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A novel transcription factor inhibitor, SP100030, inhibits cytokine gene expression, but not airway eosinophilia or hyperresponsiveness in sensitized and allergen-exposed rat

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1 We examined the effect of SP100030, a novel inhibitor of activator protein-1 (AP-1) and nuclear factor (NF)-κB transcription factors, in a rat model of asthma.

2 Sensitized Brown-Norway rats were treated with SP100030 (20 mg kg^{-1} day^{-1} for 3 days) intraperitoneally prior to allergen challenge. Allergen exposure of sensitized rats induced bronchial hyperresponsiveness (BHR), accumulation of inflammatory cells in bronchoalveolar lavage (BAL) fluid, and also an increase in eosinophils and CD2$^+$, CD4$^+$ and CD8$^+$ T-cells in the airways together with mRNA expression for IL-2, IL-4, IL-5, IL-10, and IFN-γ.

3 Pre-treatment with SP100030 inhibited BAL lymphocyte influx ($P<0.03$), specifically reduced CD8$^+$ T-cell infiltration in the airway submucosa ($P<0.03$), and mRNA expression for IL-2, IL-5, and IL-10 ($P<0.05$). Neutrophil, eosinophil, and CD4$^+$ T-cells accumulation in the airways and BHR were not affected by SP100030.

4 Our results indicate that suppression of IL-2 and IL-5 mRNA expression may not necessarily lead to suppression of BHR. The expression of IL-5 mRNA may contribute to the airway accumulation of eosinophils, but does not correlate with the extent of eosinophilia.

5 The joint AP-1 and NF-κB inhibitor, SP100030, selectively inhibits CD8$^+$ T-cells, and mRNA expression of both Th1 and Th2 cytokines *in vivo*, but does not inhibit allergen-induced airway eosinophilia and BHR.

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Abbreviations: ACh, acetylcholine; AP-1, activation protein-1; BAL, bronchoalveolar lavage; BHR, bronchial hyperresponsiveness; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-γ; Ig, immunoglobulin; IL, interleukin; MBP, major basic protein; NF, nuclear factor; OA, ovalbumin; PBS, phosphate buffered saline; PC_{200}, provocative concentration of acetylcholine needed to increase lung resistance by 200% above baseline; RT–PCR, reverse transcription-Polymerase Chain Reaction; Tc, T-cytotoxic; Th, T-helper

Introduction

Airway eosinophilic inflammation and bronchial hyperresponsiveness (BHR) are major features of bronchial asthma (Chung, 1986). CD4$^+$ T-lymphocyte may be pivotal cells mediating allergen-induced airway inflammation (Azzawi *et al*., 1990; Corrigan & Kay, 1990). In recent years, an imbalance between CD4$^+$ T-helper type 1 (Th1) and Th2 immune responses has been recognized as the major contributing factor to the pathogenesis of asthma and BHR. Expression of the Th2-derived cytokines, particularly IL-4 and IL-5, is increased in the airways of patients with asthma, especially expressed by CD4$^+$ T-cells (Hamid *et al*., 1991; Robinson *et al*., 1992). By contrast, the expression of the Th1-derived cytokine IFN-γ is not increased (Hamid *et al*., 1991; Robinson *et al*., 1992). In animal models, suppression or genetic depletion of Th2 cytokines, such as IL-4 and IL-5, may prevent the development of airway inflammation and BHR (Mauser *et al*., 1993; Brusselle *et al*., 1994; Foster *et al*., 1991; Corry *et al*., 1996). On the other hand, augmentation of Th1 response, such as mycobacterial infection (Erb *et al*., 1998), and administration of Th1-derived cytokines, such as IFN-γ (Huang *et al*., 1999b), IL-12, and IL-18 (Hofstra *et al*., 1998) may protect from allergen-induced airway inflammation. Adoptive transfer of *in vitro*-generated Th2 cells may induce airway inflammation and BHR in response to specific allergen in naïve animals (Li *et al*., 1998; Hansen *et al*., 1999), while in our previous study, Th1 cell lines may counteract Th2-mediated airway immune responses. Meanwhile, CD8$^+$ T-cells are postulated to play a protective role in the allergen-induced airway immune disorders (Olivenstein *et al*., 1993; Laberge *et al*., 1996; McMenamin & Holt, 1996), probably by promoting Th1 response (Huang *et al*., 1999a). Thus, the induction of differential expression of cytokines from T-cells may determine the subsequent immune responses and airway inflammation (Chung & Barnes, 1999).

