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Effects of the second-generation leukotriene B_4 receptor antagonist, LY293111Na, on leukocyte infiltration and collagen-induced arthritis in mice

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Abstract

The effects of the second-generation leukotriene B_4 receptor (LTB_4 receptor) antagonist, 2-[2-propyl-3-{3-[2-ethyl-4-(4-fluorophenyl)-5-hydroxy-phenoxy]-propoxy}phenoxy]benzoic acid sodium salt (LY293111Na), on leukotriene B_4-induced leukocyte infiltration and interleukin-1-accelerated collagen-induced arthritis in mice were studied. Neutrophil infiltration induced into an air pouch by leukotriene B_4 was dose-dependently inhibited by LY293111Na and strongly so by another LTB_4 receptor antagonist, 4-[5-{4-(aminoiminomethyl)phenoxy}pentoxy]-3-methoxy-*N*,*N*-bis(1-methylethyl) (*Z*)-2butenedioate (1:1) (CGS25019C). Both compounds significantly inhibited the increase of the arthritis index and the ankle bone destruction in interleukin-1-accelerated collagen-induced arthritis. Phenidone, a 5-lipoxygenase inhibitor, also inhibited interleukin-1-accelerated collagen-induced arthritis, while indomethacin and tenidap, cyclooxygenase inhibitors, had slight inhibitory effects. Injection of interleukin-1 elicited a marked increase of the leukotriene B_4 level in arthritic paws, while the prostaglandin E_2 level was slightly increased. These findings indicate clearly that leukotriene B_4 is an important mediator of interleukin-1-accelerated collagen-induced arthritis in mice. If this can be extrapolated to man, LTB_4 receptor antagonists might be useful for treatment of the acute progressive phase of human arthritis.

Keywords: Leukotriene B_4; Air pouch; Leukocyte infiltration; Collagen-induced arthritis; Interleukin-1; LY293111Na

1. Introduction

Leukotriene B_4 is a proinflammatory mediator, which is enzymatically derived from arachidonic acid via the 5-lipoxygenase pathway, and is synthesized primarily by polymorphonuclear leukocytes, monocytes and macrophages (Henderson, 1994). It works as a chemoattractant for polymorphonuclear leukocytes and its activity is comparable on a molar basis to that of *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and human complement 5a (C5a; Goetzl and Pickett, 1981). Elevated levels of leukotriene B_4 have been found in a variety of human inflammatory diseases and their animal models (Ford-Hutchinson, 1990). Increases of leukotriene B_4 levels in blood and synovial fluids of patients with rheumatoid arthritis have been detected and postulated to play an important role in the pathobiology of rheumatoid arthritis (Davidson et al., 1983; Ahmadzadeh et al., 1991).

Human neutrophils have specific membrane receptors for leukotriene B_4 which, when triggered, affect an array of in vitro activation events, including aggregation (Ford-Hutchinson, 1990) and superoxide production (Palmbled et al., 1984). Leukotriene B_4 also promotes the adhesion of neutrophils to the vascular endothelium (Gimbrone et al., 1984). In addition, neutrophils were found in large numbers in synovial tissue and synovial fluid and have been postulated to be involved in a variety of pathological conditions, such as cartilage and bone erosion in rheumatoid arthritis (Jones et al., 1991; Leirisalo-Repo et al., 1993). Therefore, interruption of neutrophil function by inhibiting leukotriene B_4 activity with its receptor antagonists would be beneficial for the treatment of rheumatoid arthritis.

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2-[2-propyl-3-{3-[2-ethyl-4-(4-fluorophenyl)-5-hydroxyphenoxy]-propoxy}phenoxy]benzoic acid sodium salt (LY293111Na) is a potent and specific LTB_4 receptor antagonist (Sawyer et al., 1995; Jackson et al., 1999). It suppresses leukotriene B_4-induced activation events in neutrophils, including superoxide production, aggregation and adhesion molecule expression in vitro. On the other hand, LY293111Na does not block human neutrophil activation responses induced by fMLP, platelet-activating factor, human recombinant interleukin-8 or C5a (Marder et al., 1995). LY293111Na also competitively inhibits leukotriene B_4-induced up-regulation of CD11b adhesive molecules on the surface of the polymorphonuclear leukocyte, presumably inhibiting the tight adhesion of polymorphonuclear leukocytes to vascular endothelial cells (Marder et al., 1995, 1996). Moreover, LY293111Na inhibits pulmonary neutrophilia and the expression of CD11b caused by inhalation of leukotriene B_4 in rhesus monkeys (Allen et al., 1996).

