

Leflunomide, a novel immunomodulating agent, prevents the development of allergic sensitization in an animal model of allergic asthma

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Summary

Background Leflunomide is a new anti-inflammatory and immunomodulating agent which is showing promise in several immune disorders, especially rheumatoid arthritis. Its activity profile suggests it may be of use in modulating allergic sensitization.

Objective To investigate the effectiveness of leflunomide in preventing the development of allergic sensitization.

Methods Fifty-three brown Norway rats were sensitized by intraperitoneal injection of ovalbumin and adjuvant (ricin) on day 0. To determine the ability of leflunomide to inhibit primary allergic sensitization six rats were treated with A77 1726, the active metabolite of leflunomide, from day 0 through day 5, six were treated from day 5 through day 10, and nine rats acted as controls. On day 14, ovalbumin-specific serum antibody levels and the magnitude of the early-phase airway response (EAR) after inhalation allergen challenge were assessed. To determine the ability of acute topical treatment with leflunomide to inhibit mast cell degranulation, three groups of five animals received either vehicle, 100 µg A77 1726, or 1000 µg A77 1726 60 min prior to aerosol allergen challenge. To determine the effects of leflunomide treatment *in vivo* on mast cell function *in vitro*, mast cells were obtained by bronchoalveolar lavage from 17 rats (nine treated with leflunomide and eight controls). Allergen-specific and non-specific degranulation (48/80 induced) were studied.

Results In the leflunomide treated rats both ovalbumin-specific IgE and IgG levels were significantly reduced, and the increases in lung resistance and lung elastance were essentially abolished, compared to those of the control group. Non significant differences were found in any of the parameters between the two leflunomide treated groups. Topical pre-treatment with leflunomide did not prevent the allergen-induced EAR. Treatment with leflunomide *in vivo* prevented allergen-induced mast cell degranulation *in vitro* because the mast cells lacked IgE on their surface. Non allergen-specific degranulation was normal and allergen-induced degranulation could be restored by passive sensitization.

Conclusions These data suggests that leflunomide can prevent primary allergic sensitization and prevent allergen-induced EAR by inhibiting production of allergen-specific IgE antibodies. Further studies in atopic conditions are warranted.

Keywords: acute-phase reaction, allergen bronchial provocation tests, immunoglobulins, immunomodulators, isoxazoles

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Introduction

Asthma is characterized by a combination of reversible

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airway obstruction, increased airway responsiveness to various bronchoconstrictive stimuli, and chronic airway inflammation [1]. Allergic sensitization and reactions to environmental allergens form an integral part of the disease process in most adults and older children with asthma. The

prevention of allergic sensitization in those at risk is the topic of much current research interest [2]. Allergen-specific IgE is produced by B lymphocytes following T cell help in the form of cytokines and cognate interactions [3]. In the sensitized subjects, the early-phase airway response (EAR) to an allergen challenge is induced by IgE-mediated mast cell degranulation with the release of bronchoconstrictor and pro-inflammatory mediators [4]. Thus, the production of allergen-specific IgE is central to the clinical symptomatology and pathogenesis of asthma. Inhibition of mast cell degranulation by cromolyns and β_2 -sympathomimetic agents has been demonstrated to prevent the acute phase reaction to allergen challenge and to be an effective treatment strategy for some atopic asthmatics [5,6].

Leflunomide, HWA 486, N-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide, is an anti-inflammatory and immunomodulating agent that is well absorbed in animals and humans and rapidly converted to its active metabolite, A77 1726 [7]. It has been shown in animal models that leflunomide is effective in the prevention and treatment of several autoimmune disorders [8–11], and in the suppression of allograft and xenograft rejection [12–15] by reducing disease-specific antibody formation. Current clinical trials show leflunomide to be safe and effective in the treatment of patients with rheumatoid arthritis [16]. As leflunomide is known to suppress antibody formation, we hypothesized that this drug could suppress the production of allergen-specific IgE and prevent the development of allergic sensitization following parenteral allergen exposure.

In addition, leflunomide has previously been shown to inhibit the release of histamine from isolated human basophils and rat peritoneal mast cells following activation by both calcium ionophore and by IgE-dependent mechanisms [17]. These properties, if reproducible *in vivo*, could indicate a potentially important role for leflunomide the treatment of atopic asthma.