The transcription of many cytokines is regulated by transcription factors (Barnes & Adcock, 1995), such as nuclear factor (NF)-κB, and activation protein (AP)-1. Activated AP-1 binding to its cognate DNA sequences may

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result in the expression of IL-2 and the T-cell receptors (Barnes & Adcock, 1995; Angel & Karin, 1991). The exact role of NF-κB and AP-1 in bronchial asthma is still unclear, but there is evidence that NF-κB may be important in the establishment of chronic inflammation in asthma. NF-κB and AP-1 are capable of activating a wide range of immunoregulatory genes (Siebenlist *et al*., 1994), including those coding for IL-1β, TNF-α, IL-2, IL-6, IL-8, and GM-CSF, as well as the IL-2 receptor, ICAM-1, MCP-1, RANTES, and E-selectin. These cytokines and adhesion molecules have been implicated in the immune responses in asthma (Chung & Barnes, 1999; Barnes *et al*., 1998). Increased activation and expression of NF-κB (Hart *et al*., 1998) and of AP-1 (Demoly *et al*., 1992) have been demonstrated in the airways of asthmatic patients. In sensitized mice deficient of NF-κB subunit p50, allergen exposure failed to elicit eosinophilic airway inflammation, compared to wild-type mice (Yang *et al*., 1998). In these $p50^{-/-}$ mice, the major defects detected were the lack of production of IL-5 and of the chemokine, eotaxin, which may cooperate in the recruitment and activation of eosinophils (Hisada *et al*., 1999).

SP100030 is a newly developed inhibitor of NF-κB and AP-1, and possesses the capacity of inhibiting gene expression induced by many different stimuli (Goldman *et al*., 1996). In *in vivo* studies on animal models of allograft rejection, SP100030 showed T-cell-specific immunosuppressive activity (Goldman *et al*., 1996; Morikawa *et al*., 1997). However, whether SP100030 acts through reducing cytokine production *in vivo* is not known. We, therefore, examined the potential effects of SP100030 on allergen-induced airway inflammation, Th1 and Th2 cytokine gene expression, and BHR in the Brown-Norway rat model of asthma.

Methods

Animal, sensitization procedures and allergen exposure

Pathogen-free inbred male Brown-Norway rats (Harlan Olac Ltd., Bicester, U.K.) (200–250 g, 9–13 weeks old) were injected with 1 ml of 1 mg ovalbumin (OA) in 100 mg Al $(OH)_3$ suspension in 0.9% (w v^{-1}) saline intraperitoneally (i.p.) on three consecutive days. OA aerosol exposure (15 min; 1% OA) to rats was performed in a 6.5 l Plexiglas chamber connected to a DeVilbiss PulmonSonic nebulizer (model No. 2512, DeVilbiss Health Care, U.K. Ltd., Middlesex, U.K.) that generated an aerosol mist pumped into the exposure chamber by the airflow supplied by a small animal ventilator (Harvard Apparatus Ltd., Kent, U.K.) set at 60 strokes per min with a pumping volume of 10 ml.

Protocol

Three groups of rats actively sensitized with OA and $Al(OH)_3$ were studied: (1) Sham-treated and saline-exposed animals (group saline, $n=8$): Sensitized animals received vehicle for SP100030 (1 ml day^{-1} of 0.25% methylcellulose) by intraperitoneal injection for 3 days from 15th to 17th days after last injection of sensitizing OA. Animals were exposed to saline aerosol for 15 min, and then studied 18–24 h thereafter; (2) Sham-treated and OA-exposed animals (group OA, $n=7$): The procedures were the same as group SS above, except the aerosol exposure was with 1% OA aerosol; (3) SP100030-treated and OA-exposed animals (group SP100030, $n=5$): The procedures were the same as for group OA, except that the compound SP100030 was used to treat animals (20 mg kg^{-1} in 1 ml 0.25% methylcellulose for 3 days).

Measurement of airway responsiveness to acetylcholine

Airway responsiveness was measured as previously described (Elwood *et al*., 1991). In brief, anaesthetized, tracheostomized, paralysed, and ventilated rats were monitored for airflow with a pneumotachograph (model F1L, Mercury Electronics Ltd., Glasgow, Scotland) connected to a transducer (model FCO40; ± 20 mmH_2O, Furness Controls Ltd., Sussex, U.K.), transpulmonary pressure *via* a transpleural catheter connected to a transducer (model FCO40; ± 1000 mmH_2O, Furness Controls) and blood pressure *via* carotid artery catheterization. Lung resistance was simultaneously calculated using LabView software (National Instruments, Austin, TX, U.S.A.). Aerosol generated from increasing half log10 concentrations of acetylcholine chloride (ACh) was administered by inhalation (45 breaths of 10 ml kg^{-1} stroke volume) with the initial concentration of 10–3.5 mol l^{-1} and the maximal concentration of 0.1 mol l^{-1} through a different circuit from the one for lung resistance measurements. The concentration of ACh needed to increase lung resistance 200% above baseline (PC_{200}) was calculated by interpolation of the log concentration-lung resistance curve.