Several LTB_4 receptor antagonists that block [^{3}H]leukotriene B_4 binding and/or inhibit subsequent functional events have been identified and used in some animal models of human arthritis. For example, CP-105,696 is a potent LTB_4 receptor antagonist (Showell et al., 1995, 1996) and inhibits collagen-induced arthritis in mice (Koch et al., 1994; Griffiths et al., 1995). However, the precise role of leukotriene B_4 in collagen-induced arthritis is still not clear, because an increase of endogenous leukotriene B_4 levels in arthritic paw has not yet been reported.

In the present study, we first compared the in vivo potency of LY293111Na on leukotriene B_4-induced leukocyte infiltration into the mouse air pouch with that of another LTB_4 receptor antagonist, 4-[5-{4-(aminoiminomethyl)phenoxy}pentoxy]-3-methoxy-*N*,*N*-bis(1-methylethyl) (*Z*)-2butenedioate (1:1) (CGS25019C; Marshall, 1994; Raychaudhuri et al., 1995). We then compared their efficacy against interleukin-1-accelerated collagen-induced arthritis with cyclooxygenase inhibitors and a 5-lipoxygenase inhibitor. Finally, we studied the changes in leukotriene B_4 levels in paws of mice subjected to collagen-induced arthritis with or without interleukin-1-acceleration to explain the apparent efficacy of LTB_4 receptor antagonists in interleukin-1-accelerated collagen-induced arthritis.

2. Materials and methods

2.1. Animals

Male BALB/c mice (6 weeks old) were purchased from Japan SLC (Hamamatsu, Japan), and male DBA/1J mice (7 weeks old) were from Charles River Japan (Yokohama, Japan). All animal experiments conducted in the present study were approved by the Animal Use and Care Committee of Shionogi.

2.2. Drugs

Leukotriene B_4 and chicken type II collagen were purchased from Sigma (St. Louis, MO, USA). Murine recombinant interleukin-1α was obtained from R&D Systems (Minneapolis, MN, USA). Freund's complete adjuvant was from Difco (Detroit, MI, USA). LY293111Na (Lot. 309MH3) was synthesized at Lilly Research Laboratories (Indianapolis, IN, USA). CGS25019C and tenidap were synthesized at Shionogi Research Laboratories. Indomethacin and phenidone were purchased from Sigma. LY293111Na was dissolved in distilled water and the other substances were suspended in 0.6% gum arabic solution, and then administered orally in a volume of 10 ml/kg.

2.3. Leukotriene B_4-induced leukocyte infiltration into air pouch

Simple air pouches were created in BALB/c mice by the method described by Edwards et al. (1981). An air

