

Oral Administration of Leflunomide (HWA486) Results in Prominent Suppression of Immunoglobulin E Formation in a Rat Type 1 Allergy Model

YUKO MIZUSHIMA, YUKIO AMANO, HIROSHI KITAGAWA and KENJI OGATA

Autoimmune Diseases Project, Discovery Research Laboratories, Hoechst Japan, Ltd., Saitama, Japan

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ABSTRACT

Leflunomide, a drug being developed for use in the treatment of rheumatoid arthritis, was evaluated in an ovalbumin (OVA)-induced rat type 1 allergy model. In a dose of 1 mg/kg/day, it strongly suppressed the formation of OVA-specific IgE, thus preventing the elevation of the plasma histamine level and induction of anaphylactic shock observed after i.v. challenge with OVA. Studies on leflunomide's effects on the secondary antibody response showed that administration during the primary immune response remarkably diminished the secondary antibody responses, except IgM, even without further drug administration. Furthermore, when administered during the secondary response after rechallenge, both the total IgE and OVA-specific IgE serum levels declined rapidly to nearly base-

line levels. Although OVA-specific IgG1, IgG2a, and IgM did not decrease from their primary response levels, these classes' secondary responses were strongly suppressed. In an in vitro study, the proliferation and antibody production of OVA-stimulated spleen cells, derived from Brown Norway rats, were strongly inhibited by **A77 1726**, leflunomide's active metabolite. When uridine was added to the cell culture, this molecule's effect on cell proliferation was completely restored, whereas the antibody production was partially restored. These findings are consistent with data indicating that leflunomide is a dihydroorotate dehydrogenase inhibitor. Taken together, the above findings suggest the therapeutic potential of leflunomide against type 1 allergic diseases.

Leflunomide (HWA486) is a novel immunoregulating drug that was originally found as an anti-inflammatory agent. Leflunomide is a pro-drug and insoluble in water. After oral administration, leflunomide is rapidly converted to its active, primary metabolite, **A77 1726**, in the intestinal wall and liver. Leflunomide has been shown to be efficacious in both preventive and curative treatment of several animal models of autoimmune diseases. These models include adjuvant arthritis in rats (Bartlett and Schleyerbach, 1985), experimental allergic encephalomyelitis in rats (Schorlemmer and Bartlett, 1994), and experimental autoimmune tubulointerstitial nephritis in rats (Thoenes et al., 1989) and MRL/lpr mice (Bartlett et al., 1988). Moreover, leflunomide prevented both acute (Mrowka et al., 1994) and chronic (Popovic and Bartlett, 1987) graft-versus-host reactions, prolonged the survival time of kidney and skin allografts in rats (Küchle et al., 1991), and induced skin allograft tolerance (Schorlemmer et al., 1993). Currently, leflunomide is under development as a drug for use in the treatment of rheumatoid arthritis. The results of a phase II clinical trial demonstrated that

leflunomide was well tolerated and efficacious in patients with rheumatoid arthritis (Mladenovic et al., 1995).

Recently, it was demonstrated that **A77 1726** inhibits de novo pyrimidine biosynthesis. This is achieved through its direct binding with high affinity to a mitochondrial protein, dihydroorotate dehydrogenase (DHO-DH) (Williamson et al., 1995), an enzyme that is responsible for the conversion of dihydroorotate to orotate during de novo pyrimidine synthesis. Thus, leflunomide's in vitro antiproliferative action on lymphocytes can be explained by its inhibition of de novo pyrimidine biosynthesis (Williamson et al., 1995; Zielinski et al., 1995). On the other hand, it has also been reported that **A77 1726** inhibited various tyrosine kinases, such as epidermal growth factor receptor (Matar et al. 1993), p56^{lck} and p59^{lyn} (Xu et al., 1995), and mitogen-activated protein kinase (Rebbaa et al., 1996), implying that the effects of leflunomide on the immune system are due to multiple molecular mechanisms. Bartlett and colleagues (Bartlett et al., 1991) reported that mouse splenic B lymphocytes, stimulated with lipopolysaccharide, showed similar sensitivity to **A77 1726** as did mouse splenic T lymphocytes stimulated with either concanavalin A or pokeweed mitogen in a proliferation assay.

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ABBREVIATIONS: leflunomide, *N*-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide; **A77 1726**, *N*-(4-trifluoromethyl)phenyl-2-cyano-3-hydroxycrotonic acid amide; DHO-DH, dihydroorotate dehydrogenase; BN, Brown Norway; alum, aluminum hydroxide gel; CMC, carboxymethylcellulose; POD, peroxidase; OVA, ovalbumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

However, the proliferation of established B cell lines and hybridomas tended to be more sensitive to **A77 1726** than the proliferation of other cell lineages, including T cell lines. Another study (Siemasko et al., 1996) also demonstrated that when splenic B lymphocytes were activated with mitogen, their antibody production was more sensitive to **A77 1726** than their proliferative activity. Due to the great suppressive potential of leflunomide on the formation of antibodies, we were interested in studying its activity on type 1 allergy. Here, we report our findings of leflunomide's effects on the primary and secondary allergic responses in an OVA-induced type 1 allergy model in Brown Norway (BN) rats.

Materials and Methods

Animals

Female BN rats, 7 weeks of age, were obtained from Charles River Japan, Inc., Yokohama, and, in principle, divided into groups of six. Studies were conducted under the guidelines of the Animal Care and Use Committee of Hoechst Japan, Ltd.

Sensitization

BN rats were primary immunized at the age of 8 weeks. OVA (Sigma, St. Louis, MO), as antigen, and aluminum hydroxide gel (alum; Pierce, Rockford, IL), as an adjuvant, were used. In studies of the primary response, a mixture of OVA (1 mg/ml) and alum (30 mg/ml) was i.p. injected at 10 ml/kg (10 mg/kg of OVA) on day 0. For challenge, 2 ml/kg (60 mg/kg) OVA in saline was i.v. injected on day 10 to induce anaphylactic shock. In studies of the secondary response, a mixture of OVA (0.1 mg/ml) and alum (1 mg/ml) was similarly injected at 1 ml/body (0.1 mg of OVA/body) in both the first (on day 0) and secondary (on day 28) immunizations.

Drug Administration

Both leflunomide (Hoechst AG, Wiesbaden, Germany) and prednisolone (Sigma) were suspended in 1% carboxymethylcellulose (CMC; Kanto Chemical, Chuou-ku, Tokyo) and administered orally.

Study of Primary Response (Anaphylactic Shock Model)

Blood was collected from the tail vein using a heparin-coated glass capillary tube on days 0, 3, 5, 7, and 9. The sera were prepared and used to determine the time courses of the total IgE level and the OVA-specific IgE titer. Anaphylactic shock was evaluated by observation of the animal's behavior just after the OVA challenge and by determining the histamine level in the plasma before and after the challenge.

Study of Secondary Response

On days 0, 5, 7, 10, 14, 21, 28, 31, 33, 35, 38, and 42 after primary immunization, blood was collected from the tail vein. The sera were prepared and used to determine the time courses of the total IgE level as well as the OVA-specific IgE, IgG1, IgG2a, and IgM titers. Table 1 summarizes the experimental design.