Bronchoalveolar lavage and cell counting

This is also described in detail elsewhere (Haczku *et al*., 1995). Briefly, after an overdose of anaesthetic, rats were lavaged with a total of 20 ml 0.9% sterile saline *via* the endotracheal tube. Total cell counts, viability and differential cell counts from cytospin preparations stained by May-Grünwald-Giemsa stain were determined under an optical microscope (Olympus BH2, Olympus Optical Company Ltd., Tokyo, Japan). At least 500 cells were counted and identified as macrophages, eosinophils, lymphocytes and neutrophils according to standard morphology under $\times 400$ magnification.

Collection of lung tissues

After opening of the thoracic cavity and removal of the lungs, the right lung without major vascular and connective tissues was cut into pieces and snap-frozen in liquid nitrogen, and then stored at $-80^{\circ}C$ for later assays for mRNA expression. The left lung was inflated with 3 ml saline/O.C.T. tissue embedding medium (1 : 1). Two blocks of half cm^3 were cut from left lung around the major bronchus, embedded in O.C.T. medium, and snap-frozen in melting isopentane and liquid nitrogen. Cryostat sections (6 μm) of the tissues were cut, air-dried, fixed in acetone, and then air-dried again, wrapped in aluminium foil and stored at $-80^{\circ}C$ for later immunohistochemical studies.

Immunohistochemistry

For detection of eosinophils, we used a mouse IgG1 monoclonal antibody against human MBP, clone BMK-13,

which has been shown to be both sensitive and specific for staining rat eosinophils in frozen sections (Haczku *et al*., 1995). The cryostat sections were incubated with BMK-13 at a dilution of 1 : 50 for 30 min at room temperature. After labelling with the second antibody, rabbit anti-mouse IgG, positively stained cells were visualized with alkaline phosphatase-anti-alkaline phosphatase method.

For staining of $CD2^+$, $CD4^+$ and $CD8^+$ T-lymphocytes in tissues, sections were incubated with mouse anti-rat monoclonal antibodies, anti-rat CD2 (pan T-cell marker), anti-rat CD4 and anti-CD8 antibodies at a dilution of 1 : 500 for 1 h. Biotin goat anti-mouse antibody and avidin phosphatase at a dilution of 1 : 200 were applied for 30 min in turn.

For all tissue sections, alkaline phosphatase was developed as a red stain after incubation with Naphthol AS-MX phosphate in 0.1 M trismethylamine-HCl buffer (pH 8.2) containing levamisole to inhibit endogenous alkaline phosphatase and 1 mg ml^{-1} Fast Red-TR salt. Sections were counterstained with Harris Hematoxylin and mounted in Glycergel. System and specificity controls were carried out for all staining. Slides were read in a coded randomized blinded fashion, under a microscope. Cells within 175 μm beneath the basement membrane were counted in all airways. Submucosal area was quantified with the aid of a computer-assisted graphic tablet. Counts were expressed as cells per mm^2 of cross-sectional subepithelial area.

Reverse transcription-polymerase chain reaction

Total RNA from lung tissue was extracted according to the method of Chomczynski & Sacchi (1987). The yield of RNA was measured by optical density at 260 nm in a spectrophotometer. RNA was analysed on a 1.5% agarose/formaldehyde gel to check for degradation, and stored at -80°C until later use. After denaturing at 70°C for 5 min, 1 μg of total RNA was used for reverse transcription in a 20 μl reaction volume containing 1 × AMV buffer (mM): Tris-HCl 50, pH 8.3, KCl 50, $MgCl_2$ 10, DTT 10, spermidine 0.5, 4 deoxynucleotide triphosphates (dNTP) 1, including deoxyadenosine triphosphate (dATP) deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and thymidine 5′-triphosphate (dTTP), ribonuclease inhibitor 32 U, 0.2 μg random primer pd(N)6 sodium salt, 8 U AMV reverse transcriptase at 42°C for 60 min. Complementary DNA (cDNA) product was diluted to 100 μl in water. PCR was performed on 5 μl of diluted cDNA product in a total volume of 25 μl with a final concentration of 1 × KCl or NH4 buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μg each of sense and anti-sense primers, and 1 U Taq polymerase. The primers were designed according to published sequences as in our previous study (Huang *et al*., 1999a). The PCR reagents were overlaid with mineral oil and amplification was carried out through 20 – 40 cycles of denaturation at 94°C for 30 s, annealing at individual temperature for 30 s and extension at 72°C for 30 s, followed by final extension at 72°C for 10 min. The optimal PCR conditions, in terms of suitable buffer, annealing temperature and number of cycles, were determined by PCR with pooled cDNA from all samples. Annealing temperatures were 62°C for GAPDH, IL-4 and IFN-γ, 58°C for IL-5, and 65°C for IL-2 and IL-10. Serial sampling every two cycles through 20 – 42 cycles was used to determine the exponential phase of the product amplification curve. The cycle numbers we used for PCR were 26 for GAPDH, 36 for IL-2, IL-4, and IL-5, and 34 for IL-10 and IFN-γ.