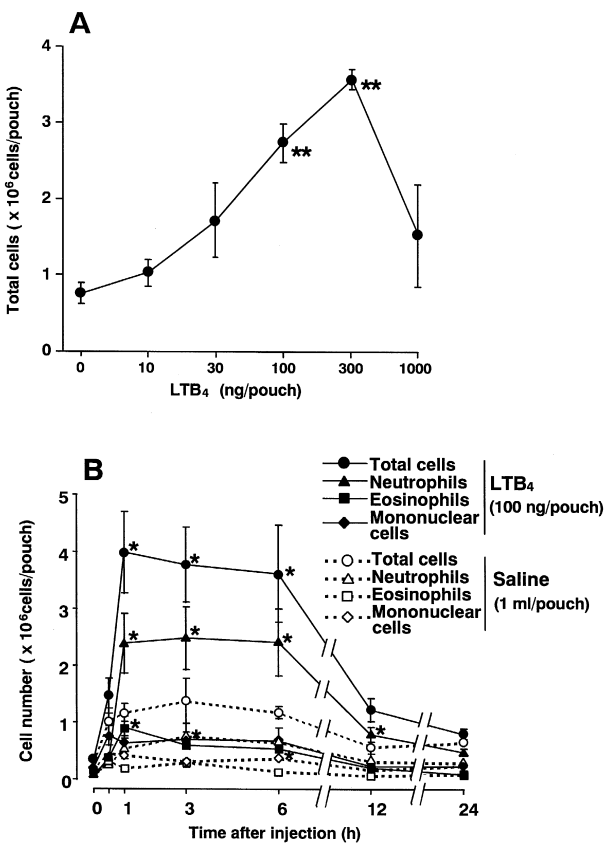

Fig. 1. Leukotriene B_4-induced cell infiltration into air pouch in mice. (A) Dose–response: 1 ml of leukotriene B_4 saline solution (0–1000 ng per pouch) was injected into the air pouch. Infiltrating cells were harvested 3 h after leukotriene B_4 injection. Each value represents the mean ± S.E.M. for four animals. $^{**}P < 0.01$ vs. saline-injected group (0 ng; Student's *t*-test). (B) Time course: infiltrating cells were harvested 0, 1, 3, 6, 12 or 24 h after injection of 100 ng leukotriene B_4 per pouch. Each value represents the mean ± S.E.M. for four to seven animals. *Significant increase ($P < 0.05$) vs. corresponding cell types in the saline-injected group (Student's *t*-test). LTB_4: leukotriene B_4.

pouch was formed by the subcutaneous injection of 5 ml of air (through a 0.22 μm filter) into the backs of mice. Three days later, the pouch was reinflated with 3 ml of sterile air. Six days after the initial injection of air, 1 ml of ice-cold leukotriene B_4 saline solution was injected into the air pouch immediately after preparation. At specified times after the leukotriene B_4 injection, the animals were bled under pentobarbital anesthesia and the infiltrating cells were harvested by washing the pouch with 2 ml of saline containing 20 U/ml of heparin. The total number of cells was determined with a hemocytometer with Turk's stain and differential cell counts were done for cytocentrifuged preparations (Cytospin 3; Shandon Southern Instruments, Pittsburgh, PA, USA) stained with May–Grunwald–Giemsa. Four hundred cells were counted and classified as neutrophils, eosinophils and mononuclear cells, based on normal morphological criteria. Since most of the injected leukotriene B_4 solution was absorbed by the air pouch tissue, there was almost no collectable exudate in the pouch.

2.4. Iiterleukin-1 accelerated collagen-induced arthritis

Collagen arthritis was induced in male DBA/1J mice according to the method described by Trentham et al. (1977) and Hom et al. (1988). Chicken type II collagen and an equal volume of Freund's complete adjuvant were emulsified by stirring in a homogeniser for 15 min 4°C at 10,000 rpm. Mice were immunized intracutaneously with 100 μg of type II collagen (100 μl of emulsion) on day 0, and a booster injection of type II collagen was given on day 21. The development of arthritis was then accelerated by subcutaneous injection of 0.3 μg of interleukin-1α on days 25 and 26. Drugs were administered orally once or twice daily from the day before the acceleration of arthritis (day 24).

The severity of the arthritis was assessed using a visual scoring system (Nickerson-Nutter and Medvedeff, 1996). Each paw was scored on a graded scale from 0 to 4: 0 = normal, 1 = swelling in 1–2 single digital joints, 2 = mild swelling of the entire paw, 3 = severe swelling of the entire paw, 4 = gross deformity and/or ankylosis. The arthritic index was the sum of the scores for the individual paws, making 16 the maximal score for each mouse (score of 4 for each paw) under blind investigation.

Radiographs of the four paws were taken at the end of experiment (40 kV, 3 mA, 40 s: OHMIC, Type OM-55G, Tokyo, Japan) and radiographic scoring was assessed by the extent of joint space narrowing, bone destruction and periosteal new bone formation. Scores were assigned as integers from 0 to 3 per joint (with 0 equaling normal and 3 equaling maximum joint destruction) and were determined by blind investigation. The radiographic index was the sum of scores for the individual paws, making 12 the maximal score for each mouse (score of 3 for each paw).

