergen exposure was not inhibited by the 5-LO inhibitor nor by either of the receptor antagonists, indicating that factors other than leukotrienes are involved in this response.

The increase in airway smooth muscle cell DNA synthesis following repeated allergen exposure was accompanied by a

significant increase in airway smooth muscle thickness. Increased ASM thickness may be due to ASM cell hyperplasia or hypertrophy, or a combination of both these events (Heard & Hossain 1973; Ebina *et al.*, 1993). Leukotriene-enhanced ASM cell proliferation may contribute to this increased ASM thickening. Treatment with the 5-LO inhibitor or either the BLT or CysLT₁ receptor antagonist significantly attenuated allergen-induced increases in ASM thickness to control levels. This indicates that leukotrienes may be important mediators in allergen-induced ASM thickening.

Eosinophil recruitment into the lungs has been reported following both single and repeated allergen exposure in sensitized Brown Norway rats (Elwood *et al.*, 1991). In the present study, eosinophil recruitment was increased both in the airways and in parenchyma of repeatedly allergen-exposed rats. There was an attenuation of eosinophil recruitment to the airways and parenchyma after treatment with the 5-LO inhibitor (SB 210661), and BLT (SB 201146) or CysLT₁ (pranlukast) receptor antagonists. The cysteinyl leukotrienes LTD₄ and LTE₄ stimulate human eosinophil chemotaxis *in vitro* (Spada *et al.*, 1994), and stimulate the recruitment of pulmonary eosinophils in humans (Laitinen *et al.*, 1993; Diamant *et al.*, 1997). Further evidence in guinea-pigs indicates that this chemotactic role of cysteinyl leukotrienes for eosinophils is mediated *via* an interleukin-5 dependent mechanism (Underwood *et al.*, 1996). Our data suggest that LTB₄ may also be involved in eosinophil recruitment. This concept is supported by previous *in vivo* studies in guinea-pigs in which LTB₄ administered directly to the lungs induces pulmonary eosinophilia (Silbaugh *et al.*, 1987). Treatment with a BLT receptor antagonist, U-75302, has been demonstrated to attenuate allergen-induced eosinophil recruitment (Richards *et al.*, 1989). Therefore, both cysteinyl leukotrienes and leukotriene B₄ appear to contribute to eosinophil recruitment in the airways following chronic allergen challenge in guinea-pigs and rats.

Bronchial responsiveness to acetylcholine was significantly increased in the repeatedly allergen-exposed rats compared to the saline-exposed controls, 24 h after the final challenge. Treatment with SB 210661, SB 201146 or pranlukast caused a significant attenuation of ASM thickening and eosinophil recruitment without having a significant influence on bronchial hyperresponsiveness. Two previous studies have reported a significant attenuation of airway hyperresponsiveness following a single allergen challenge in 5-LO-deficient mice (Irvin *et al.*, 1997), or after treatment with a CysLT₁ receptor antagonist following repeated allergen challenge of sensitized rats (Wang *et al.*, 1993). The discrepancy in these findings from our own may be due to the species and experimental differences between the studies. In both our study and that of Wang *et al.* (1993) attenuation of ASM thickness is reported following treatment with a CysLT₁ receptor antagonist. The model we used has, however, a much more extreme protocol, with more challenges in a shorter time-period, and is one where the IgE levels of rats are still high at the time of the final allergen challenge (Pauwels *et al.*, 1979). This may therefore produce a much greater inflammatory response, with the potential for many mediators to be involved in the genesis of bronchial hyperresponsiveness. Our data indicate that additional factors must contribute to bronchial hyperresponsiveness in this model, as attenuation of ASM thickening and eosinophil infiltration did not prevent bronchial hyperresponsiveness. This phenomenon, however, warrants further investigation.