Southern blotting and Cerenkov counting

Each PCR product (10 μl) of was size-fractionated and visualized with ethidium bromide on 1.5% agarose gel electrophoresis, followed by Southern blotting to Hybond-N membrane and hybridization to the appropriate cloned cDNA in order to confirm the identity of the product and, because all primer pairs cross at least one intron, to check for possible genomic contamination. Hybridizations were carried out at 65°C overnight with the appropriate cloned cDNA, which had been ^{32}P labelled, in 6 × standard saline citrate, 10 × Denhardt's solution (0.2% w v^{-1} each of bovine serum albumin, ficol and polyvinylpyrolidone), 5 mM EDTA, 0.5% sodium dodecyl sulphate and 0.2% sodium pyrophosphate, 100 μg ml^{-1} sonicated salmon sperm DNA. In addition, 5 μl of each PCR reaction was dot-blotted on to Hybond-N membrane and also hybridized to cDNA probe. Dot blots were excised and radioactivity measured by Cerenkov counting. All measurements were made below the saturation level of a Packard 1900CA liquid scintillation analyser (Packard Instrumentation BV, Groningen, The Netherlands). Results were generated from the counting of dot blots and expressed as a ratio of cytokine to GAPDH count, the latter used as an internal control.

Western blot analysis

Whole cell proteins were extracted from frozen lung tissue. Forty μg $lane^{-1}$ of whole-cell proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose filters (Hybond-ECL, Amersham Pharmacia Biotech, Amersham, U.K.) by blotting. Filters were blocked for 1 h at room temperature in Phosphate-buffered saline (PBS), 0.05% Tween 20, 5% non-fat dry milk. The filters were then incubated with Rabbit anti-human phospho-Jun antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 1 h at room temperature in PBS, 0.05% Tween 20, 5% non-fat dry milk at dilution of 1 : 1000. This antibody cross-reacts with both mouse and rat phospho-Jun and non-phosphorylated Jun proteins. Filters were washed three times in PBS, 0.05% Tween 20 and subsequently incubated for 60 min at room temperature with anti-rabbit antibody conjugated to horseradish peroxidase (Dako, Ely, U.K.) in PBS, 0.05% Tween 20 and 5% non-fat dry milk, at a dilution of 1 : 4000. After a further three washes in PBS/Tween, visualization of the immunocomplexes was performed using ECL as recommended by the manufacturer (Amersham Pharmacia Biotech).

Drugs and reagents

OA (Grade V, salt-free), methylcellulose, Ach, Naphthol AS-MX phosphate in 0.1 M trismethylamine-HCl buffer (pH 8.2) containing levamisole, Fast Red-TR salt, ethidium bromide, 10 × Denhardt's solution, EDTA, sodium dodecyl sulphate, sodium pyrophosphate, and sonicated salmon sperm DNA were purchased from Sigma (Dorset, U.K.). Al $(OH)_3$, isopentane, Harris Hematoxylin were from BDH (Dorset,

U.K.). SP100030 was a gift from Signal Pharmaceuticals (San Diego, U.S.A.). Liquid nitrogen was supplied by BOC (Luton, U.K.). O.C.T. medium was from Raymond A Lamb (London, U.K.); mouse IgG1 monoclonal antibody against human MBP, clone BMK-13, from Monosan®, Bradsure Biologicals Ltd. (Leicestershire, U.K.). Mouse anti-rat monoclonal antibodies, and biotin goat anti-mouse antibody were products from Pharmingen, Cambridge Bioscience, (Cambridge, U.K.). Avidin phosphatase and Glycergel were from DAKO Ltd (High Wycome, U.K.). In RT–PCR, random primer pd(N)6 sodium salt was purchased from Pharmacia (Milton Keynes, U.K.); Taq polymerase from Bioline (London, U.K.). All other reagents used in RT–PCR were purchased from Promega (Southampton, U.K.). Hybond-N membrane and ^{32}P were obtained from Amersham (Bucks, U.K.).