TABLE 1
Experimental design of the study of the inhibitory effect of leflunomide on the secondary immune response in the BN rat model of type 1 allergy

Group ^a	No. of Rats/Group	Immunization		Leflunomide Treatment	
		Primary (day 0)	Secondary (day 28)	Days 0–24	Days 28–42
1	6				
2	6	OVA	OVA	2 mg/kg	
3	6	OVA	OVA		1% CMC
4	6	OVA	OVA		2 mg/kg

^a Group 1: untreated control; group 2: group receiving leflunomide during the period of the primary immune response; group 3: CMC control receiving 1% CMC alone; group 4: group receiving leflunomide during the period of the secondary immune response.

Determination of Plasma Histamine Level

Blood was collected from the jugular vein of conscious rats before and 3 to 5 min after challenge with OVA, transferred into an ice-cold tube containing EDTA, and immediately subjected to cold centrifugation to separate the plasma. The plasma was stored at –20°C until analyzed. The plasma histamine levels were determined by radioimmunoassay using a histamine kit (Eiken Chemical, Bunkyo-ku, Japan).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used for determination of the total IgE concentration and the titers of OVA-specific IgE, IgG1, IgG2a, and IgM antibodies. Plates coated with an anti-rat IgE monoclonal antibody were used to measure the total IgE and OVA-specific IgE titers. Plates coated with OVA were used for assay of the OVA-specific IgG1, IgG2a, and IgM titers. For the total IgE and OVA-specific IgE assays, the sera used as standards and the serum samples were diluted 100 times, whereas for IgG1, IgG2a, and IgM, they were diluted 1000 times with casein buffer. The standards were adjusted so that each contained the same concentration of serum as the samples. For preparation of the sequential dilutions of the standards and test samples with higher titers than the standards, casein buffer including 1% IgE-free rat serum was used for the total IgE and OVA-specific IgE, whereas 0.1% rat serum was used for IgG1, IgG2a, and IgM. All binding reactions were conducted at room temperature and with shaking. Washing was done three times, using phosphate-buffered saline (PBS) containing 0.1% Tween 20 in an ELISA autoprocessor, BEP-II (Behringwerke AG, Marburg, Germany). Tetramethylbenzidine solution (Behringwerke AG) was added to the wells for color development, and immediately after adding the stop solution (0.5 N H₂SO₄), the absorbance was measured at 450 and 650 nm, the latter as a reference. Standards and samples were determined in duplicate. The data were analyzed using either a log-log curve-fit (total IgE) or, for OVA-specific antibodies, a 4-parameter curve fit software package (Delta Soft).

Determination of IgE Level in Serum

Round-bottomed microtiter plates for ELISA (96-well Maxisorp plate; Nunc, Inc., Naperville, IL) were coated with 50 μl/well of 10 μg/ml anti-rat IgE antibody (mouse monoclonal antibody; Zymed Laboratories, Inc., South San Francisco, CA) in PBS and incubated for 90 min at room temperature. The volume of each solution used in the rest of this assay was 50 μl. After the plate was washed, the IgE standards or test samples were added to the wells and incubated for 1 h. The IgE standards were prepared by adding a definite amount of purified rat IgE (Chemicon International, Temecula, CA) to IgE-free, Sprague-Dawley rat serum (IgE-free serum) which had been obtained by adsorption of the IgE antibody onto a protein G-Sepharose 4B column (Pharmacia Biotech, Uppsala, Sweden) coated with an anti-rat IgE antibody (Nordic Immunological Laboratories, Tilburg, the Netherlands). After washing the plate, peroxidase (POD)-labeled anti-rat IgE antibody (Nordic Immunological Laboratories) was added to each well, and the plate was incubated on a shaker for 1 h at room temperature to allow serum IgE to react with the antibody.

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After the plate was washed, color development, addition of the stop reagent, and measurement of the optical density were carried out.

Determination of OVA-Specific IgE Titer

For the determination of OVA-specific IgE, plates coated at room temperature for 90 min with 100 μ l/well of 10 μ g/ml monoclonal anti-rat IgE antibody in PBS were used. The volume of each solution used in the rest of this assay was 100 μ l. After washing the plate, the standards or test samples were added to duplicate wells. The standards were prepared as follows: serum collected on day 9 from a sensitized female BN rat was diluted 1:100 with casein buffer and arbitrarily assigned a value of 32 U. Then 2-fold serial dilution to 0.5 U was performed with casein buffer containing 1% IgE-free serum. The samples were allowed to react on a shaker for 1 h at room temperature. Then the plates were washed, biotin-labeled OVA was added to each well, and the plates were incubated on a shaker for 90 min at room temperature. After washing, 1000-times diluted POD-labeled streptavidin (Amersham International, Buckinghamshire, England) was added to the wells and the plates were incubated on a shaker for 30 min. After the plates were washed, color development with tetramethylbenzidine, addition of stop reagent, and measurement of the optical density were carried out. The limit of detection in this experimental system was 0.5 U.

Determination of OVA-Specific IgG1, IgG2a, and IgM Titers

ELISA plates were coated with 50 μ l/well of 5 mg/ml OVA in PBS and incubated at 4°C overnight to prepare OVA-coated plates. The volume of each solution used was 50 μ l in the rest of these assays. After the OVA-coated plates were washed, the standards or samples were added to the wells and allowed to react for 1 h. The standard serum used in the ELISA of OVA-specific IgE was also used in these assays. That is, the antiserum diluted 1:1000 with casein buffer was arbitrarily assigned a value of 32 U, and 2-fold serial dilution to 0.5 U or 0.125 U was performed with casein buffer containing 0.1% serum of normal female Sprague-Dawley rats and used as the standards. After the plates were washed, POD-labeled anti-rat IgG1 antibody (mouse monoclonal antibody; Zymed Laboratories), POD-labeled anti-rat IgG2a antibody (mouse monoclonal antibody; Zymed Laboratories), and POD-labeled anti-rat IgM antibody (mouse monoclonal antibody; Zymed Laboratories) were diluted 1:4000, 1:4000, and 1:1000, respectively, with casein buffer and added to the wells for the respective OVA-specific IgG1, IgG2a, and IgM assays. The plates were incubated on a shaker for 1 h and washed. Color development, addition of the stop solution, and measurement of the optical density were carried out. The limit of detection was 0.5 U for OVA-specific IgG1 and IgG2a, and 0.1 U for OVA-specific IgM.

Antibody Titer

The mean and S.D. of the antibody titer in each group of six rats were calculated and are shown in the figures and tables. For samples whose titer was out of the quantitatively detectable range, the mean and S.D. were calculated using the detection limit value.

In Vitro Experiments

Spleen Cell Preparation. A spleen cell suspension was prepared from BN rats. Briefly, BN rats were i.p. injected with 10 mg of OVA/300 mg of alum/kg for the primary immunization. The secondary immunization consisted of injection of 10 μ g of OVA/100 μ l of saline/body to the foot pad 10 days after the primary immunization. More than 1 week after the last immunization, the spleen was removed from the anesthetized rats. The spleen cells were suspended in RPMI 1640 containing 2% fetal bovine serum (2% FBS-RPMI 1640), washed three times in 2% FBS-RPMI 1640, and resuspended in 10% FBS-RPMI.

