

Effects of long-term oral treatment with leflunomide on allergic sensitization, lymphocyte activation, and airway inflammation in a rat model of asthma

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Summary

Background Short-term treatment with leflunomide is effective in suppressing antigen-specific antibody production and allergen-induced bronchoconstriction after sensitization. This agent may thus have a role in future primary prevention strategies in allergic disease.

Objective The current study aimed to determine whether long-term oral treatment with leflunomide prevents allergic sensitization permanently.

Methods After sensitization with ovalbumin, six groups of rats ($n = 31$) were treated daily with leflunomide or diluent for up to 30 days. Ovalbumin-specific IgE and IgG were determined weekly for at least 2 weeks after cessation of treatment. T lymphocytes from another 21 animals were stimulated *ex vivo* with ovalbumin or concanavalin A.

Results Ovalbumin-specific IgE and IgG were lower during treatment with leflunomide compared with controls ($P < 0.002$) but increased after the cessation of treatment. Antigen-specific T-cell proliferation was decreased in cells obtained from leflunomide treated animals ($P < 0.05$), but not when stimulated with concanavalin A. Eosinophil ($P < 0.0001$) and neutrophil ($P < 0.02$) numbers in bronchoalveolar lavage 24 h after allergen challenge were lower in the leflunomide treated animals.

Conclusions Leflunomide prevents antigen-specific immunoglobulin production after sensitization during treatment, inhibits allergen-induced airway inflammation and diminishes antigen-specific T lymphocyte proliferation.

Keywords: leflunomide, acute-phase reaction, bronchoalveolar lavage fluid, immunoglobulins, allergic sensitization, immunomodulators, isoxazoles

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Introduction

The interaction between environmental allergens and the immune system is critical to the development of human allergy. Allergic asthma can be considered to be a combination of reversible airway obstruction, increased airway responsiveness to various stimuli, and airway inflammation [1], and is characterized by the production of antigen-specific immunoglobulins (Ig), mostly IgE. T lymphocytes and their products have been shown to be intimately

involved in the regulation of IgE production [2] and play an important role as immunoregulators in asthma [3,4]. With increasing knowledge of the cellular and molecular signals responsible for the induction of allergic inflammation, efforts have been made to develop agents which may be able to interfere with these pathways.

The isoxazole derivative leflunomide is a novel and effective immunomodulatory and anti-inflammatory substance that holds promise as a therapeutic agent. It has been demonstrated to have prophylactic and therapeutic effects in the treatment of several animal models of autoimmune disease [5,6] and graft rejection [7–9] as well as

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being safe and effective in clinical trials of human rheumatoid arthritis [10]. While its full spectrum of activity is unknown, leflunomide is known to inhibit the proliferation of T lymphocytes [11,12]. It is well absorbed orally in rats [13] and in humans [10] and undergoes rapid biotransformation, preferentially to the active metabolite A77 1726 [14].

In our previous studies [15], A77 1726, the primary metabolite of leflunomide, has been shown to be effective in suppressing antigen-specific antibody production when given for a period of 5 days after sensitization with ovalbumin. In addition, the early phase airway response to allergen challenge (which occurs within minutes after inhalation of antigen and lasts up to 1 h [16] was largely diminished in the A77 1726 treated rats, and the sensitization of mast cells *in vivo* was inhibited in these animals. These data strongly suggest that A77 1726 is able to prevent allergic sensitization and to decrease the heightened airway responsiveness seen following allergen challenge. However, it is not known whether these effects are long-lasting or whether leflunomide only temporarily decreases the immunological and physiological responses to sensitization. Addressing this question, we designed a study to investigate whether long-term oral treatment abolishes the antigen-specific immunoglobulin production after sensitization; whether this effect persists after stopping the drug; and what is the optimal duration of treatment.

The production of cytokines from TH₂-like T cells are central to the production of allergen-specific IgE antibodies and have been shown to play an important role in the development of eosinophilic inflammation of the airways [17]. The relevant allergen is presented to the T cells, which then become activated and undergo proliferation. Leflunomide has been shown not to inhibit T cell production of IL-4 [18, 19], but to inhibit the production of antigen-specific IgE [15]. Protein antigens, such as ovalbumin, house dust mite and grass pollens, are T cell dependent and cannot directly activate B cells. In order to understand how leflunomide decreases the production of allergen-specific IgE, we studied both allergen specific and non-specific T cell proliferation *ex vivo*. Finally, as the definite proof that leflunomide inhibits allergic sensitization, we measured the influx of inflammatory cells into the airways 24 h after allergen challenge *in vivo*.