To determine the potential usefulness of leflunomide for modifying the process of allergic sensitization and allergen-induced bronchoconstriction, we used a brown Norway (BN) rat model of allergic sensitization and assessed antigen-specific antibody levels, the magnitude of the early-phase airway response after allergen challenge, and the ability of leflunomide to inhibit mast cell degranulation.

Methods

Animals and sensitization

Experiments were performed in 53 inbred male BN rats (age: 14–17 wks; weight 220–315 g). The animals were housed under specific pathogen free conditions and were allowed to feed and drink *ad libitum*. All rats were actively

sensitized on d0 by intraperitoneal (i.p.) injection with 100 μ g ovalbumin (OA) in PBS, along with 50 ng of the IgE selective adjuvant ricin. The study protocol was approved by the Institutional Animal Ethics Committee.

Animal preparation

To measure airway responses to OA challenge, the rats were weighed and subsequently anaesthetized by i.p. injection of ketamine, 60 mg/kg bodyweight, and xylazine, 3 ng/kg bodyweight. After placing the animals in the supine position, the cervical trachea was surgically exposed and a cannula was then inserted via a tracheostomy and secured with a silk ligature. A polyethylene catheter was inserted into the right femoral vein for administration of intravenous (i.v.) drugs. The rats were then connected to a small animal respirator (Harvard Rodent Ventilator 683, MA, USA) and ventilated with 10 mL/kg air at a rate of 90 strokes/min with sighs given every 5 min/ Spontaneous respiratory movements were interrupted by i.v. injection of pancuronium bromide, 0.4 mg/kg bodyweight. Subsequently, supplemental anaesthesia was given i.v. in the form of ketamine in every 15–45 min, and muscle relaxation was maintained by supplemental doses of pancuronium bromide as required. Heart rate and blood pressure were monitored via a standard electrocardiogram (78342A, Hewlett Packard Co., ID, USA). At the end of each experiment, 1.5 mL blood was collected via cardiac puncture for the estimation of OA-specific serum antibody titres; subsequently, the animals were killed with 1 mL pentobarbitone sodium.

Measurement of respiratory mechanics

Flow (V') and pressure at the airway opening (Pao) were measured using a heated pneumotachograph (8420, Hans Rudolph Inc., MO, USA) coupled to a differential pressure transducer (DP45-16, Validyne, CA., USA and a piezo-resistive pressure transducer (8507C-2, Endevco, MA, USA), respectively. Both signals were low passed filtered ($F_c = 10$ Hz, 6 pole butterworth, Applied Measurements, Victoria, Australia), digitized (12 bit DT2801-A, Data Translation, Ma., USA) and recorded on computer using a commercial acquisition and analysis package (Anadat 5.2, RHT-Infodat Inc., Que., Canada). Flow was digitally integrated with respect to time to give volume (V).

Respiratory mechanics were calculated from the Pao, V' and V signals using a multilinear regression implementation of the equation of motion of a single compartment model of the respiratory system, as follows:

$$Pao = E_{RS} \cdot V + R_{RS} \cdot V' + P_{A,EE}$$

where E_{RS} represent the elastance of the respiratory system, R_{RS} represents the resistance of the respiratory system and

$P_{A,EE}$ represents the alveolar pressure at end-expiration [18]. We have found that E_{RS} frequently varies with volume during mechanical ventilation [19]. We therefore, allow E_{RS} to vary during ventilation by including a volume-dependent term, ie:

$$E_{RS} = E_1 + E_2 \cdot V$$

where E_1 represents the volume-independent component of elastance and E_2 represents the volume-dependent component. At each measurement time respiratory mechanics were calculated from discrete 20s data epochs.

Measurement of airway responses to OA

Ovalbumin challenges were performed on d14. Initially, the rats were challenged with a 0.9% saline aerosol for 5 min; this was followed by measurements at 1 min intervals until they indicated a stable baseline. Subsequently, the animals were challenged with an aerosol of OA (50 mg/mL in 5 mL of 0.9% saline) for 5 min. Fresh antigen solution was used for each animal. Aerosols were generated with a jet nebulizer (LC PLUS, Pari-Werk GmbH, Germany) at an airflow of 5 L/min, and administered to the airways through a separate ventilatory system bypassing the pneumotachograph to prevent deposition of aerosol droplets. After exposure to the aerosol the tracheal cannula was wiped dry before being reconnected to the pneumotachograph circuit. Subsequently, measurements were performed at 1 min intervals for 1 h. The pulmonary response to OA was assessed by taking the peak value of R_{RS} and the corresponding E_{RS} value; for the purposes of comparison between animals, these values were expressed as percentage of the values after saline aerosolization.