Treatment of Spleen Cells with A77 1726 and Uridine. A77 1726 (27 mg) was dissolved in 10 ml of 2% NaHCO₃ containing 0.9%

NaCl and sterilized by filtration through a 0.2- μ m filter to prepare a 10 mM A77 1726 stock solution. This stock solution was diluted with PBS to 1 mM and then further diluted to 0.2 mM, 0.04 mM, and 0.008 mM with 10% FBS-RPMI 1640. Uridine was dissolved and further diluted to 1 mM, 0.2 mM, and 0.04 mM with 10% FBS-RPMI 1640. The OVA-primed spleen cells were adjusted to a concentration of 2×10^6 cells/ml in 10% FBS-RPMI 1640. To each well of 96-well flat-bottomed culture plates, 0.1 ml of the spleen cell suspension, 0.02 ml of 10 mg/ml OVA, 0.02 ml each of various concentrations of A77 1726 and uridine, and 0.04 ml of 10% FBS-RPMI 1640 were then added. This stimulation of the spleen cells was performed in triplicate for 72 h. Six hours before harvest, 18.5 kBq of [³H]thymidine was added to each well. Proliferation was quantitatively evaluated on the basis of the uptake of [³H]thymidine by rat spleen cells.

Antibody Production

The same spleen cell preparation as for the proliferation assay above was used. Twenty-four-well culture plates were used for this experiment. Briefly, 0.5 ml of the spleen cell suspension adjusted to 1×10^7 cells/ml in 10% FBS-RPMI was added to each well. Then, 0.1 ml of A77 1726 (0.008, 0.04, and 0.2 mM), 0.1 ml of OVA (2 mg/ml), 0.1 ml of uridine (0.04, 0.2, and 1 mM), and 0.2 ml of 10% FBS-RPMI 1640 were added to each well. The plates were then incubated at 37°C for 3 days. Three days later, the cultured spleen cells were collected from duplicate wells into separate 15-ml tubes. The cells were washed five times with 2% FBS-RPMI 1640 and once with 10% FBS-RPMI 1640. Finally, the spleen cells were resuspended in 1 ml of 10% FBS-RPMI 1640 and dispersed into 12-well culture plates. Next, 0.6 ml of 10% FBS-RPMI 1640, 0.2 ml of A77 1726 (0.008, 0.04, and 0.2 mM), and 0.2 ml of uridine (0.04, 0.2, and 1 mM) were added to each well. The plates were incubated for 4 days. Four days later, the culture medium was collected from each well and filtered through a 0.2- μ m filter membrane. The antibody in each medium was determined by ELISA.

Statistical Analysis

Statistically significant differences were determined by Welch's *t* test for validation of the results derived from the anaphylactic shock model. For analysis of the secondary response, Wilcoxon's signed rank test was applied to the time course change within the same group, Steel's test was used for comparison of groups assayed on the same day, and Mann-Whitney's *U* test was used for comparison between two groups assayed on different days.

Results

Inhibitory Effects of Leflunomide on Primary Immune Response

Therapeutic Dose. An anaphylactic shock model in rats was used to determine the effective dose of leflunomide under 10-day repeated administration.

The total serum IgE levels were measured over time in OVA-sensitized BN rats that were orally administered leflunomide at 0.5, 1, or 2 mg/kg/day from day 0 to 9 after sensitization. The control group, administered 1% CMC alone (CMC control group, *n* = 6), showed an increase in the IgE levels beginning from day 5, and on day 7 the value was significantly higher than the initial value (day 0) of the same group (Fig. 1). The mean value on day 9 reached nearly 500 μ g/ml (Table 2, experiment 1). In the leflunomide 0.5 mg/kg group (*n* = 5), the IgE antibody level significantly increased on and after day 7 compared with day 0 (Fig. 1). The mean value in the leflunomide 0.5 mg/kg group on day 9 was 358 μ g/ml, and the reduction of the IgE antibody level was not significant (Table 2, experiment 1). On the other hand, the

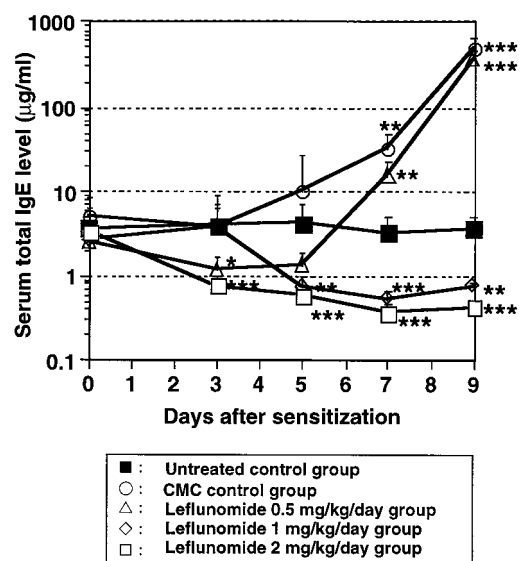


Fig. 1. Time course of serum total IgE level after sensitization with OVA and the inhibitory effect of leflunomide. The serum total IgE level was determined for blood collected from BN rats before and after sensitization with OVA. Leflunomide was administered daily from days 0 to 9 after sensitization. The symbols and vertical lines show the means and S.D.s, respectively. *, **, and *** indicate a significant difference from the value on day 0 of the same group, calculated by Welch's *t* test. **P* < .05; ***P* < .01; ****P* < .001

IgE level became significantly lower than the initial level (3 μg/ml, day 0) on day 5 and on day 3 in the leflunomide 1 mg/kg group (*n* = 4) and 2 mg/kg group (*n* = 6), respectively, and these low IgE levels continued until day 9 (Fig. 1). The reduction in the total IgE level showed dose dependence on leflunomide.

The OVA-specific IgE titer in sera on day 9 was undetectable in the untreated group, and the mean was around 25 U in the CMC control group. The mean titer in the 0.5 mg/kg group was around 21 U on day 9, showing no significant difference from the CMC control group. In the 1- and 2-mg/kg leflunomide groups, on the other hand, the OVA-specific IgE titer was below the limit of detection (0.5 U) in all animals, suppressed to about 1:50 or lower than in the CMC control group (Table 2, experiment 1).

On day 10, 60 mg/kg OVA was injected into the jugular vein of the sensitized animals to examine the effect of leflunomide on the development of anaphylactic shock. Blood was sampled before and after the challenge with OVA to measure the plasma histamine level as an index of the activation of mast cells and basophils. In the untreated control group, the plasma histamine level was hardly changed before and after the challenge, whereas in the CMC control group and 0.5-mg/kg leflunomide group, the animals showed a remarkable rise in the histamine level after challenge and then decreased spontaneous behavior, and they died quietly (Table 2, experiment 1). In the 1-mg/kg leflunomide group, on the contrary, the mean histamine level was significantly lower than that in the CMC control group, although a slight increase was seen in some animals after the challenge. Anaphylactic shock did not develop in any of the animals, and the survival rate was 100%. In the 2-mg/kg group, the histamine level did not increase even after the challenge with OVA.