Methods

Animals and sensitization

Our study was approved by the Institutional Animal Ethics Committee. Experiments were performed in 52 inbred male Brown Norway (BN) rats (age 14–17 weeks). The animals were housed under specific pathogen free conditions and were allowed to feed and drink *ad libitum*. All rats were

Sensitization (0.1 mg OA +50 ng ricin i.p.)

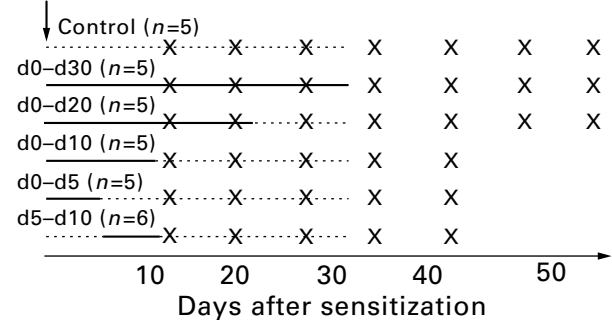


Fig. 1. Study design. Animals were sensitised on day 0 and subsequently received daily oral doses of either leflunomide 5 mg/kg in 0.2 mL carboxymethyl cellulose (CMC) [solid lines] or CMC alone [dotted lines]. X, time points where ovalbumin-specific IgE and IgG titres were determined.

actively sensitized on day 0 (d0) by intraperitoneal (i.p.) injection of 100 µg ovalbumin (OA) in PBS, along with 50 ng of the IgE selective adjuvant ricin.

Long-term oral dosing of leflunomide

Five groups of rats ($n = 26$) were treated with daily doses of leflunomide 5 mg/kg bodyweight, dissolved in 0.2 mL sterile carboxymethyl cellulose (CMC) given orally for periods up to 30 days (Fig. 1). The control group consisted of five animals, receiving 0.2 mL CMC daily for 30 days. Blood was obtained from tail veins, beginning on day 12 after sensitization, and subsequently weekly for at least 2 weeks after cessation of drug administration. For bleeding the animals were anaesthetized.

Serum antibody measurements

OA-specific IgE titres were estimated by ELISA according to the method of van Halteren *et al.* [20]. Briefly, microtitre plates (Falcon flexible assay plate 3912, Becton Dickinson, CA, USA) were coated overnight at 4 °C with mouse antirat IgE in PBS (1 µg/mL). Doubling dilutions of standards (rat serum identified to be of high IgE titre by passive cutaneous anaphylaxis assay) and samples were added and incubated for 3 h. Digoxigenin conjugated OA was added (1:1000) and incubated for 1 h, followed by sheep antidigoxigenin 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). Titres were expressed as reciprocal log₂ titres. OA-specific IgG were determined by haemagglutination of OA-coupled sheep red blood

cells and titres expressed as reciprocal $\log_2$ titre as previously described [21].

Titres of IgG1, IgG2a and IgG2b were estimated by ELISA. After coating the plates overnight with OA in PBS fetal calf serum was added for blocking. Samples and standards (rat IgG1, IgG2a and IgG2b, PharMingen, San Diego, CA, USA) were added in doubling dilutions. Biotinylated anti-IgG 1, anti-IgG2a and anti-IgG2b (PharMingen, San Diego, CA, USA) were added and incubated for 1 h. After incubation with streptavidine-HPO conjugate for 1 h TMB was added and the reaction was stopped with 1 M H_2SO_4 . The plates were read at 450 nm.

Total IgG was determined by ELISA. After coating microtitration plates (Linbro/Titertek E.I. A. ICN Biomedicals Inc., Costa Mesa CA, USA) with rabbit antirat IgG for 6 h and blocking with 2% BSA in PBS overnight doubling dilutions of standards and samples were added and incubated for 2 h. Subsequently, biotinylated sheep antirat IgG was added for 1 h, followed by incubation with streptavidin-horseradish peroxidase conjugate. TMB was used for colour development and the plates were read at 450 nm.