Data analysis

Data were presented as mean ± s.e.mean. For multiple comparison of different groups, Krüskal-Wallis test for analysis of variance was used. If the Krüskal-Wallis test for analysis of variance was significant, Mann–Whitney *U*-test was used for comparison between two individual groups. Data analysis was performed utilizing SPSS for Windows statistical software package. A P value of <0.05 was considered to be significant.

Results

Bronchial responsiveness to ACh

There was no significant difference in baseline lung resistance between the groups. Sensitized, sham-treated and OA-exposed rats had a significant increase in mean $-\log PC_{200}$ compared to sensitized saline-exposed rats ($P<0.002$, Figure 1). The drug SP100030 showed no effect on the baseline responsiveness to ACh, and also no effect on allergen-induced BHR, as reflected by the significant leftward shift of ACh concentration-lung resistance response curve and elevated PC_{200} compared to saline exposed rats ($P<0.02$, Figure 1).

Inflammatory cell responses

There was a significant increase in the numbers of total cells, eosinophils, lymphocytes and neutrophils recovered in BAL fluid of sensitized rats exposed to OA compared to sensitized rats exposed to saline ($P<0.005$, Figure 2). OA exposure or treatment with SP100030 had no effect on the numbers of macrophages in BAL fluid, while OA-induced increase in lymphocyte counts was significantly reduced by SP100030 ($P<0.03$). The increase of other inflammatory cells, such as eosinophils, and neutrophils in BAL fluid induced by OA exposure of sensitized rats was not affected by SP100030.

Allergen exposure of sensitized rats caused a significant increase in the airway submucosa infiltration of eosinophils, $CD2^+$, $CD4^+$, and $CD8^+$ T-cellslymphocytes ($P<0.05$, Figure 3), among which only $CD8^+$ T-cells infiltration was partially, but significantly inhibited by treatment with SP100030 ($P<0.03$).

Cytokine expression in lungs

In sensitized rats, OA exposure induced a significant increase in IL-2, IL-4, IL-5, IL-10, and IFN-γ mRNA expression ($P<0.03$, Figure 4). SP100030 suppressed the OA-induced increase in IL-2, IL-5, and IL-10 mRNA expression ($P<0.05$).

Phospho-jun expression

Western blot analysis of phospho-jun expression was induced in lung tissue isolated from ovalbumin stimulated sensitized animals (Figure 5). In contrast, there was no induction of phospho-jun in saline-exposed ovalbumin sensitized animals. SP100030 (20 mg kg^{-1} for 3 days) inhibited both phospho-jun and non-phosphorylated jun expression in control non-ovalbumin-exposed and ovalbumin-treated sensitized animals. The additional effect on non-phosphorylated jun expression was due to the feedback effect of AP-1 inhibition on the AP-1 sensitive Jun expression.

Discussion

In the Brown-Norway rat model, allergen exposure of sensitized animals induced airway accumulation of inflammatory cells, such as eosinophils, lymphocytes, and neutrophils in BAL fluid, eosinophils, $CD2^+$, $CD4^+$ and $CD8^+$ T-cells in the airway submucosa, and also increased BHR to ACh and expression of mRNA for Th1 (IFN-γ) and Th2 (IL-4, IL-5, and IL-10) cytokines. Pre-treatment with SP100030, a dual AP-1 and NF-B inhibitor (Goldman *et al*., 1996), attenuated BAL lymphocyte accumulation and airway submucosal infiltration of $CD8^+$ T-cells, together with suppression on the allergen-induced upregulation of mRNA expression for both IL-2, IL-5 and IL-10. SP100030 inhibited the induction of phosho-jun expression induced by allergen exposure, indicating that this compound acted as an inhibitor of AP-1 induced in this rat model. However, there was no effect of SP100030 on BHR.

Little is known about the bioactivity of SP100030. *In vivo* studies in animal models of allograft transplantation (Goldman *et al*., 1996; Morikawa *et al*., 1997), delayed type hypersensitivity, and Mycobacteria-induced adjuvant arthritis (Goldman *et al*., 1996), reveal that SP100030 may protect against T-cell-mediated immunoinflammatory responses. In our study, SP100030 inhibited T-cell accumulation, particularly $CD8^+$ T-cells, in the airways following allergen challenge, but had no effect on eosinophils and neutrophils, indicating a lymphocyte-specific mechanism of action. However, selective inhibition of $CD8^+$ T-cells may be useful in the context of allograft rejection, which is mediated largely through $CD8^+$ T-cells and Th1 responses, but may not be helpful in allergen-induced airway inflammation, in which $CD8^+$ T-cells were shown to possess protective activity (Olivenstein *et al*., 1993; Laberge *et al*., 1996; McMenamin & Holt, 1996; Huang *et al*., 1999a). This reduction of BAL lymphocytes and airway infiltrating $CD8^+$ T-cells may be associated with the significant inhibition of IL-2 mRNA expression by SP100030, since IL-2 is important in causing proliferation of T-cell populations (Smith, 1988).