Serum antibody measurements

OA-specific IgE titres were estimated by ELISA according to the method of van Halteren [20]. Briefly, microtitre plates (Falcon flexible assay plate 391, Becton Dickinson, CA, USA) were coated overnight at 4°C with mouse anti-rat IgE in PBS (1 µg/mL). Doubling dilutions of standards and samples were added and incubated for 3 h. Digoxigenin conjugated OA was added (1:1000) and incubated for one hour, followed by sheep anti-digoxigenin POD conjugate (1:1000) for 1 h. The peroxidase substrate TMB was used for colour development and the plates were read spectrophotometrically at 450 nm with an ELISA plate reader (automated Microplate Reader EL311, BIO-TEK Instruments Inc., VT, USA). Titers were expressed as reciprocal $\log_2$ titres. OA-specific IgG titres were determined by haemagglutination of OA-coupled sheep red blood cells and titres expressed as reciprocal $\log_2$ titres as previously described [21].

Secretion of histamine in vitro

Experiments were performed to determine if the isolated lung mast cells had been actively sensitized to *in vivo* by surface bound anti-OA IgE and were capable of releasing histamine upon challenge with soluble allergen *in vitro*. In addition, isolated lung mast cells were passively sensitized *in vitro* by incubation (2 h at 37°C) with high titre anti-OA IgE serum (1:10 dilution of a serum with a titre of > 1280) to ascertain if lack of histamine release was due to lack of surface bound anti-OA IgE or some other mechanism of inhibition.

Release of histamine *in vitro* was according to the method of Pearce *et al.* [22]. Briefly, equal numbers of mast cells ($\sim 1\text{--}5 \times 10^6$ lavage cells/tube) were incubated in a 1 mL volume of Hepes-Tyrodes buffer containing BSA (0.1%) buffer for 10 min at 37°C in the presence of various stimuli: (i) medium alone (spontaneous release), (ii) frozen, thawed and sonicated (total release), (iii) 1% OA (antigen-specific release), (iv) 48/80 (10 µg/mL, a mast cell degranulator), (v) anti-OA IgE (1:10 of a serum with a titre of > 1280, passive sensitization control), (vi) anti-OA IgE + 1%OA (passive sensitization) having been equilibrated for 5 min at 37°C. Ice-cold buffer was then added (2 mL) to stop the reaction and the tubes were spun at 3000 rpm for 10 min. The supernatant was collected and samples were stored frozen (−20°C) until the histamine assays were performed. A commercial kit was used to estimate the methyl-histamine content of the supernatants generated in the above procedures (Kabi Pharmacia Diagnostics, Uppsala, Sweden). The principle of the procedure is a double antibody radioimmunoassay where methyl-histamine in the sample competes with a fixed amount of ^{125}I -labelled methyl-histamine for the binding sites of the monoclonal antibodies. Antibody bound material was separated by the addition of a second antibody immunoabsorbent followed by incubation, centrifugation and decanting. The radioactivity in the pellet was then measured and is inversely proportional to the quantity of methyl-histamine in the sample. Quantification was achieved by reference to a standard curve generated from the methyl-histamine standard provided. Histamine release is expressed as a percentage of that in the supernatant over the total histamine content of the cells and supernatant and is corrected for spontaneous release.

Study protocols

Inhibition of allergic sensitization Six rats were treated with i.p. injections of A77 1726, the active metabolite of leflunomide, from d0 through d5 (lef d0–d5), and six animals from d5 through d10 (lef d5–d10), respectively, with daily doses of 5 mg/kg bodyweight, dissolved in 0.2 mL sterile phosphate buffered saline (PBS). The control group consisted

of nine rats, four of which received 0.2 mL sterile PBS i.p. from d0 through d5 while the other five did not receive any without further injections after sensitization. Lung function was measured before and during an inhaled OA challenge, performed on d14.