Lymphocyte proliferation

Preparation of cells

Twenty-one additional animals were sensitized with OA and ricin on day 0 (see above). The control group ($n = 10$) received 0.2 mL CMC daily from day 0 to day 10. Eleven animals were treated with leflunomide 5 mg/kg body weight in 0.2 mL CMC daily from day 0 to day 10. Five of the control and five of the leflunomide treated animals were bled on day 10 for the determination of OA-specific IgE and IgG levels, and then were sacrificed with 1 mL pentobarbitone sodium. The other five control and six leflunomide treated animals were bled and sacrificed on day 14, i.e. 4 days after stopping treatment. Lymph nodes (parathymic, mediastinal and mesenteric) were aseptically removed from animals into cold DAB/0.2% BSA (DAB – PBS + 0.1 $\mu\text{g}/\text{mL}$ Mg and Ca chloride). Single cell suspensions were prepared by passage of tissues through sterile gauze (125 μm) followed by centrifugation and lysis of red blood cells with NH_4Cl and used for T cell proliferation assays.

Proliferation assay

Lymph node cells were cultured at 4×10^5 per microtitre well with RPMI containing 10^{-5} M 2-ME plus antibiotics and supplemented with 5% normal rat serum. Cells were stimulated *in vitro* with OA at 100 $\mu\text{g}/\text{mL}$ or concanavalin A (ConA) at 5 $\mu\text{g}/\text{mL}$ (as positive control), or with media alone. Cultures were pulsed with tritiated thymidine at 0.5 $\mu\text{Ci}/\text{well}$ after 4 days and then incubated for a further 18 h prior to harvesting and counting in a protocol optimized

for antigen response. Experiments were performed in triplicate for each variable and means were determined.

Inhalational allergen challenge and isolation of cells in bronchoalveolar lavage fluid

Ten of the above animals, five of which had been treated with leflunomide and five with CMC orally for 10 days, were exposed to 1% OA (diluted in PBS) for 30 min within a TRI-R respiratory infection system (TRI-R Instruments Inc., NY, USA) 24 h prior to bronchoalveolar lavage (BAL). BAL was performed according to the method of Holt *et al.* [22]. Total and differential cell counts were determined.

Drugs and chemicals

Leflunomide was a generous gift from Hoechst AG (Wiesbaden, Germany). OA (grade V, crystallized and lyophilized), ricin, Tween 20, rabbit antirat IgG, and CMC were obtained from Sigma Chemical Co. (St Louis, MO, USA). Mouse (monoclonal) antirat IgE was supplied by Biosource (Camarillo, CA, USA), BSA from CSL (Parkville, VIC, Australia), rabbit antirat IgG from RPMI 1640 from GIBCO BRL (Glen Waverley, VIC, Australia), antirat IgG1, antirat IgG2a and antirat IgG2b from PharMingen (San Diego, CA, USA), Digoxigenin-3-*o*-methylcarbonyl-aminocaproic acid-N-hydroxysuccinimide ester and 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). Biotinylated sheep antirat IgG and streptavidin-horseradish peroxidase conjugate were bought from Amersham, Sydney, NSW, Australia. Pentobarbitone sodium (Lethabarb^R) was purchased from Virbac (Peakhurst, NSW, Australia). Sterile gauze for passage of tissues was obtained from Plastok, UK. Well plates for bulk cultures were purchased from Costar and human recombinant IL-2 from Cetus corporation, CA, USA.

Statistical analysis

The data are presented as means $\pm$ SEM, and statistical comparisons among several means were performed using single-factor or two-factor ANOVA as appropriate. To analyse the relationship between variables, linear regression analysis was performed using the technique of least squares. A *P*-value < 0.05 was considered statistically significant.