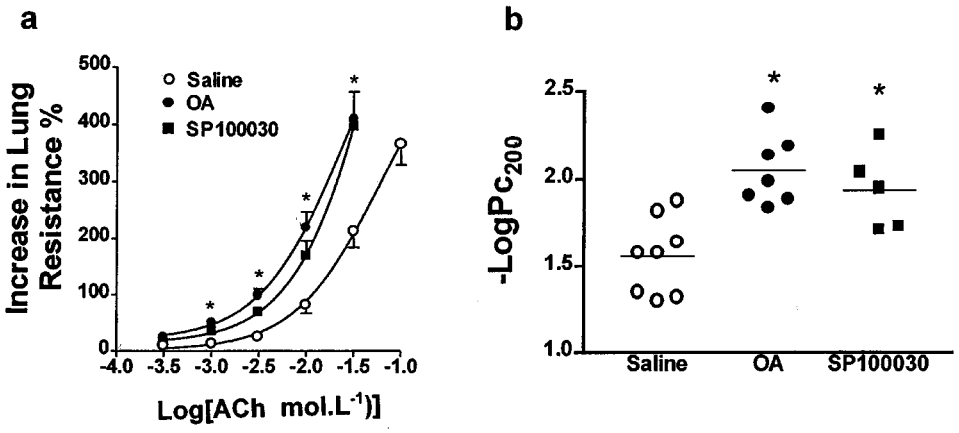

Figure 1 (a) Mean per cent increase in lung resistance to increasing concentrations of acetylcholine (ACh) for three groups of sensitized rats: sham-treated and saline aerosol exposed, $n=8$; sham-treated and OA aerosol-challenged, $n=7$; treated with SP100030 and challenged with OA aerosol, $n=5$. The concentration-response curves are significantly shifted leftward for both group OA and SP100030, compared to Saline group, as reflected by the increased lung resistance at the ACh concentrations of 10^{-3}, $10^{-2.5}$, 10^{-2}, and $10^{-1.5}$ M. (b) Mean $-\log PC_{200}$, which is the negative logarithm of the provocative concentration of ACh needed to increase baseline lung resistance by 200%, for the three groups of rats detailed in (a). Treatment with SP100030 did not have significant effect on allergen-induced increase in $-\log PC_{200}$. $^*P<0.05$ as group OA or SP100030 compared to group Saline. Data shown as mean$\pm$s.e.mean.

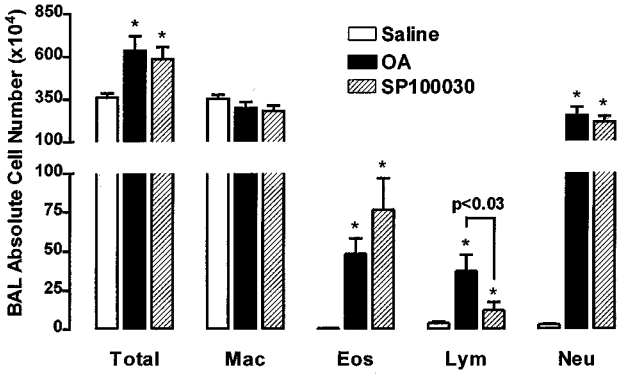

Figure 2 Mean numbers of total cells, eosinophils (Eos), lymphocytes (Lym) and neutrophils (Neu) in BAL fluid in groups of rats as specified in Figure 1. The counts of total cells, eosinophils, lymphocytes, and neutrophils were significantly increased in sensitized rats exposed to OA aerosol. SP100030 treatment suppressed the increase in counts of lymphocytes. $^*P<0.05$ as compared to saline group. Data shown as mean$\pm$s.e.mean.