In experiment 2 in Table 2, the leflunomide-treated group showed a slightly larger value for the mean and S.D. because

a rise in the histamine level after challenge was observed in one of six animals. However, OVA-specific IgE was not detected, and no animals died of anaphylactic shock. In the prednisolone-treated group, although the total IgE level and OVA-specific IgE titer decreased to one-fourth and one-half of that in the CMC control group, respectively, the plasma histamine level after challenge was not decreased. All animals in this group died of anaphylactic shock. Based on these results, a dose of 1 to 2 mg/kg leflunomide was shown to be enough to inhibit elevation of the OVA-specific IgE and histamine levels and inhibit the induction of anaphylactic shock by OVA challenge.

Optimal Dosing Time. We investigated the optimal time for administration of leflunomide to the anaphylactic shock model to efficiently achieve its inhibitory effect on IgE production and anaphylactic shock.

The inhibitory effect of leflunomide at 2 mg/kg was compared between 10-day dosing (days 0–9), 7-day dosing (days 0–6 and days 3–9), and 4-day dosing (days 0–3 and days 6–9) (Table 3, experiment 1). In the 7-day dosing, for both of the periods, the OVA-specific IgE titer on day 9 was below the limit of detection in all animals, and the increase in the histamine level, determined 5 min after challenge, was very slight in degree. Therefore, it became clear that a 7-day dosing period, either days 0 to 6 or days 3 to 9, was sufficient for leflunomide to be effective at 2 mg/kg. No immediate anaphylactic shock was seen in either of the two groups, although one of six animals in the days 0 to 6 group showed a slight decrease in spontaneous behavior some time after challenge and then died. On the other hand, dosing for 4 days was insufficient for inhibition of IgE production. Although the degree of OVA-specific IgE suppression was statistically significant, the level of plasma histamine after OVA challenge was only slightly depressed in all of the animals of these groups, resulting in death in one of six animals in the days 0 to 3 group and in four of six animals in the days 6 to 9 group. Next, we examined shortening the effective dosing period to less than 7 days. A dose of 2 mg/kg of leflunomide was administered for 4 days from days 3 to 6 based on the experimental results that an effective response was observed by dosing for days 0 to 6 and for days 3 to 9, but not by dosing for days 0 to 3 or for days 6 to 9. Administration of leflunomide for 4 days from days 3 to 6 at a dose of 2 mg/kg resulted in sufficient suppression of OVA-specific IgE production, thus preventing the increase in histamine level and anaphylactic shock (Table 3, experiment 2). On the other hand, in the aforementioned experiment in which 2 mg/kg leflunomide was dosed from days 0 to 3, the total IgE level on day 9 rose to about 7 times that on day 0, but the levels on days 3, 5, and 7 were significantly lower than on day 0 (data not shown). Then, to examine whether a higher dose of leflunomide might suppress Ig production more strongly and prevent a rise in the total IgE level and OVA-specific IgE formation on day 9, the dose was increased to 10 mg/kg. As a result, the total serum IgE level on day 9 was the same as on day 0, the OVA-specific IgE titer was also below the limit of detection in all of the animals in this group, and the histamine level did not increase after challenge (Table 3, experiment 2). The cause of death of four animals in the days 0 to 3 group was unclear. The possibility of adverse effects of leflunomide is unlikely because these rats were observed to be normal until the second challenge of OVA and because the

TABLE 2

Inhibitory effects of leflunomide in the anaphylactic shock model of BN rats and comparison of the effects with prednisolone

Antigen	Drug	Total IgE on Day 9	OVA-Specific IgE on Day 9	Histamine Level on Day 10		No. Survived/ Total
				Before challenge	After challenge	
		$\mu\text{g/ml}$	U	μM		
Expt. 1						
		$3.62 \pm 1.40^{***}$	$<0.5^{***}$	0.46 ± 0.22	$0.45 \pm 0.03^{***}$	6/6
OVA	1% CMC	491.51 ± 184.34	25.47 ± 5.34	0.65 ± 0.13	25.70 ± 6.09	0/6
OVA	Leflunomide (0.5 mg/kg)	357.72 ± 119.05	20.78 ± 6.33	0.68 ± 0.14	37.27 ± 10.06	0/5
OVA	Leflunomide (1 mg/kg)	$0.75 \pm 0.09^{***}$	$<0.5^{***}$	0.57 ± 0.17	$1.19 \pm 0.81^{***}$	4/4
OVA	Leflunomide (2 mg/kg)	$0.42 \pm 0.07^{***}$	$<0.5^{***}$	$0.46 \pm 0.09^*$	$0.27 \pm 0.08^{***}$	6/6
Expt. 2						
		$4.34 \pm 2.33^{***}$	ND	0.62 ± 0.14	$0.35 \pm 0.08^{***}$	5/5
OVA	1% CMC	466.15 ± 211.88	18.68 ± 7.45	0.73 ± 0.45	43.37 ± 10.63	0/5
OVA	Leflunomide (2 mg/kg)	$0.85 \pm 1.20^{***}$	$<0.5^{***}$	0.49 ± 0.08	$6.34 \pm 14.09^{***}$	6/6
OVA	Prednisolone (4 mg/kg)	$95.12 \pm 38.84^{**}$	$9.38 \pm 3.62^*$	0.53 ± 0.19	39.47 ± 5.26	0/6

Values represent the mean $\pm$ S.D. When the OVA-specific IgE titer was below the limit of detection (0.5 U) in all animals per group, a provisional value of < 0.5 was used. When a value below the limit of detection was obtained in some animals per group, statistical analysis was performed using a provisional value of 0.5. Differences from the CMC control group were tested for significance by Welch's t test (* $P < .05$; ** $P < .01$; *** $P < .001$). ND, not done.

TABLE 3

Dosing period and inhibitory effect of leflunomide in anaphylactic shock model

Antigen	Leflunomide	Dosing Period	Total IgE on Day 9	OVA-Specific IgE on Day 9	Histamine level on Day 10		No. Survived/ Total
					Before challenge	After challenge	
	<i>mg / kg</i>	<i>days</i>	<i>µg / ml</i>	<i>U</i>	<i>µM</i>		
Expt. 1							
			2.02 ± 0.61***	<0.5***	0.55 ± 0.14	0.43 ± 0.13***	5/5
OVA	1% CMC	0–9	322.48 ± 88.10	22.17 ± 8.51	0.50 ± 0.19	30.38 ± 11.90	0/5
OVA	2	0–3	24.68 ± 18.81***	1.12 ± 0.88***	0.56 ± 0.14	12.47 ± 8.96*	5/6
OVA	2	0–6	0.50 ± 0.13***	<0.5***	0.47 ± 0.17	0.99 ± 0.56***	5/6
OVA	2	0–9	0.38 ± 0.04***	<0.5***	0.76 ± 0.37	0.44 ± 0.19***	5/5
OVA	2	3–9	1.56 ± 2.78***	<0.5***	0.47 ± 0.10	0.66 ± 0.57***	6/6
OVA	2	6–9	44.84 ± 46.31***	1.37 ± 0.87***	0.62 ± 0.18	19.92 ± 6.77	2/6
Expt. 2							
			3.72 ± 2.42***	<0.5***	0.39 ± 0.20	0.28 ± 0.07***	6/6
OVA	1% CMC	0–9	420.60 ± 194.00	24.69 ± 4.34	0.62 ± 0.16	46.55 ± 6.78	0/5
OVA	2	0–9	0.45 ± 0.06***	<0.5***	0.42 ± 0.10*	0.30 ± 0.17***	6/6
OVA	2	3–6	2.53 ± 2.93***	<0.5***	0.67 ± 0.23	2.80 ± 4.07***	6/6
OVA	10	0–3	2.86 ± 1.20***	<0.5***	0.51 ± 0.20	0.53 ± 0.17***	2/6