Results

Long-term oral treatment with leflunomide

Antibody levels

No OA-specific IgE and IgG antibodies were detectable

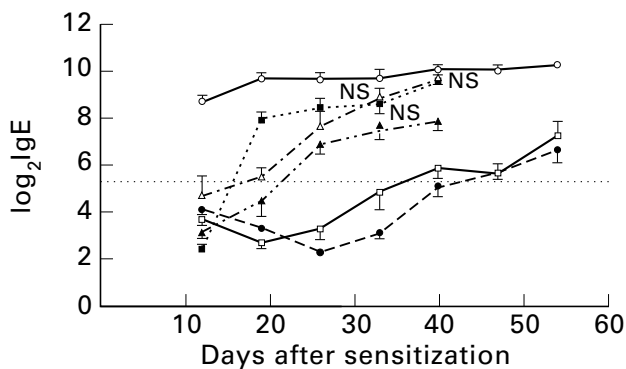


Fig. 2. Ovalbumin-specific IgE levels during long-term daily oral treatment with leflunomide. Control animals received the diluent carboxymethyl cellulose orally, whilst five groups of animals were treated with leflunomide for different periods. The dotted line represents the IgE level below which no significant changes in resistance in response to inhalational ovalbumin challenge have been found (unpublished data). IgE titres of leflunomide treated animals were lower compared with controls at all time points unless marked ns ($P < 0.005$).

before sensitization. OA-specific IgE and IgG levels were significantly lower on day 12 in all leflunomide treated groups compared with controls ($P < 0.0001$, Figs 2 & 3). During the first phase of treatment with leflunomide specific IgE levels further decreased in the groups treated from d0 to d20 and d0 to d30, respectively, and remained low during treatment in all groups. After cessation of treatment IgE and IgG titres increased in all leflunomide treated animals (Figs 2 & 3). Treatment with leflunomide starting 5 days after sensitization resulted in the shortest suppression of specific antibody production whereas the effect was most prolonged in the animals treated from d0 to d30. OA-specific IgE and IgG levels in the groups treated from d0 to d20 and from d0 to d30 were still significantly lower compared with controls on day 54 post sensitization ($P < 0.001$), but were clearly

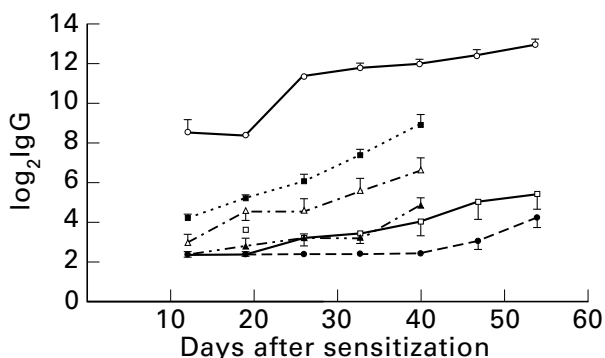


Fig. 3. Ovalbumin-specific IgG levels during long-term daily oral treatment with leflunomide. For a description of the groups of animals see Fig. 2. IgG titres of leflunomide treated animals were lower compared with controls at all time points ($P < 0.005$).

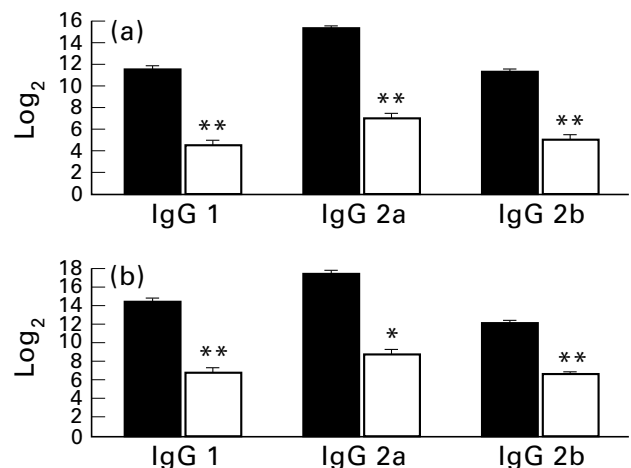


Fig. 4. Ovalbumin (OA)-specific IgG isotype levels in animals treated with leflunomide (open bars) from day 0 to day 20 after sensitization and control animals (filled bars). (a) Day 12 after sensitization, (b) Day 33 after sensitization. All three OA-specific IgG isotypes were significantly decreased compared with controls on both occasions. * $P < 0.0002$, ** $P < 0.0001$.

increasing and had achieved levels above that previously associated with an acute phase response to allergen challenge (Fig. 2).