Topical administration of leflunomide prior to allergen challenge Fifteen animals were studied on day 14 (d14) following sensitization to determine whether topical administration of A77 1726 had any protective effect on bronchial responsiveness to a subsequent allergen challenge with OA. Five control animals received intra-tracheal instillation of 0.1 mL sterile PBS 60 min before the allergen challenge, while A77 1726 (100 µg or 1 mg in 0.1 mL PBS), was administered to two other groups of five animals, respectively.

Effect of leflunomide on mast cell degranulation A group of 17 animals was actively sensitized with OA/ricin as above. Nine rats were injected with A77 1726 (5 mg/kg) i.p. in 0.2 mL sterile PBS daily from d0 through d5 and eight rats acted as controls. Bronchoalveolar lavage was performed according to the method of Holt *et al.* [23] to obtain lung mast cells on d14 following sensitization. Total and differential cell counts were performed, and the percentages and absolute numbers of each cell type were calculated.

Drugs and chemicals

A77 1726 was a generous gift from Hoechst AG (Wiesbaden, Germany). OA (grade V, crystallized and lyophilized), ricin, 48/80, HEOPES and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Mouse (monoclonal) anti-rat IgE was supplied by Biosource (Camarillo, CA, USA), digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester, sheep anti-digoxigenin-POD Fab fragments from Boehringer Mannheim GmbH (Mannheim, Germany) and TMB Peroxidase Substrate and Solution B from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA), BSA was from CSL (Parkville, VIC, Australia) and RPMI 1640 from GIBCO BRL (Glen Waverley, VIC, Australia). Ketamine (Ketamil®) was purchased from Troy Laboratories (Smithfield, NSW, Australia), xylazine (Rompun®) from Bayer (Pymble, NSW, Australia), pancuronium bromide (Pancuronium®) from Astra Pharmaceuticals (North Ryde, NSW, Australia), and pentobarbitone sodium (Lethabarb®) from Virbac (Peakhurst, NSW, Australia).

Statistical analysis

The data presented as means ± SEM, and statistical comparisons among several means were performed using single-factor or two-factor ANOVA as appropriate. To analyse the

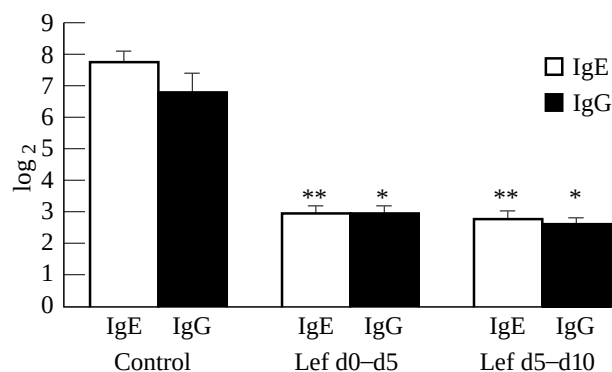


Fig. 1. OA-specific serum antibody levels in the control and leflunomide treated groups. After sensitization with OA and the adjuvant ricin on d0, the group lef d0–d5 ($n = 6$) received daily i.p. injections of 5 mg/kg A77 1726 in 0.2 mL PBS from d0 through d5, and the group lef d5–d10 ($n = 6$) from d5 through d10, respectively. The control group consisted of nine rats; four rats received daily i.p. injections with 0.2 mL PBS from d0 through d5, and five did not get any injections after sensitization. Blood was collected on d14; IgE was estimated by ELISA, and IgG by haemagglutination assay, respectively, with the titres expressed as reciprocal log₂ titres. Values are mean ± SEM. * $P < 0.0005$ vs control; ** $P < 0.00001$ vs control.

relationship between variables linear regression analysis was done using the technique of least squares. A P -value < 0.05 was considered statistically significant.

Results

Inhibition of allergic sensitization

There were no significant differences in antigen-specific serum antibody production, baseline lung function, or airway responses to OA between the two subgroups of the control animals; thus, these two subgroups were pooled for comparisons between control and leflunomide treated animals.