There has been little work regarding SP100030 in the rat species, and most of the published data has been obtained in mice or on human cell lines (Goldman *et al*., 1996; Morikawa *et al*., 1997; Gerlag *et al*., 2000). However, in one study in an adjuvant arthritis model in the rat, an inhibition of paw oedema was observed at doses of 20–30 mg kg^{-1} (Goldman *et al*., 1996). More convincingly, in the mouse, SP100030 at a dose of 10 mg kg^{-1} inhibited arthritis induced by collagen, together with evidence of suppression of NF-κB binding in the synovium. We chose a dose of 20 mg kg^{-1} from our current studies based on these previous observations, and showed at this dose in the rat, there was inhibition of the induction of phospho-jun in the lungs following allergen exposure of sensitized rats, indicating that SP100030 acted as an inhibitor of AP-1.

The marked inhibition of IL-2 mRNA expression is compatible with the inhibitory effects of SP100030 on transcription factors, NF-κB and AP-1, which are important in IL-2 and IL-2R transcription (Angel & Karin, 1991; Siebenlist *et al*., 1994). The suppression of IL-5 mRNA expression was also observed in NF-κB p50 subunit-deficient mice (Yang *et al*., 1998), in which the expression of IL-5 and eotaxin in CD4$^+$ T-cells was significantly reduced compared to wild-type mice following allergen exposure. However, in that study, the BAL eosinophilia was significantly inhibited in p50$^{-/-}$ mice. It is possible that NF-κB may be essential in the development and generation of particular mediators other than IL-5, such as IL-1β, TNF-α, GM-CSF, eotaxin, and adhesion molecules, which are also important for triggering eosinophilic recruitment, survival and activation (Chung & Barnes, 1999), but may be less sensitive to SP100030. It is also possible that there is a differential inhibitory effect of SP100030 on AP-1 and NF-κB. Considering the prominent inhibitory effect on IL-2, and relatively less IL-5 mRNA expression in our study and the striking inhibition of IL-5 in NF-κB-deficient mice, we may speculate that SP100030 may be more active in suppressing AP-1 than NF-κB. Our data indicate that SP100030 indeed does inhibit AP-1 expression in this rat model of allergic inflammation.

We found a dissociation between IL-5 suppression by SP100030 and eosinophil infiltration. IL-5 has been shown to closely related to the terminal differentiation and prolonged survival of eosinophils (Sanderson, 1992). Administration of anti-IL-5 monoclonal antibody may prevent allergen-induced airway eosinophilia in animal models (Milne & Piper, 1994; Hamelmann *et al*., 1997; Mauser *et al*., 1995), while IL-5 gene knock-out mice failed to recruit eosinophils in response to allergen (Foster *et al*., 1996). Nevertheless, the levels of IL-5 mRNA expression in the lungs from SP100030-treated rats exposed to OA were still significantly higher than those exposed to saline aerosol. Probably, the role of IL-5 in allergen-induced eosinophilic inflammation may be most important in the initial recruitment and basal proliferation, and the further bulk proliferation and activation may be

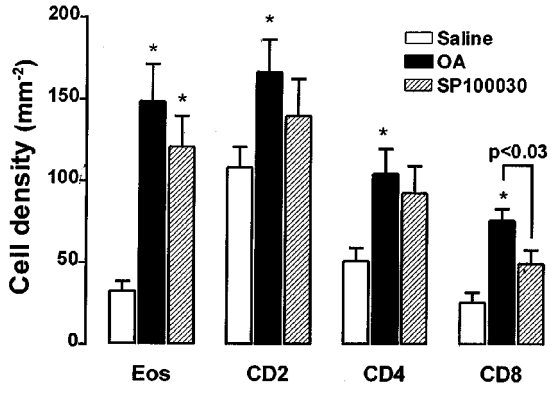

Figure 3 Mean numbers of eosinophil and T lymphocyte subset ($CD2^+$, $CD4^+$ and $CD8^+$) counts in airway submucosa expressed as per mm^2 in groups as detailed in Figure 1. Allergen challenge caused a significant increase in total number of T lymphocytes expressing $CD2^+$, $CD4^+$, and also $CD8^+$ T lymphocytes. SP100030 significantly reduced $CD8^+$ T-cell counts. $^*P<0.05$ as compared to group Saline. Data shown as mean ± s.e.mean.

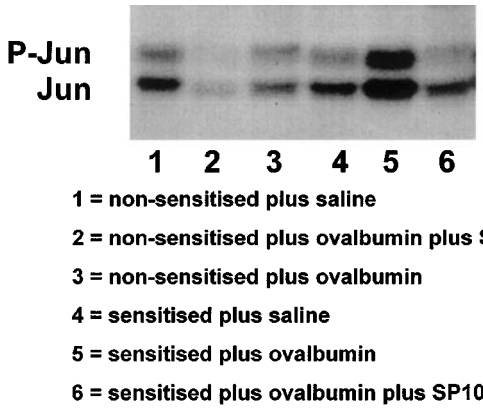

Figure 5 Western blot analysis of phospho-jun and of jun expression in lung tissue isolated from non-sensitized and sensitized rats exposed to ovalbumin, and the effect of SP100030. There was an induction of phospho-jun and jun expression in sensitized and ovalbumin-exposed rats. This phospho-jun and non-phosphorylated jun induction were inhibited by SP100030. A representative example of three experiments is shown.