In the animals treated with leflunomide from d0 to d20 all isotypes of ovalbumin-specific IgG were significantly diminished on two different time points (day 12 and day 33) after sensitization compared with controls (Fig. 4a&b). However, the concentration of total IgG was not significantly different between the control group and the leflunomide treated group on day 12 (3.32 ± 0.16 mg/mL vs 3.99 ± 0.25 mg/mL).

Lymphocyte proliferation

Lymphocytes obtained from animals treated with leflunomide (day 10) showed a fourfold lower proliferation when stimulated with OA compared with controls (13336 ± 271 cpm vs 53669 ± 11083 cpm, $P < 0.03$). In contrast, after stimulation with concanavalin A no significant differences were found between both groups (13305 ± 1617 vs 13163 ± 453 , respectively). Ninety-six hours after cessation of treatment (day 14), lymphocyte proliferation was not significantly different between treated and control animals after *in vitro* stimulation with OA (8657 ± 523 cpm vs 10935 ± 737 cpm, $P = 0.07$).

Cellular content of BAL fluid

BAL fluid recovered from leflunomide treated animals which had been exposed to a 1% aerosol of OA 24 h before, had 15-fold lower total numbers of eosinophils compared with controls. Total neutrophil and mononuclear

Table 1. Inflammatory cells in bronchoalveolar lavage fluid recovered from sensitized rats 24 h after inhalational challenge with ovalbumin. Percentages of the total cell counts are shown in parentheses

	TCC (10^3)	Macrophages ($\times 10^3$)	Neutrophils ($\times 10^3$)	Monocytes ($\times 10^3$)	Eosinophils ($\times 10^3$)	Lymphocytes ($\times 10^3$)
Leflunomide	1090 $\pm$ 120	834 $\pm$ 92 (76.6 $\pm$ 1.8%)	15.5 $\pm$ 5.1 ** (1.3 $\pm$ 0.3%)	5.3 $\pm$ 1.9 (0.5 $\pm$ 0.2%)	*25.7 $\pm$ 6.4 *** (2.2 $\pm$ 0.4%)	198.4 $\pm$ 31.8 (18.2 $\pm$ 1.9%)
Controls	1638 $\pm$ 230	967.8 $\pm$ 146.9 (58.8 $\pm$ 1.9%)	61.3 $\pm$ 15.1 (4.0 $\pm$ 1.1%)	10.7 $\pm$ 3.4 (0.9 $\pm$ 0.2%)	409.0 $\pm$ 50.4 (25.3 $\pm$ 1.3)	185.4 $\pm$ 43.4 (11.0 $\pm$ 1.2%)

* $P < 0.05$, ** $P < 0.02$, *** $P < 0.0001$

leucocyte counts were also significantly decreased (Table 1). BAL total cell counts tended to be lower in the leflunomide treated group ($1.09 \pm 0.12 \times 10^6$ vs $1.64 \pm 0.23 \times 10^6$, $P < 0.07$).

Discussion

In the present study, utilizing a rat model of asthma, we have shown that long-term oral treatment with leflunomide transiently inhibits specific antibody production after allergic sensitization and diminished the influx of inflammatory cells into the airways 24 h following inhalational allergen challenge. During treatment with leflunomide, antigen-specific proliferation of T cells was inhibited. T cell proliferation in response to mitogenic stimuli was unaltered. Although a critical role for T cell clonal expansion has not been established in allergen-induced airway inflammation, our observations that leflunomide inhibits both the production of allergen-specific antibodies and the influx of inflammatory cells following allergen challenge suggests that the T cell system may be directly involved in this process.

The central regulatory role of the T cell in allergy and IgE production is well established [23] and it has been suggested that the development and maintenance of allergic inflammation is due to T lymphocyte activation [24,25]. T cell activation in these circumstances leads to an increase in the production of TH₂ type cytokines such as IL-4 which is crucial to the B cell switch to IgE production and also leads to an increase in production of cytokines and chemokines crucial for cellular infiltration.