Effects of leflunomide on allergen specific antibody production Prior to sensitization, the sera were negative for OA-specific IgE and IgG. Following sensitization the OA-specific IgE and IgG levels increased in the control animals to 7.77 ± 0.34 and 6.77 ± 0.60 , respectively. leflunomide treatment resulted in a marked suppression of both OA-specific IgE and IgG antibody production, with these levels being 2.99 ± 0.21 and 2.99 ± 0.21 , respectively, for group lef d0–d5, and 2.82 ± 0.22 and 2.66 ± 0.21 , respectively for group lef d5 ± ± d10 (Fig. 1).

Effects of leflunomide on airway responses to OA Baseline lung function parameters were not significantly different

Table 1. Baseline lung function parameters of the three groups

Group	n	Resistance (cm H ₂ O·mL ⁻¹ ·s)	Elastance (cm H ₂ O·mL ⁻¹ ·s)
Lef d0–d5	6	0.162 ± 0.012	4.137 ± 0.259
Lef d5–d10	6	0.177 ± 0.026	4.540 ± 0.511
Control	9	0.160 ± 0.012	4.271 ± 0.239

Mean ± SEM

between the three groups Table 1). All rats showed their peak response to OA during the first 15 min after the challenge. The control group showed highly significant increased in R_{RS} (by $154.2 \pm 17.4\%$ to 0.406 ± 0.040 cm H₂O·mL⁻¹·s; $P < 0.0001$) and in E_{RS} (by $92.1 \pm 15.2\%$ to 8.171 ± 0.819 cm H₂O·mL⁻¹; $P < 0.001$) after the OA challenge (Fig. 2). In contrast, there were no increases in R_{RS} (by $4.1 \pm 2.9\%$ to 0.169 ± 0.013 in group lef d0–d5, and by $6.8 \pm 3.0\%$ to 0.189 ± 0.027 cm H₂O·mL⁻¹ in group lef d5–d10), or in E_{RS} (by $6.0 \pm 0.5\%$ to 4.385 ± 0.271 cm H₂O·mL⁻¹ in group lef d0–d5 and by $6.2 \pm 2.3\%$ to 4.792 ± 0.484 cm H₂O·mL⁻¹ in group lef d5–d10) in either leflunomide treated group (Fig. 2).

Topical administration of leflunomide prior to allergen challenge

Immunoglobulin production and lung mechanics Baseline values There were no significant differences in antigen-specific serum antibody production (IgE and IgG) at the

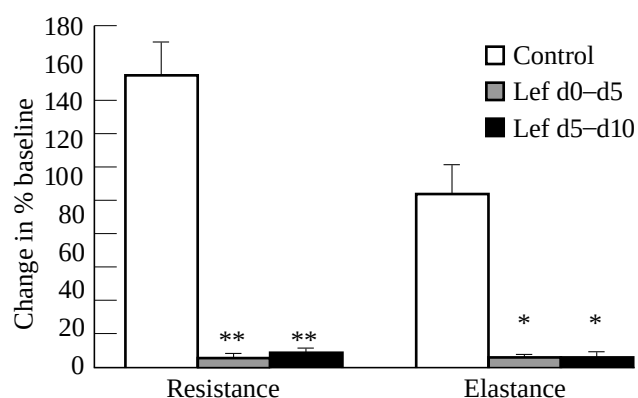


Fig. 2. Airway responses to OA challenge in the control and leflunomide treated groups on d14. Lung function after an initial challenge with 0.9% saline was taken as baseline. The airway response to OA challenge was assessed by taking the peak value of lung resistance during the first 15 min after challenge and the corresponding value of lung elastance. Values are mean ± SEM. * $P < 0.001$ vs control; ** $P < 0.00005$ vs control.

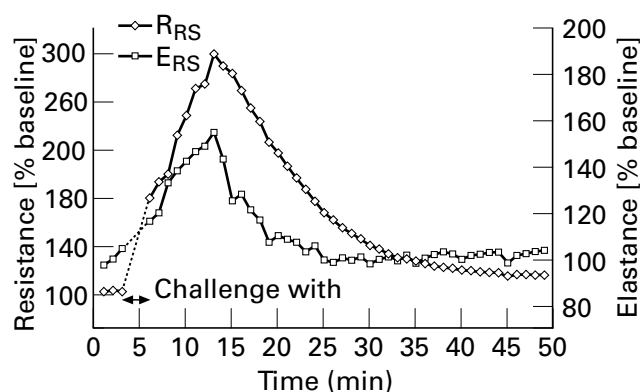


Fig. 3. Airway responses to OA challenge in one sensitized animal. R_{RS} and E_{RS} : resistance and elastance of the respiratory system.

time of lung function tests between the three groups of animals undergoing challenge with OA (Table 2). Furthermore, baseline R_{RS} and E_{RS} were not different between the two groups that received A77 1726 on one hand and the control group on the other.