2.5. Measurement of delayed type hypersensitivity and anti-type II collagen antibody

To investigate the cell-mediated immune response, delayed-type hypersensitivity to type II collagen, we estimated the delayed-type hypersensitivity described as follows. Two days before the animals were killed, the thickness of both ears was measured with a dial thickness gauge (Teclock, Tokyo, Japan). Type II collagen was dissolved in phosphate buffered saline (PBS) which contained 0.05 M of acetic acid, and then the delayed-type hypersensitivity was induced by intradermal injection of 10 μg of type II collagen diluted in PBS into each ear in a volume of 10 μl. At 24 h after induction of the delayed-type hypersensitivity reaction, the ear thickness of each animal

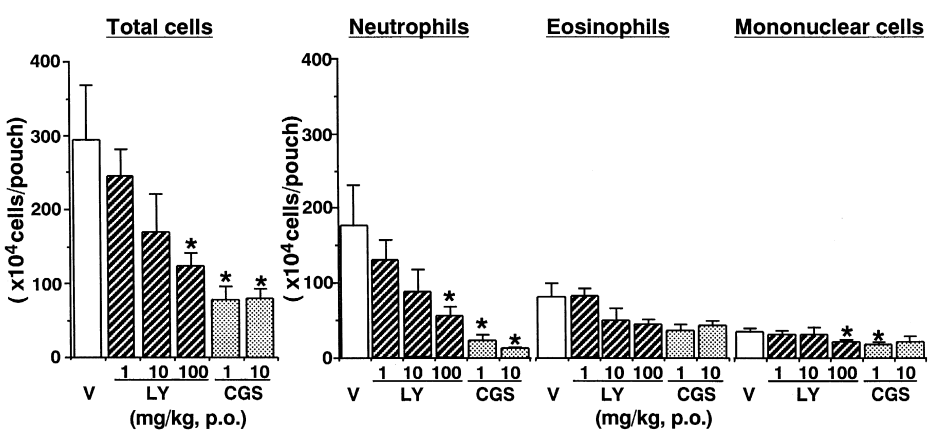

Fig. 2. Effects of LY293111Na and CGS25019C on leukotriene B_4-induced cell infiltration into air pouch in mice. Infiltrating cells were harvested 3 h after injection of 100 ng leukotriene B_4 per pouch. Drugs were administered p.o. 1 h before leukotriene B_4 injection. Each value represents the mean ± S.E.M. for six animals. * $P < 0.05$ vs. vehicle control (Dunnett's test). V: Vehicle, LY: LY293111Na, CGS: CGS25019C.

was measured. The ear swelling of each mouse was used as an index of the cell-mediated immune response.

At the end of the experiment, the mice were bled under ether anesthesia and the serum level of anti-type II collagen antibody was measured with the enzyme-linked immunosorbent assay (ELISA) system described below. Serum samples were diluted with PBS containing 1% bovine serum albumin, then added to an immune-plate (Nunc, Roskilde, Denmark), which was coated with 10 μg of type II collagen. After 2-h incubation at 36°C, the plates were washed and affinity-purified peroxidase conjugated goat anti-mouse immunoglobulin (Ig) G and IgM (Cappel Research Products, Durham, NC, USA) were added. After another 30 min incubation at room temperature, substrate was added to the plates. The color was allowed to develop for 20 min and the optical density (OD) was read at 492 nm.