We have previously shown that during treatment with leflunomide OA-specific IgE and IgG production are reduced to levels equivalent to those of naive, nonsensitized control animals [15]. All isotypes tested of OA-specific IgG were equally effected whilst total IgG levels were not. In the rat the production of IgG1 is associated with TH₂ type responses whilst IgG2a and 2b are associated with TH₁ [26,27]. Our results with IgG isotyping of this response

suggest that leflunomide does not function selectively on either TH₁ or TH₂ type responses to effect the balance between these populations. This is consistent with studies showing that synthesis and release of cytokines from TH₁ or TH₂ lymphocytes is not effected by leflunomide although the ensuing action of these cytokines may be antagonised by the drug [18]. Since total IgG levels are not effected we suggest that its action is antigen-specific but not generally suppressive of antibody responses. Evidence in the literature suggests that leflunomide acts on B cells during the active antibody-secreting stage not in the early differentiation stage of development [28]. Recovery of antibody levels on stopping the drug (Figs 2 and 3) is supportive of a temporary block of cell cycling rather than a terminal or toxic effect on B cells. IgE antibody levels are seen to start increasing almost immediately on stopping treatment and are heading for control levels after 10 days (Fig. 2), with a longer lag for IgG antibodies (Fig. 3).

We also observed decreased antigen specific T cell proliferation in response to OA stimulation *in vitro* during administration of leflunomide, whereas stimulation with the T cell mitogen ConA resulted in normal lymphocyte proliferation. These data also suggest that the effects of leflunomide are antigen specific. It might be suggested that the decreased production of antigen-specific antibody in this model is a direct effect of this antigen-specific inhibition of T cell proliferation leading to inadequate production of cytokines such as IL2 and IL4 and T cell help for antibody production. However, previous studies by others indicate that inhibition of cytokine production is not the mechanism by which leflunomide effects cellular proliferation with the production of IL2 and level of expression of IL2- receptor being relatively unaffected [29]. It has also been demonstrated that whilst leflunomide does not impair the ability of T cells to produce various cytokines it is capable of blocking the clonal expansion of T cells [19] and it is this activity of the drug which may explain the effect on antigen-specific proliferation seen in our model.

Since leflunomide appears to be effecting antigen-specific

responses there are several levels at which it could be acting. It is possible that it could be effecting antigen presenting cell function through expression or interaction of costimulatory molecules or it may be acting at the level of membrane events of antigen-specific interactions, alternatively it may be acting at the level of intracellular cell signalling events. It has been demonstrated that leflunomide inhibits tyrosine kinase activity and this activity may be responsible for its antiproliferative effects [11]. It has also been suggested that its antiproliferative properties are due to effects on dihydroorotate dehydrogenase activity [12] or due to interruption of *de novo* pyrimidine nucleotide biosynthesis [30] but the antigen-specific nature of its effects in this model remain to be adequately explained and are under current investigation by this group.

The inhibition of antigen-specific proliferation is rapidly reversible since 96 h after cessation of leflunomide treatment no significant difference in proliferation is found between treated and control groups. The cessation of drug administration and the recovery of antigen stimulated T cell proliferation is rapidly followed by an increase in the production of antibody. Therefore, continuous treatment is required to bring about these effects which are relatively readily reversed once treatment is stopped. Although we were not able to measure tissue concentrations of A77 1726, cessation of treatment presumably results in a rapid decrease below the antiproliferative threshold. The mean residence time of A77 1726 after oral administration of leflunomide in rabbits is 10.5 ± 2.6 h [31]. These data indicate that the mechanism of action of leflunomide is not cytotoxic/apoptotic since its effects are reversible and this confirms previous studies which demonstrated that its antiproliferative effects were reversible [32].

It has been recently demonstrated in a mouse model that mast cells play an important role in the recruitment of eosinophils into the lung after sensitization and allergen challenge [33], suggesting that the release of mast cell mediators activates and recruits eosinophils. We have demonstrated here that leflunomide treatment reduces IgE production and therefore, presumably, IgE activation of mast cells. We have previously shown that IgE dependent mast cell degranulation is inhibited *in vitro* by leflunomide via prevention of synthesis of allergen-specific IgE [15]. Mast cell mediators are also known to have an effect on the integrity of the endothelial barrier through which these cells pass when migrating from the blood stream into the lung. Thus it seems possible that the suppression of antigen-specific IgE demonstrated here is the initial effect also leading to the reduction observed in cellular infiltration, although other mechanisms may also be involved and are currently under investigation by this group.