Values represents the mean $\pm$ S.D. When the OVA-specific IgE titer was below the limit of detection (0.5 U) in all animals per group, a provisional value of < 0.5 was used. When a value below the limit of detection was obtained in some animals per group, statistical analysis was performed using a provisional value of 0.5. Differences from the CMC control group were tested for significance by Welch's t test (* $P < .02$; *** $P < .001$).

preliminary experiment clearly demonstrated that the same animal model was well tolerated with dosing 10 mg/kg/day for days 0 to 9 (data not shown). However, these findings were not sufficient to exclude the possibility of the toxicity of leflunomide.

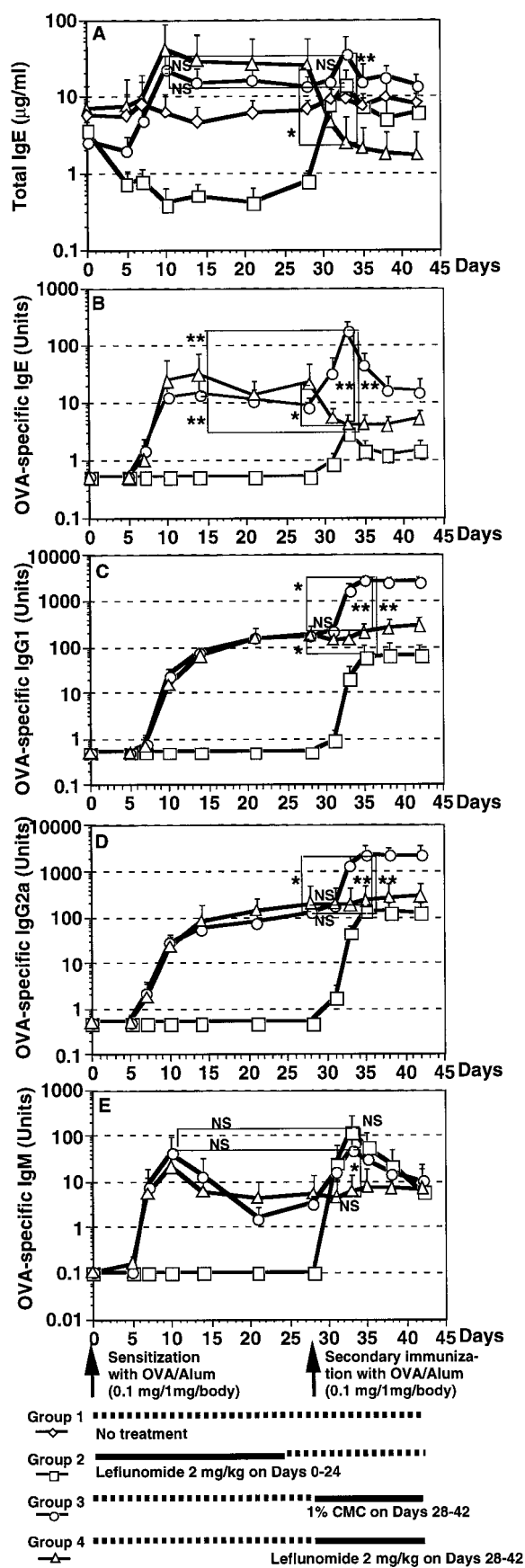
Inhibition of Antibody Production by Leflunomide in the Secondary Immune Response

In this experiment, the doses of OVA and alum were reduced to one-fifteenth and one forty-fifth, respectively, of those used in the primary response study. This was to attenuate the immunizing condition, because the previous condition was considered to be too severe for long-term drug administration.

Serum Total IgE Levels. First, the effect of leflunomide on the total IgE level in serum was examined. The results are expressed as the mean and the S.D. in each group (Fig. 2A). There were large individual differences due to the weak immunizing conditions used. In group 3 (CMC control group), the total IgE level peaked on day 10 ($21.5 \mu\text{g/ml}$), decreased gradually thereafter, and showed a sharp peak ($35.1 \mu\text{g/ml}$) 5 days after the secondary immunization on day 28. Group 4, which was administered leflunomide on and after the second-

ary immunization, showed the peak value for the total IgE level ($38.7 \mu\text{g/ml}$) on day 10 and then a gradual decrease, similar in fashion to that observed in the CMC control group. In contrast to group 3, however, the total serum IgE level in group 4 decreased rapidly to nearly the baseline level of nonsensitized BN rats (3 days after secondary immunization and at the start of leflunomide treatment). Thereafter, due to leflunomide administration, the IgE level remained continuously low. In group 2, which received leflunomide for 25 consecutive days beginning from day 0, the total IgE level began to decrease on day 5 and did not increase during the entire dosing period. These antibody concentrations were significantly lower than those of the untreated control group. The secondary immunization, performed on day 28, after a 4-day rest period of leflunomide administration, resulted in a rise in the total IgE value, which peaked 5 days later ($13.4 \mu\text{g/ml}$). This was also the case in the CMC control group. Although there was no significant difference, the mean value of the total IgE in group 2 was as low as one-third of the concentration detected in the CMC control group.

OVA-Specific IgE Titers. Leflunomide exhibited a very interesting inhibitory effect on the OVA-specific IgE titer (Fig. 2B). In the CMC control group (group 3), the titer



increased remarkably from day 7 through day 10, then increased gradually until day 14 when the titer peaked (13.2 U), and thereafter decreased gradually. Furthermore, responding to the secondary immunization, a sharp peak was observed 5 days after rechallenge (day 33) and reached a titer of 164 U. This was 12 times higher than that documented on day 14. On the contrary, in the group given leflunomide on and after the secondary immunization (group 4), the titer decreased significantly within 5 days after the secondary immunization (day 33) to about one-fifth (4.0 U) of that observed on day 28 (20.4 U), and it did not increase thereafter.

On the other hand, in the group given leflunomide from the day of primary immunization, day 0, to day 24 (group 2), the primary immune response was completely suppressed and the specific IgE titer was below the limit of detection (0.5 U). After the secondary immunization on day 28, after a 4-day resting period of leflunomide administration, the OVA-specific IgE titer increased and peaked on day 33 (2.9 U), but it was significantly lower when compared with that on day 33 in the CMC control group (about 1:60), and also with the primary response (day 14) in the CMC control group (about 1:5). In group 2, however, the time period necessary to reach the peak titer was the same as in the CMC control group.