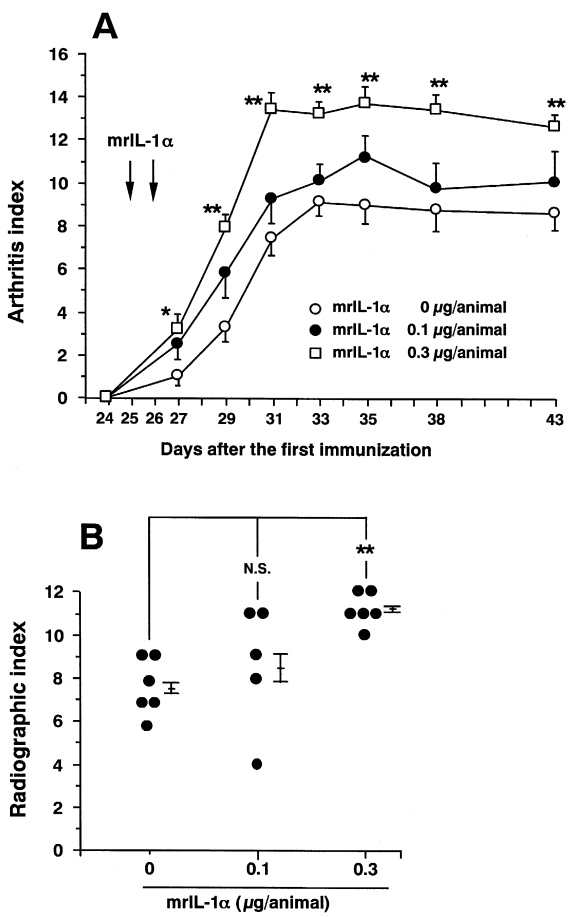

Fig. 3. Effect of interleukin-1 on collagen-induced arthritis in DBA/1J mice. Mice were immunized with 100 μg per animal of chicken type II collagen on days 0 and 21. Next, 0, 0.1 or 0.3 μg per animal of murine recombinant interleukin-1α was injected s.c. on days 25 and 26. (A) Change of arthritis index. Each value represents the mean ± S.E.M. for five to six animals. (B) Radiographs of mice were taken on day 43 and radiographic scoring was assigned as integers from 0 to 3 per joint (0 = normal, 3 = maximum joint destruction) and represents the sum of four paws of each animal. N.S., not significant, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. saline-injected (0 μg) group (Wilcoxon *U*-test). mrIL-1α: murine recombinant interleukin-1α.

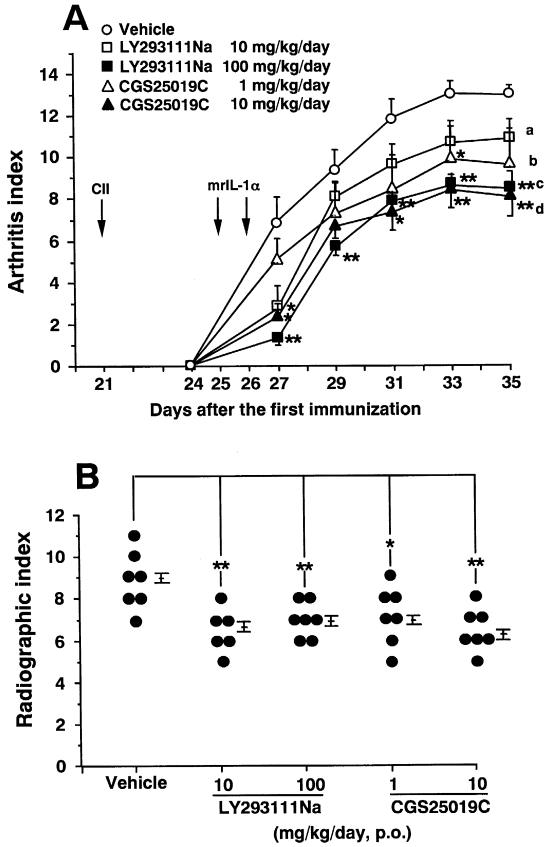

Fig. 4. Effects of LY293111Na and CGS25019C on interleukin-1-accelerated collagen-induced arthritis in mice. Arthritis was induced as described for Fig. 3. Drugs were administered p.o. twice a day from day 25 to day 35 (doses, as given per day). (A) Changes of arthritis index. Each value represents the mean ± S.E.M. for six to seven animals. (B) Radiographs of mice were taken on day 35 and radiographic scoring was assigned as in Fig. 3. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. vehicle control (Wilcoxon *U*-test). $^{a}P < 0.05$, ^{b}not significant ($P = 0.1065$), $^{c}P < 0.01$ and $^{d}P < 0.01$ vs. vehicle control (two-way ANOVA). CII: chicken type II collagen, mrIL-1α: murine recombinant interleukin-1α.

2.6. Measurement of leukotriene B_4 and prostaglandin E_2 levels in paws

Leukotriene B_4 and prostaglandin E_2 levels in paws were measured in additional sets of animals as described earlier (Hori et al., 1996). With the mice under ether anesthesia, the paws were cut off at the level of the ankle and wrist joints, immediately frozen, and kept in liquid nitrogen for at least 10 min. About 100–150 mg paw samples were pulverized by hitting a cold piston with a hammer five times, then the samples were stored at −80°C until assay. Leukotriene B_4 and prostaglandin E_2 were extracted by a procedure based on the method of Suzuki et al. (1990). The pulverized samples were mixed with 5 ml of 80% cold ethanol (high performance liquid chromatography (HPLC) grade; Wako, Osaka, Japan) and homoge-

nized for 30 s using a Polytron homogenizer, and left standing in an ice bath for 60 min. The samples were then centrifuged at 2000 × *g* for 20 min at 4°C. Supernatants were diluted to obtain 10% ethanol solution by adding