The actions of leflunomide described above make this agent a potentially useful agent for use in prevention and

treatment of allergic sensitization. Successful prevention of allergic sensitization may result in the prevention of atopic conditions, including atopic dermatitis and asthma [34]. From a theoretical point of view, an agent which inhibits T cell proliferation in a non-specific fashion may be dangerous as T cell proliferation is central to both cell mediated and humoral immunity. The results of the present study demonstrate that leflunomide inhibits antigen-specific T cell proliferation but not proliferation to mitogen.

Together with our previous study [15], this report indicates that oral treatment with leflunomide effects the inflammatory cascade in allergic asthma through inhibition of antigen-specific T cell proliferation, antigen-specific immunoglobulin production and cellular infiltration of the lung. Thus, this drug may have considerable benefits in preventative and therapeutic approaches to asthma treatment and warrants further investigation.

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References

- 1 Djukanovic R, Roche WR, Wilson JW et al. Mucosal inflammation in asthma. *Am Rev Respir Dis* 1990; 142:434-57.
- 2 Leung DYM, Geha RS. Regulation of the human IgE antibody response. *Intern Rev Immunol* 1987; 2:75-91.
- 3 Corrigan CJ, Hartnell A, Kay AB. T lymphocyte activation in acute severe asthma. *Lancet* 1988; 1:1129-32.
- 4 Walker C, Kaegi MK, Braun P, Blaser K. Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J Allergy Clin Immunol* 1991; 88:935-42.
- 5 Vidic-Dankovic B, Kosec D, Damjanovic M et al. Leflunomide prevents the development of experimentally induced myasthenia gravis. *Int J Immunopharmacol* 1995; 17:273-81.
- 6 Robertson SM, Lang LS. Leflunomide: inhibition of S-antigen induced autoimmune uveitis in Lewis rats. *Agents Actions* 1994; 42:167-72.
- 7 Mrowka C, Thoenes GH, Langer KH, Bartlett RR. Prevention of the acute graft-vs.-host disease (GVHD) in rats by the immunomodulating drug leflunomide. *Ann Hematol* 1994; 68:195-9.
- 8 Xiao F, Chong AS, Foster P et al. Leflunomide controls rejection in hamster to rat cardiac xenografts. *Transplantation* 1994; 58:828-34.
- 9 McChesney LP, Xiao F, Sankary HN et al. An evaluation of