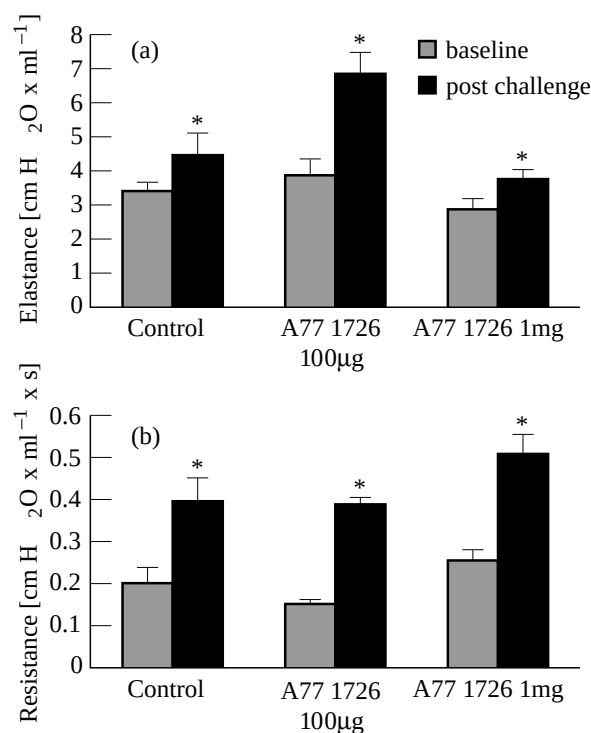


Fig. 4. Airway responses to ovalbumin challenge. A771726 100 µg and A771726 1 mg; 100 µg and 1 mg of A771726 in 0.1 mL sterile PBS, respectively, were instilled into the distal trachea 60 min before exposure to allergen. The control animals received 0.1 mL sterile PBS. *significantly higher than baseline values ($P < 0.05$).

Table 2. Specific antibody production in the three groups of actively sensitized animals prior to topical administration of A77 1726, or PBS only

Group	n	A-specific IgE (log ₂)	OA-specific IgG (log ₂)
A771726 (100 µg)	5	7.82 ± 0.35	7.22 ± 0.46
A771726 (1 mg)	5	7.92 ± 0.29	7.32 ± 0.45
Control	4	8.07 ± 0.43	7.20 ± 0.80

Mean ± SEM

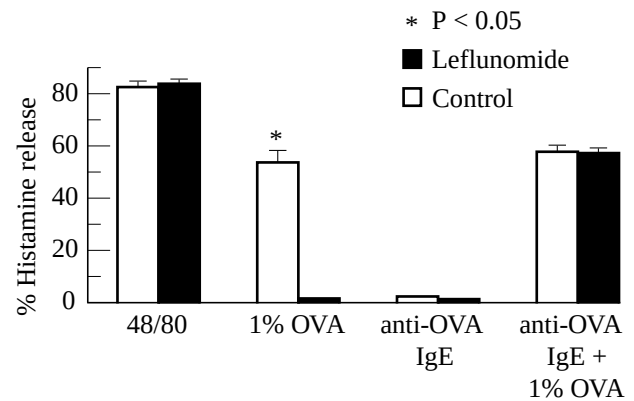
Airway responses OA One animal from the control group died shortly after the OA challenge whilst measurements were being recorded and had to be excluded from the data analysis. A representative example of changes in pulmonary mechanics after OA-induced bronchoconstriction is shown in Fig. 3. After challenge with OA all groups showed a significant increase in resistance and elastance (Fig. 4). The peak response was seen in all animals within 15 min. Topical pre-treatment with A77 1726, either with 100 µg or 1 mg did not inhibit the response to OA challenge (Fig. 4).