Our evidence implicates cysteinyl leukotrienes in allergen-induced airway smooth muscle cell DNA synthesis and

increases in airway smooth muscle thickening. The attenuation of increased airway smooth muscle cell DNA synthesis and thickening by the CysLT₁ receptor antagonist pranlukast could be mediated through two distinct mechanisms. Firstly, this could have occurred by inhibiting direct receptor-mediated effects of cysteinyl leukotrienes to induce airway smooth muscle cell DNA synthesis. Secondly, if airway smooth muscle cell DNA synthesis was secondary to cysteinyl leukotriene release from infiltrating eosinophils, inhibition of eosinophil recruitment into the lung by cysteinyl leukotriene antagonists may indirectly reduce the capacity for further release of cysteinyl leukotrienes from migrating eosinophils. Differential effects have recently been described for three structurally different CysLT₁ receptor antagonists: pranlukast, pobilukast and zafirlukast. All three compounds inhibited LTD₄-induced contraction of human bronchus but only pranlukast and pobilukast inhibited the effect of LTD₄ on epidermal growth factor (EGF)-induced proliferation of human ASM cells (Panettieri *et al.*, 1998b). This indicates that subtypes of the CysLT₁ receptor may exist which augment human ASM cell mitogenesis but are distinct from those which mediate LTD₄-induced contraction of ASM. It is not known if this is also the case in rat ASM cells but, to date, evidence suggests that the CysLT₁ receptor in rat and human have similar characteristics especially with respect to agonist and antagonist affinities. CysLT₁ receptor antagonists from different structural classes have been shown to have similar potencies against cysteinyl leukotriene-induced contractions in rat lung compared to those reported for human bronchus (Tudhope *et al.*, 1994).

Little is known about the mechanisms and the mediators influencing airway smooth muscle thickening, or the cellular processes underlying these events. Growth factors such as EGF (Tomlinson *et al.*, 1994), platelet-derived growth factor (Hirst *et al.*, 1992) and thrombin (Panettieri *et al.*, 1995), and contractile agonists such as histamine (Panettieri *et al.*, 1990) and endothelin-1 (Glassberg *et al.*, 1994) have been demonstrated to induce airway smooth muscle cell proliferation. Leukotriene D₄ has also been shown to augment the influence of the growth factors insulin-like growth factor (Cohen *et al.*, 1995) and EGF (Panettieri *et al.*, 1998b) to potentiate airway smooth muscle cell proliferation. A combination of the cytokines IL-1 β and IL-6 induces both guinea-pig tracheal smooth muscle cell hyperplasia and hypertrophy *in vitro* (De *et al.*, 1995). A study with human airway smooth muscle cells has implicated a role for angiotensin II in cellular hypertrophy but not hyperplasia (McKay *et al.*, 1998). In our model, treatment with the 5-LO inhibitor and either BLT or CysLT₁ receptor antagonists significantly inhibited increases in airway smooth muscle thickening, suggesting that both LTB₄ and cysteinyl leukotrienes may influence this process. In a previous *in vivo* study in rats, the CysLT₁ receptor antagonist MK-571 also significantly inhibited allergen-induced increases in airway smooth muscle mass (Wang *et al.*, 1993). The BLT receptor antagonist had a significant inhibitory effect on allergen-induced increases in airway smooth muscle thickness but not on increased DNA synthesis, suggesting that factors other than cell proliferation may contribute to airway smooth muscle thickening. The possibility remains therefore that ASM cell proliferation and ASM thickening may be differentially regulated through the release of different types of mediators.

One confounding factor in this study is that, following repeated allergen exposure, rats exhibited a significant increase in ASM cell DNA synthesis but not a significant increase in the total number of ASM cells, although there was an overall increase compared to the saline-exposed control group. The reason for this may be related to the temporal delay between

detection of DNA synthesis by BrdU incorporation and the actual onset of cell proliferation. Alternatively, it is possible that ASM cell proliferation may be accompanied by a similar rate of ASM cell apoptosis, such that no overall increase in cell number is observed.

In summary, we demonstrate that cysteinyl leukotrienes mediate increased airway smooth muscle cell DNA synthesis induced by repeated allergen exposure in Brown Norway rats. In addition, the appearance of airway smooth muscle thickening is also dependent on products of the 5-LO pathway, including both LTB₄ and cysteinyl leukotrienes. Both LTB₄ and cysteinyl leukotrienes are also involved in allergen-induced

airway eosinophilia, but are not directly responsible for bronchial hyperresponsiveness. These results support the concept that leukotrienes participate in increased ASM cell proliferation and ASM thickening following repeated allergen exposure, processes underlying airway wall remodelling in chronic asthma.

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