OVA-Specific IgG1 Titers. In the CMC control group (group 3, Fig. 2C), the antigen-specific IgG1 titer increased greatly from day 7 through day 10, and then increased gradually until day 21 thereafter. Then it reached a plateau, which was maintained until day 28. In the secondary response, the titer increased greatly, especially 3 to 5 days after the secondary immunization, and reached about 2700 U 7 days after rechallenge (day 35). This was a 17-fold increase from 163 U on day 28. In contrast, the group receiving leflunomide during the period of the secondary response (group 4) did not demonstrate such remarkable findings. This group showed neither a drastic decrease, as was observed in the specific IgE titer, nor a remarkable increase after the secondary immunization, as was observed in the CMC control group, and only had a 1.2-fold increase in the specific IgG1 titer on day 35. On the other hand, in the group administered leflunomide during the primary induction period (group 2), the primary response was completely inhibited and the titer was below the limit of detection (0.5 U). After a 4-day rest period, the secondary immunization was carried out. The specific antibody production was weaker than observed in the primary immune response of the CMC control group. After the secondary immunization, the time point at which the antibody titer increased in group 2 was the same as that in the CMC control group.

OVA-Specific IgG2a Titers. When the antigen-specific IgG2a titer was measured, a similar time course to that of IgG1 was observed except that the suppressive effect of leflunomide on the secondary immune response seemed slightly less potent

Fig. 2. Effect of leflunomide on IgE or OVA-specific Ig production in the secondary immune response of type 1 allergy model in BN rats. Each symbol shows the mean, and error bar shows S.D. of six rats. Collected serum samples were analyzed for the concentrations of total IgE (A) and OVA-specific IgE (B), IgG1 (C), IgG2a (D), and IgM (E). Statistically significant differences were analyzed by Wilcoxon's signed rank test for the time course change within the same group, by Steel's test for comparison of groups assayed on the same day, and by Mann-Whitney's *U* test for the comparison between two groups assayed on different days. **P* < .05; ***P* < .01

than on IgG1 in group 2 (Fig. 2D). That is, the antibody titer in the CMC control group (group 3) was increased 18-fold, from 120 U on day 28 to a maximum of 2100 U on day 35. In group 4, on the other hand, the increase was small in degree, i.e., there was a 1.1-fold increase between the titer on day 28 and that on day 35. In group 2, the primary immune response was completely inhibited, being below the limit of detection (0.5 U). After the secondary immunization, the titer of the specific IgG2a on day 35 was almost the same as that determined in the primary immune response (day 28) in the CMC control group. The increase in the IgG2a antibody titer occurred at the same time point as observed in the CMC control group.

OVA-Specific IgM Titers. A characteristic of antigen-specific IgM is that the titer of the secondary response never exceeds that of the primary immune response. Thus, the effect of leflunomide on the antigen-specific IgM titer differed from its effects on the antibodies of the other classes (Fig. 2E). In the CMC control group (group 3), the IgM titer from day 7 to day 10 increased 5-fold and peaked. The titer then decreased promptly. After the secondary immunization, the titer peaked after 5 days (day 33) but then stayed almost the same as that seen after the primary immunization. In the group administered leflunomide during the secondary response (group 4), the titer did not rise after the secondary immunization, and titers of 4 to 7 U remained from day 28 through day 42. In group 2, on the other hand, the primary immune response was completely inhibited and the IgM titer stayed below the limit of detection (0.1 U). After the secondary immunization, after a 4-day rest period, the antigen-specific IgM titer determined in the animals of group 2 was almost the same as that observed in the primary immune response of the CMC control group. The times of increase and decrease in the IgM titer in the secondary response of group 2 were the same as those in the CMC control group.

Administration of leflunomide during the secondary immune response significantly suppressed the production of OVA-specific IgE, IgG1, IgG2a, IgM, and total IgE after

secondary immunization. In the case of administration of leflunomide during the primary immune response and stopped 4 days before the secondary immunization, the times of increase and decrease in the antibody titer of each examined class were just the same as those determined for the CMC control group, but the titers of OVA-specific IgE, IgG1, and IgG2a were significantly lower than in the CMC control group. This suppression was especially remarkable for OVA-specific IgE. It is noteworthy that no adverse effects relating to the administration of leflunomide at 0.5 to 10 mg/kg/day were observed during these studies.

Effect of Uridine on Suppressed Cell Proliferation and Antibody Production In Vitro

Inhibition of dihydroorotate dehydrogenase (DHO-DH) has been found to be one of the major molecular mechanisms by which leflunomide exerts its activity on immunocompetent cells (Williamson et al., 1995; Zielinski et al., 1995). To elucidate whether the suppressive effects of leflunomide on antibody production were primarily due to DHO-DH inhibition, the following experiments were performed.

Before conducting experiments to determine the suppressive effects of **A77 1726** in vitro, we determined the optimal concentration of OVA for stimulating BN rat spleen cells from rats previously immunized with OVA in vivo. It was found that 1 mg/ml OVA was the most effective concentration for primed rat spleen cell proliferation and, under the same conditions, nonprimed splenocytes were not stimulated by this concentration of OVA (data not shown).

The effects of **A77 1726** on the proliferation of rat spleen cells were evaluated in the above described cell activation system. The results are shown in Table 4. The OVA-specific proliferation of rat spleen cells was completely inhibited by 0.004 mM and nearly 90% inhibited by 0.0008 mM **A77 1726**. This suppressed proliferation of spleen cells was greatly restored by the addition of 0.02 mM uridine to the culture medium (Table 5). This indicates that, for cell proliferation, it is most likely that inhibition of DHO-DH is the major mechanism by which **A77 1726** exerts its effects, and other molecular mechanisms might be negligible.

As shown in Table 6, the formation of OVA-specific IgG1, IgG2a, and IgM was greatly suppressed by **A77 1726** at concentrations of 0.0008 to 0.02 mM. This was very similar to the results observed in the proliferation assay. However, contrary to the results obtained in the proliferation assay, Ig production could not be restored by the addition of 0.02 mM uridine to the culture, but it was partially restored when 0.1 mM uridine was added. As is widely known, not only protein synthesis by cells but also other factors, such as stimulation with lymphokines and signal transduction, play important roles in the formation and secretion of immunoglobulins. The

TABLE 4
Effects of **A77 1726** on OVA-specific proliferation of rat spleen cells

A77 1726 Final concentration	Expt. 1		Expt. 2	
	Average	S.D.	Average	S.D.
mM	cpm		cpm	
0.0	14930	176	15341	1907
0.0008	ND	ND	1578	98
0.004	266	28	258	77
0.02	122	5	142	42
0.1	122	13	ND	ND

ND, not done.

TABLE 5
Restoration of suppressed proliferation of spleen cells by coculturing with uridine

A77 1726	Uridine, mM							
	0		0.004		0.02		0.1	
mM	cpm	% ^a	cpm	%	cpm	%	cpm	%
0.0	15341	100.0	15918	100.0	14679	100.0	12834	100.0
0.0008	1578	10.3	4071	25.6	17726	120.8	12961	101.0
0.004	258	1.7	1967	12.4	14222	96.9	11290	88.0
0.02	142	0.9	1458	9.2	15689	106.9	11235	87.5

^a Values represent the percentage of the cpm in the 0 mM **A77 1726** treatment group at each concentration of uridine.