Effect of leflunomide on mast cell degranulation

In the A77 1726 treated group (5 mg/kg i.p. daily from d0–d5) both OA-specific IgE and IgG levels were significantly reduced compared to those of the control animals (IgE: 4.10 ± 0.22 & 9.45 ± 0.44 ; IgG: 2.77 ± 0.18 & 9.32 ± 0.33 respectively, $P < 0.0001$). However, there were no significant differences in the BAL between the A77 1726 treated group and controls with regard to total cell counts ($3.37 \pm 0.41 \times 10^6$ & $2.81 \pm 0.42 \times 10^6$ respectively) and differential cell counts (Table 2).

No significant differences were observed between A77 1726 treated group and control groups with respect to total histamine content (10.50 ± 2.45 pg & 11.00 ± 2.62 pg respectively) nor following stimulation with non-specific degranulating agent, 48/80 (Fig. 5). However, when stimulated with 1% OA a significant difference was apparent between the two groups: the A77 1726 treated group released significantly less histamine than the control group, $P < 0.05$ (Fig. 5).

Passive sensitization of mast cells in vitro To investigate whether the reduction was due to direct stabilization of mast cells by A77 1726 or due to non-sensitization since the IgE titre was much reduced in the A77 1726 treated group (see above) the cells were passively sensitized with anti-OA IgE and challenged with 1% OA. Following this procedure no significant difference was found between the A77 1726

**Fig. 5.** Histamine release from lung mast cells. BAL cells from A771726 and control groups were stimulated with 48/80 (10 µg/mL), 1% OA, anti-OA IgE (titre > 1280) or anti-OA IgE (titre > 1280) + 1% OA. Results are mean ± SEM * $P < 0.05$.

treated group and the control group (Fig. 5). Sensitization with anti-OA IgE without challenge with OA did not induce histamine release.

Discussion

The results of the present study demonstrate that leflunomide is capable of preventing the development of allergic sensitization after parenteral allergen exposure in the BN rat model of allergic asthma. The agent suppressed specific antibody production, and the early-phase airway response to allergen challenge by preventing the arming of mast cells with antigen-specific IgE antibodies. However, topical administration of leflunomide before allergen challenge in sensitized animals failed to prevent allergen-induced bronchoconstriction.

The BN rat model is a well characterized animal model of allergic asthma [24–26]. These animals almost uniformly respond to sensitization with the development of high levels of circulating antigen-specific IgE with a peak around 14 days after sensitization [27,28]. Furthermore, they reliably develop EARs and often also late-phase airway responses to inhalational challenge [24,26]. Ricin, a toxic lectin from castor beans, enhances OA-specific IgE production and augments antigen-induced airway inflammation, but does not potentiate antigen-induced airway hyperreactivity in BN rats [29]. It has been shown that ricin promotes IgE production by depletion of a subpopulation of T cells which are potent suppressors of the primary IgE response, and by increasing the synthesis of IL-4 splenic T cells [30].

In the lung of a sensitized individual, exposure to allergens causes an EAR induced by IgE-mediated mast cell degranulation with the release of bronchoconstrictor and

proinflammatory mediators [4]. This immediate reaction with a decrease in airflow generally occurs within 15 after allergen challenge and subsides within the next 30–60 min. All our control animals showed very intense airway responses to OA. In contrast, there were only small and physiologically insignificant challenge-induced increases in RRS and in ERS in both leflunomide treated groups.

The inflammatory response of the airways is one of the principal features of asthma [31]. It is characterized by the presence of activated mast cells, T lymphocytes, and eosinophils in the airway mucosa. In addition, neutrophils, macrophages, platelets, and epithelial cells have been suggested to contribute to triggering and modulating the inflammatory responses [32–34]. *In vitro* and *in vivo* studies have provided evidence that IgE-triggered mediator release from mast cells is largely responsible for the early airway response (EAR) provoked by allergen [35]. Pro-inflammatory cytokines, IL-4, IL-5, IL-6, and TNF α have been shown to be released from preformed stores by a number of cell types but mast cells in particular. In addition, increased *de novo* synthesis of these cytokines has been reported [36–38]. IgE production by allergen-specific B cells is regulated by an array of cytokines but IL-4 in particular has been shown to promote synthesis whilst IFN γ is suppressive in function for this isotype [39,40]. Thus, activation of mast cells promotes a pro-inflammatory milieu in which further IgE production is enhanced.