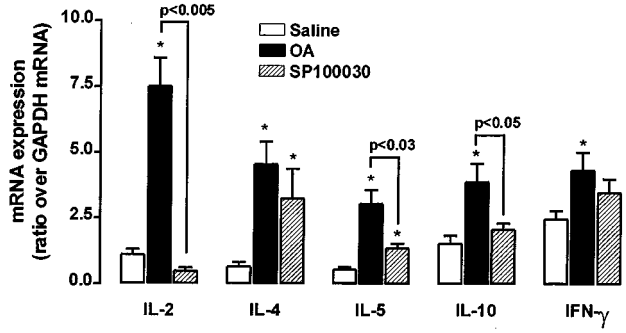

Figure 4 Mean IL-2, IL-4, IL-5, IL-10 and IFN-γ mRNA expression in rat lung expressed as a ratio to GAPDH mRNA as determined by RT–PCR, followed by Southern blot analysis. The expression was obtained on a radioactive probe-hybridized dot-blot of the PCR products. An increase in the mRNA expression for all five cytokines was noted after OA exposure of sensitized rats (group OA). SP100030 significantly reduced mRNA expression of IL-2, IL-5 and IL-10. $^*P<0.03$ as compared to group Saline. Data shown as mean ± s.e.mean.

attributed to other mediators, such as eotaxin, released in response to allergen exposure. The necessity for IL-5 to cooperate with other mediators in activation of eosinophils and induction of BHR was shown in studies using IL-5 transgenic mice (Hisada *et al*., 1999; Iwamoto & Takatsu, 1995), in which BHR and further increase in eosinophil infiltration were observed only after exposure to eotaxin or allergen. These studies also provided evidence that IL-5 gene expression does not necessarily lead to the development of BHR. It is reasonable to hypothesize that the suppression of IL-5 gene expression by SP100030 was not enough to inhibit BHR and eosinophilia in this study, even with a concomitant suppression of IL-2 mRNA. Again, it is possible that higher doses of SP100030 might further inhibit IL-5 and reduce eosinophilic inflammation. Our current data with SP100030 are similar to those we have previously reported with cyclosporin A which inhibited allergen-induced expression of IL-2, IL-4 and IL-5 without affecting allergen-induced BHR, while eosinophilic inflammation was suppressed (Huang *et al*., 1999c). Therefore, the relationship between suppression of BHR and eosinophilic infiltration is inconsistent.

Other possible factors underlying the failure of SP100030 to suppress BHR includes the suppressed expression of IL-10 mRNA and persistent neutrophilia. Although IL-10 is classified as a Th2 cytokine, it can be secreted from other cell types, such as epithelial cells (Bonfield *et al*., 1995) and alveolar macrophages (Moore *et al*., 1993), and has a inhibitory effect on both Th1 and Th2 cells (Moore *et al*., 1993; Fiorentino *et al*., 1991), and also on eosinophils (Takanashi *et al*., 1994), neutrophils (Cassatella *et al*., 1993), and monocytes/macrophages (Berkman *et al*., 1995). In animal models of allergen-induced airway inflammation and late airway responses, the overall effect of IL-10 appeared to be protective (Woolley *et al*., 1994; Zuany-Amorim *et al*., 1995). The suppression of IL-10 mRNA expression may have contributed to the airway inflammation and BHR. However, whether the mechanism by which SP100030 suppressed IL-10 mRNA was through suppression of transcription factors in T-cells remains to be determined. The neutrophils have been previously implicated in BHR, and products of neutrophils such as LTB_4 can induce BHR (O'byrne *et al*., 1984; Murphy *et al*., 1986). This also emphasizes the multi-faceted mechanisms of BHR that blockade of a single mechanism or mediator can not inhibit the ongoing immune events in the complicated inflammatory network.

In conclusion, our study demonstrated the inhibitory effects of SP100030 on lymphocyte infiltration, particularly $CD8^+$ T-cells, probably through inhibition of IL-2 mRNA expression. However, the inhibition in IL-2, IL-5, and IL-10 mRNA did not result in a suppression of airway infiltration of eosinophils and neutrophils, or BHR. It is, therefore, essential to dissect further the interactions between different cells and mediators involved in allergen-induced inflammation.

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