TABLE 6
Partial restoration of suppressed OVA-specific IgG1, IgG2a, and IgM production of spleen cells by coculturing with uridine

A77 1726, mM		Uridine, mM							
		0		0.004		0.02		0.1	
		U	% ^a	U	%	U	%	U	%
IgG1	0.0	138.0	100.0	53.0	100.0	123.5	100.0	134.1	100.0
	0.0008	6.4	4.6	2.3	4.3	1.8	1.5	64.3	47.9
	0.004	4.7	3.4	3.9	7.4	2.0	1.6	75.4	56.2
	0.02	0.7	0.5	0.5	0.9	2.2	1.8	60.7	45.3
IgG2a	0.0	57.0	100.0	35.5	100.0	41.8	100.0	148.7	100.0
	0.0008	7.7	13.5	3.0	8.5	1.2	2.9	52.3	35.2
	0.004	5.5	9.6	4.4	12.4	1.2	2.9	82.7	55.6
	0.02	2.5	4.4	2.9	8.2	1.8	4.3	83.4	56.1
IgM	0.0	96.8	100.0	105.2	100.0	49.7	100.0	185.5	100.0
	0.0008	2.6	2.7	5.0	4.8	16.8	33.8	91.6	49.4
	0.004	0.2	0.2	0.3	0.3	3.5	7.0	95.2	51.3
	0.02	0.2	0.2	0.2	0.2	0.8	1.6	39.1	21.1

^a Values represent the percentage of the U in the 0 mM A77 1726 treatment group at each concentration of uridine.

results of this study suggest that perhaps other modes of action than just the inhibition of DHO-DH are also involved in the suppression of Ig production by leflunomide.

Discussion

In this study, leflunomide showed strong suppressive effects, not only on IgE production, but also on the induction of anaphylactic shock in BN rats by challenge with OVA. However, the precise mechanism behind this prevention of anaphylactic shock has not yet been totally elucidated. The strong suppression of IgE formation in these animals was obviously involved in preventing this reaction. An additional possibility for the prevention of anaphylactic shock may be leflunomide’s reported inhibitory effects on the release of chemical mediators, such as histamine (Bartlett et al., 1989). However, this effect most likely plays a minor role, because the administration of leflunomide (2 mg/kg/day), from day 3 to day 6, was more effective in preventing anaphylactic shock than when the drug was given from day 6 to day 9. Since the half-life of leflunomide in rats was reported to be about 8 h (Silva et al., 1996), the amount of A77 1726 on day 10 in the rat bodies of the days 3 to 6 administration group had to be less than that in the days 6 to 9 administration group. These findings indicate only a minor contribution of leflunomide to any direct inhibition of chemical mediators leading to the prevention of anaphylactic shock. Taken together, it is conceivable that the strong suppression of IgE formation was the dominant means by which leflunomide prevented anaphylactic shock in the rats, rather than a direct effect of the drug on the release of chemical mediators from mast cells and basophils. Furthermore, the doses (1–2 mg/kg/day) used in this study were nearly 5 to 10 times less than those used in rheumatoid arthritis models (Bartlett and Schleyerbach, 1985), and this was consistent with previous reports (Siemasko et al., 1996; Chong et al., 1996) demonstrating that Ig production was more sensitive than cell proliferation to leflunomide’s activity. This suggests that leflunomide might have great potential for curing type 1 allergic diseases, which are caused by the production of excess amounts of IgE. It is thus possible that this could be achieved with fewer adverse effects due to the effectiveness of leflunomide at relatively low dose levels.

The dosing period of day 3 to day 6 was critical for strong

suppression of IgE production and for prevention of anaphylactic shock in the animals. As shown in Fig. 1, the total IgE production began to increase from day 5. The period from day 3 to day 6 was considered to be the time of active proliferation and differentiation of IgE-producing B lymphocytes. A similar degree of IgE suppression was not obtained by administering leflunomide at 2 mg/kg/day from day 0 to day 3 (Table 3, experiment 1), which should be the time period in which initial antigen processing and T lymphocyte activation take place. This was, although, able to be inhibited by increasing the dose of leflunomide to 10 mg/kg/day from day 0 to day 3 (Table 3, experiment 2). These early stages of the immune response might be less sensitive to leflunomide’s activity than the later stages, in which B lymphocyte proliferation and antibody production take place.

Going a step further, the effects of leflunomide were investigated by using the secondary immune response model of hypersensitivity in BN rats. It was found that the production of all Ig classes measured in this experiment was strongly suppressed after administration of 2 mg/kg/day of leflunomide during the primary immune response period from day 0 to day 24. In the secondary immune response of these rats, antibody production except for the total IgE and OVA-specific IgM was also significantly suppressed without further administration of leflunomide in comparison with the CMC control group. These results indicate that leflunomide might suppress not only Ig production, but also the increase in memory cells during the primary response.

When leflunomide was administered during the period of the secondary response to OVA from day 28 to day 42, the production of all Ig classes was strongly suppressed and no secondary response was observed. Interestingly, quick significant decreases in the OVA-specific IgE titer and the total IgE level were observed in comparison to their levels on day 28 in this group, but not for the OVA-specific IgG1, IgG2a, and IgM titers. Certainly, the very short half-life of IgE in the serum might be the cause of the decrease in the total IgE and OVA-specific IgE, but the period of observation of the secondary response was long enough to detect decreases in the OVA-specific IgG1, IgG2a, and IgM titers, whose half-lives in the serum are 6 days, 6 days, and 3 days, respectively. Therefore, these results indicate that IgE production might be more sensitive than IgG1, IgG2a, and IgM productions to leflunomide. And in comparison to the IgG and IgM titer, the

remarkable fast decrease of IgE titer after leflunomide administration in the period of the secondary response was observed, suggesting that leflunomide might have the potential for curing type 1 allergic diseases.

Leflunomide's active metabolite, **A77 1726**, has been reported to antagonize interleukin-4 ($ED_{50} = 3 \mu M$) (Bartlett et al., 1989), a multipotent lymphokine that is essential in T lymphocyte-dependent Ig production, induction of the Th2 subset, and class switch from IgM to IgG1 as well as IgG1 to IgE (Kopf et al., 1993). This might be one possible explanation for the relatively stronger suppression of IgE production by leflunomide compared with the other classes of immunoglobulins. Additionally, it was demonstrated that disease development in BALB/c mice, which are susceptible to *Leishmania major*, was prevented by administration of leflunomide before or in the very early stage of infection with *L. major* (Solbach et al., 1995). This suggests conversion of the predominant cell type in the immune response from Th2 to Th1, possibly by suppressing Th2 cells.