- leflunomide in the canine renal transplantation model. *Transplantation* 1994; 57:1717–22.
- 10 Mladenovic V, Domljan Z, Rozman BJ et al. Safety and effectiveness of leflunomide in the treatment of patients with active rheumatoid arthritis. *Arthritis Rheum* 1995; 38:1595–603.
 - 11 Xu X, Williams JW, Bremer EG, Finnegan A, Chong AS. Inhibition of protein tyrosine phosphorylation in T cells by a novel immunosuppressive agent, leflunomide. *J Biol Chem* 1995; 270:12398–403.
 - 12 Greene S, Watanabe K, Braatz-Trulson J, Lou L. Inhibition of dihydroorotate dehydrogenase by the immunosuppressive agent leflunomide. *Biochem Pharmacol* 1995; 50:861–7.
 - 13 He G, McAlister VC, Lee TD et al. Oral leflunomide prevents small bowel allograft rejection in the rat. *Transplantation Proc* 1994; 26:1613.
 - 14 Leflunomide Investigator's Brochure. Wiesbaden, Germany, Hoechst AG, March 1995.
 - 15 Eber E, Uhlig T, McMenamin C, Sly PD. Leflunomide, a novel immunomodulating agent, prevents the development of allergic sensitisation in an animal model of allergic asthma. *Clin Exp Allergy* 1998 28:376–84.
 - 16 Hargreave FE, Dolovich J, Robertson DG, Kerigan AT. The late asthmatic response. *Can Med Assoc J* 1974; 110:415–24.
 - 17 Copple AJ, Wagner K, Bertrand C et al. Central role of immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and T helper 2 cell cytokine production: inhibition by a non-anaphylactogenic anti-IgE antibody. *J Exp Med* 1996; 183:1303–10.
 - 18 Bartlett RR, Campion G, Musikic P et al. Leflunomide: A novel immunomodulating drug. In: Lewis AJ, Furst DE, eds *Non-steroidal anti-inflammatory drugs. Mechanisms and clinical uses*. New York, Basel, Hong Kong: Marcel Dekker, Inc. 1994: 349–366.
 - 19 Lang R, Wagner H, Heeg K. Differential effects of the immunosuppressive agents cyclosporine and leflunomide in vivo. Leflunomide blocks clonal T cell expansion yet allows production of lymphokines and manifestation of T cell-mediated shock. *Transplantation* 1995; 59:382–9.
 - 20 Van Halteren AGS, van der Cammen MJF, Biewenga J, Savelkoul HFJ, Kraal G. IgE and mast cell responses upon intestinal allergen exposure. A murine model to study the onset of food allergy. *J Allergy Clin Immunol* 1997; 99:94–9.
 - 21 Sedgwick JD, Holt PG. Induction of IgE. -isotype specific tolerance by passive stimulation of the respiratory mucosa. *Immunol* 1983; 50:625–30.
 - 22 Holt PG, Warner LA, Papadimitriou JM. Alveolar macrophages: functional heterogeneity within macrophage populations from rat lung. *Aust J Exp Biol Med Sci* 1982; 60:607–18.
 - 23 Kay AB, Corrigan CJ, Frew AJ. The role of cellular immunology in asthma. *Eur Resp J* 1991; 4:105s–112s.
 - 24 Rawle FC, Mitchell EB, Platts-Mills AET. cell responses to the major allergen from the house dust mite *Dermatophagoides pteronyssinus*, antigen P1: comparison of patients with asthma, atopic dermatitis, and perennial rhinitis. *J Immunol* 1984; 133:195–201.
 - 25 Kapsenberg ML, Wierenga EA, Bos JD, Jansen HM. Aberrant T cell regulation of IgE production in atopy. *Eur Resp J* 1991; 13:27s–30s.
 - 26 Gracie JA, Bradley JA. Interleukin 12 induces interferon-gamma dependent switching of IgG alloantibody subclass. *Eur J Immunol* 1996; 26:1217–21.
 - 27 Savudi A, Kuhn J, Huygen K et al. TH2 activated cells prevent experimental autoimmune uveoretinitis, a TH1 dependent autoimmune disease. *Eur J Immunol* 1993; 23:3096–103.
 - 28 Siemasko KF, Chong ASF, Williams JW, Bremer EG, Finnegan A. Regulation of B cell function by the immunosuppressive agent Leflunomide. *Transplantation* 1996; 61:635–42.
 - 29 Cherwinski HM, McCarley D, Schatzman R, Devens B, Ransom JT. The immunosuppressant leflunomide inhibits lymphocyte progression through cell cycle by a novel mechanism. *J Pharmacol Expr* 1995; 272:460–8.
 - 30 Cao WW, Kao PN, Chao AC et al. Mechanism of the antiproliferative action of leflunomide. A77 1726, the active metabolite of leflunomide, does not block T-cell receptor-mediated signal transduction but its antiproliferative effects are antagonized by pyrimidine nucleosides. *J Heart Lung Transpl* 1995; 14:1016–30.
 - 31 Lucien J, Dias VC, LeGat DF, Yatscoff RW. Blood distribution and single-dose pharmacokinetics of leflunomide. *Ther Drug Monit* 1995; 17:454–9.
 - 32 Bartlett RR, Dimitrijevic M, Mattar T et al. Leflunomide (HWA 486), a novel immunomodulating compound for the treatment of autoimmune disorders and reactions leading to transplantation reaction. *Agents Actions* 1991; 32:10–21.
 - 33 Kung TT, Stelts D, Zurcher JA et al. Mast cells modulate pulmonary eosinophilia in mice. *Am J Respir Cell Mol Biol* 1995; 12:404–9.
 - 34 Holt PG, Sly PD. Allergic respiratory disease: strategic targets for primary prevention during childhood. *Thorax* 1997; 52:1–4.