The EAR can be attenuated by topical administration of several drugs which interfere with different stages of the asthmatic inflammatory cascade. Nedocromil sodium (NES) and sodium cromoglycate are commonly known to elevate the threshold to bronchoconstrictive stimuli by interfering with the release of mediators from mast cells. Both drugs inhibit the release of TNF α from mast cells [41]. In addition NES has been shown to inhibit IL-2 induced histamine release from mast cells [42]. The immunosuppressive agent cyclosporin A (CsA) has been shown to inhibit IgE-dependent histamine release from human basophils [43] and so suppress mast cell- and cytokine-dependent inflammatory reactions [44]. All animals in our study reached their peak in resistance within 15 min and returned to baseline within 45 min after allergen challenge. In the present study leflunomide, given as a topical pretreatment prior to allergen challenge, did not inhibit the EAR. We chose an interval of 60 min between A771726 administration and allergen challenge. Other anti-inflammatory substances which are known to inhibit mediator release and prevent bronchoconstriction like nedocromil sodium are effective within 30 min [45].

Mast cells recovered from *in vivo* OA-sensitized, leflunomide treated animals indicated that it was the lack of OA-specific IgE that prevented histamine release on further challenge with allergen *in vitro* since upon passive sensitization with anti-OA IgE and challenge with OA there was

no significant difference between treated and control groups (Fig. 5). Bartlett *et al.* [17] demonstrated that leflunomide (2×10^{-6} M) can inhibit both IgE and A23187-dependent histamine release from isolated rat peritoneal mast cells when treated *in vitro* by direct mast cell membrane stabilization. Under the treatment regimen employed in this study (leflunomide given d0–d5 after sensitization) the local concentrations in the airways would be much less than this and thus no direct stabilization of the mast cell membrane would be expected.

Leflunomide has previously been shown, in animal models, to be effective in the prevention and treatment of several autoimmune disorders [8–11] and in the suppression of allograft and xenograft rejection [12–15] by reducing disease-specific antibody formation. The precise way in which leflunomide works is not known, however, due to the demonstrated effectiveness in several animal models, representing different aspects of the immune response, and its proven clinical efficacy in patients with rheumatoid arthritis, leflunomide's mechanism of action is of considerable interest. Presently, there are two known biochemical activities of this drug. The first reported was inhibition of tyrosine kinase [46,47] and, more recently, suppression of *de novo* pyrimidine biosynthesis [48] via inhibition of dihydroorate dehydrogenase activity [49,50]. Currently, it is not known whether one or both of these biochemical effects are responsible for the many immunomodulating activities of leflunomide.

In the present context, leflunomide is known to interfere with the activities of several cytokines, but does not inhibit the formation or the release of cytokines formed by T cells [7]. It strongly suppresses the effects of IL-3 and IL-4 as well as the activities of IL-2, GM-CSF, and TNF α . Thus, leflunomide suppresses the effects of the main cytokine promoting production of IgE and IgG1, namely IL-4 [3]. Inhibition of the effects of IL-2 on B lymphocytes would also be expected to result in suppression of OA-specific IgE and IgG [51]. The data from the present study are consistent with these findings. Indeed, our data show very clearly that leflunomide is capable of suppressing antibody responses even when given some time following primary immunization.

As patients with allergic diseases such as allergic asthma, allergic rhinitis, and atopic dermatitis show abnormalities in immune regulation, there have been attempts to develop drugs that could correct these immune dysfunctions. Several immunomodulators have recently been studied in the treatment of atopic dermatitis and asthma. These include thymopentin, a synthetic pentapeptide that promotes T cell production of IL-2 and IFN γ , and cyclosporine, which reduces IgE production and inflammation possibly by inhibiting cytokine production by T cells [52,53]. With better understanding of the

mechanisms underlying allergic diseases, immunological approaches to the treatment of these disorders may become more important in the future, e.g. the regulation of IL-4 production or action may be useful for the prevention of therapy of immediate hypersensitivity disorders. Leflunomide holds promise as a therapeutic agent in humans in transplantation and autoimmune diseases [7,16]. The data presented herein strongly suggest that this agent can also prevent allergic sensitization. While current treatment of allergic diseases is aimed at pharmacological control of symptoms due to inflammatory processes, primary prevention strategies are becoming increasingly favoured and it is here that leflunomide may have a role.

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