In 1995, Williamson et al. demonstrated that DHO-DH, an enzyme which catalyzes the fourth step in pyrimidine de novo biosynthesis, is a target molecule of **A77 1726**. On the other hand, it has been also reported that **A77 1726** inhibited the tyrosine kinase activity involved in epidermal growth factor receptor signaling (Mattar et al., 1993) p56^{lck} and p59^{lyn} (Xu et al., 1995; Xu et al., 1996). In our experiment using spleen cells from BN rats, the impaired proliferation of spleen cells caused by **A77 1726** was completely reversed by addition of uridine to the culture medium (Table 5). However, Ig production was only partially restored even after adding 5 times the amount of uridine required to completely restore the proliferation of splenocytes (Tables 5 and 6). If the inhibition of DHO-DH was leflunomide's only molecular mechanism of action, one would expect that the administration of uridine would be capable of completely restoring the suppressed antibody production in in vitro experimental situations. The findings presented here argue against DHO-DH as the sole mechanism of action for leflunomide, and these findings suggest that leflunomide might have suppressed antibody formation also by some other mechanism. This could be through blocking the activity of tyrosine kinases (Mattar et al., 1993; Williamson et al., 1995; Zielinski et al., 1995; Xu et al., 1995, 1996). Leflunomide has demonstrated remarkable suppressive activity on IgE formation, thus preventing type 1 allergic disease in the present rat model of hypersensitivity.

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References

Bartlett RR, Dimitrijevic M, Mattar T, Zielinski T, Germann T, Rüde E, Thoenes GH, Küchle CCA, Schorlemmer HU, Bremer E, Finnegan A and Schleyerbach R (1991) Leflunomide (HWA 486), a novel immunomodulating compound for the treatment

of autoimmune disorders and reactions leading to transplantation rejection. *Agents Actions* **32**:10–21.

Bartlett RR, Mattar T, Weithmann U, Anagnostopoulos H, Popovic S and Schleyerbach R (1989) Leflunomide (HWA 486): A novel immunorestoring drug, in *Therapeutic Approaches to Inflammatory Diseases* (Lewis AJ, Doherty NS and Ackerman NR eds) pp 215–228, Elsevier Science Publishing, New York.

Bartlett RR, Popovic S and Raiss RX (1988) Development of autoimmunity in MRL/lpr mice and the effects of drugs on this murine disease. *Scand J Rheumatol Suppl* **75**:290–299.

Bartlett RR and Schleyerbach R (1985) Immunopharmacological profile of a novel isoxazol derivative, HWA 486, with potential antirheumatic activity-I. Disease modifying action on adjuvant arthritis of the rat. *Int J Immunopharmacol* **7**:7–18.

Chong ASF, Rezai K, Gebel HM, Finnegan A, Foster P, Xu XL and Williams JW (1996) Effects of leflunomide and other immunosuppressive agents on T cell proliferation in vitro. *Transplantation* **61**:140–145.

Kopf M, Gros GL, Bachmann M, Lamers MC, Bluethmann H and Köhler G (1993) Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature (Lond)* **362**:245–248.

Küchle CCA, Thoenes GH, Langer KH, Schorlemmer HU, Bartlett RR and Schleyerbach R (1991) Prevention of kidney and skin graft rejection in rats by leflunomide, a new immunomodulating agent. *Transplant Proc* **23**:1083–1086.

Mattar T, Kochhar K, Bartlett R, Bremer EG and Finnegan A (1993) Inhibition of the epidermal growth factor receptor tyrosine kinase activity by leflunomide. *FEBS Lett* **334**:161–164.

Mladenovic V, Domljan Z, Rozman B, Jajic I, Mihajlovic D, Dordevic J, Popovic M, Dimitrijevic M, Zivkovic M, Campion G, Musikic P, Löw-Friedrich I, Oed C, Seifert H and Strand V (1995) Safety and effectiveness of leflunomide in the treatment of patients with active rheumatoid arthritis. Results of a randomized, placebo-controlled, phase II study. *Arthritis & Rheum* **38**:1595–1603.

Mrowka C, Thoenes GH, Langer KH and Bartlett RR (1994) Prevention of the acute graft-versus-host disease (GVHD) in rats by the immunomodulating drug leflunomide. *Ann Hematol* **68**:195–199.

Popovic S and Bartlett RR (1987) The use of the murine chronic graft Vs host (CGVH) disease, a model for systemic lupus erythematosus (SLE), for drug discovery. *Agents Actions* **21**:284–286.

Rebbaa A, Hurh J, Yamamoto H, Kersey DS and Bremer EG (1996) Ganglioside GM3 inhibition of EGF receptor mediated signal transduction. *Glycobiology* **6**:399–406.

Schorlemmer HU and Bartlett RR (1994) Therapeutic activity of leflunomide in acute and chronic relapsing experimental allergic encephalomyelitis. *Agents Actions* **41**:C271–C273.

Schorlemmer HU, Seiler FR and Bartlett RR (1993) Prolongation of allogeneic transplanted skin grafts and induction of tolerance by leflunomide, a new immunosuppressive isoxazol derivative. *Transplant Proc* **25**:763–767.

Siemasko KF, Chong ASF, Williams JW, Bremer EG and Finnegan A (1996) Regulation of B cell function by the immunosuppressive agent leflunomide. *Transplantation* **61**:635–642.

Silva HT, Shorthouse R and Morris RE (1996) Single- and multiple-dose pharmacokinetics and pharmacodynamics of leflunomide's active metabolite A77 1726 in normal Lewis rats. *Transplant Proc* **28**:3092–3094.

Solbach W, Asmuth PA, Zimmermann S, Humborg C and Röllinghoff M (1995) Protective effect of leflunomide on the natural course of *Leishmania major*-induced disease in genetically susceptible BALB/c mice. *Int J Immunopharmacol* **17**:481–488.

Thoenes GH, Sitter T, Langer KH, Bartlett RR and Schleyerbach R (1989) Leflunomide (HWA 486) inhibits experimental autoimmune tubulointerstitial nephritis in rats. *Int J Immunopharmacol* **11**:921–929.

Williamson RA, Yea CM, Robson PA, Curnock AP, Gader S, Hambleton AB, Woodward K, Bruneau JM, Hambleton P, Moss D, Thomson TA, Spinella-Jaegle S, Morand P, Courtin O, Sautés C, Westwood R, Hercend T, Kuo EA and Ruuth E (1995) Dihydroorotate dehydrogenase is a high affinity binding protein for A77 1726 and mediator of a range of biological effects of the immunomodulatory compound. *J Biol Chem* **270**:22467–22472.

Xu X, Williams JW, Bremer EG, Finnegan A and Chong AS-F (1995) Inhibition of protein tyrosine phosphorylation in T cells by a novel immunosuppressive agent, leflunomide. *J Biol Chem* **270**:12398–12403.

Xu X, Williams JW, Gong H, Finnegan A and Chong AS-F (1996) Two activities of the immunosuppressive metabolite of leflunomide, A77 1726: Inhibition of pyrimidine nucleotide synthesis and protein tyrosine phosphorylation. *Biochem Pharmacol* **52**:527–534.

Zielinski T, Zeitter D, Müllner S and Bartlett RR (1995) Leflunomide, a reversible inhibitor of pyrimidine biosynthesis? *Inflamm Res* **44**(Suppl 2):S207–S208.

Send reprint requests to: Yuko Mizushima, Marketed Products Department, Drug Regulatory Affairs, Hoechst Marion Roussel, Ltd., 17–51, Akasaka 2-chome, Minato-ku, Tokyo 107-8465, Japan. E-mail: yuko.mizushima@hmrag.com