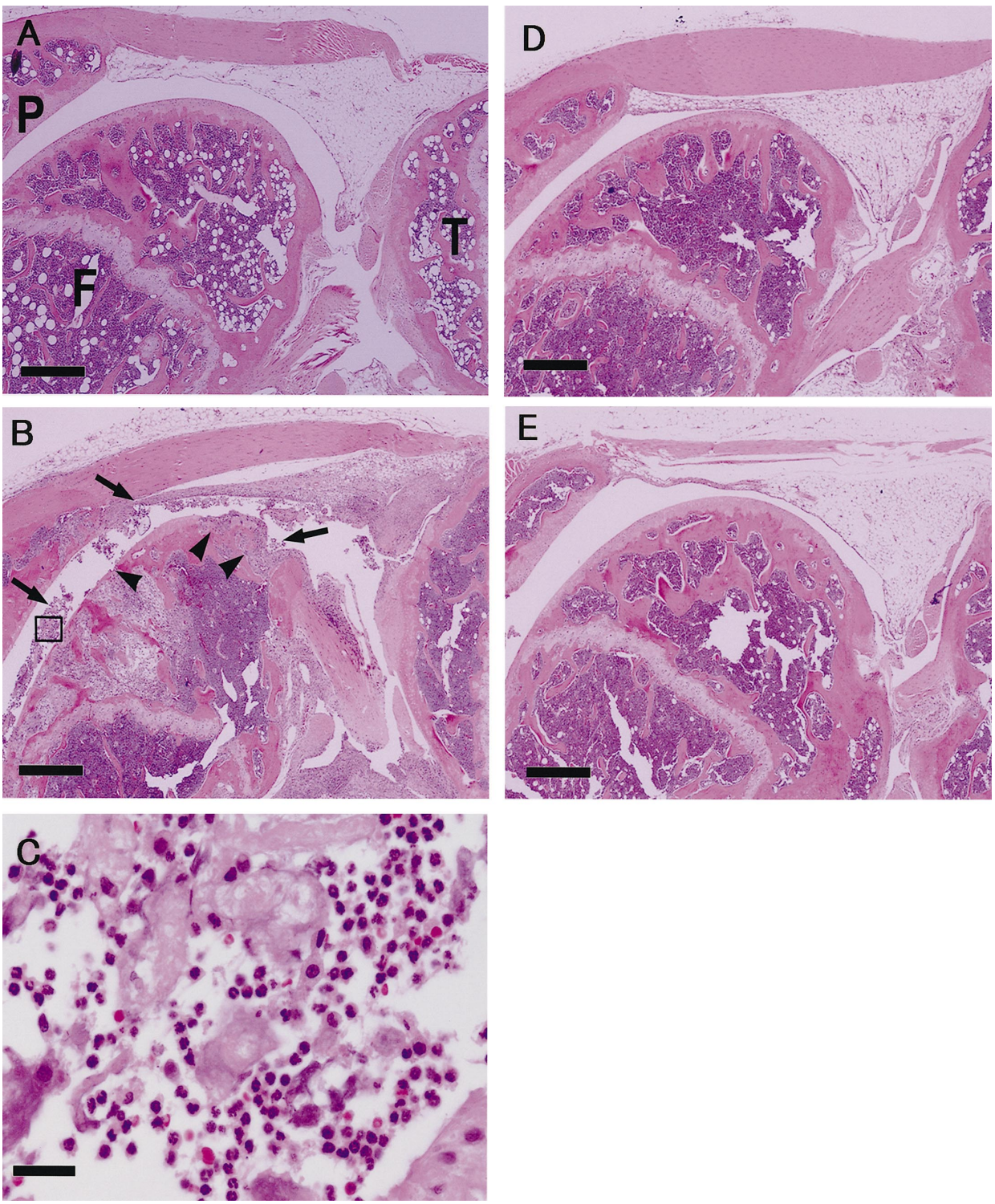

Fig. 5. Effects of LY293111Na and CGS25019C on the histological change of the lateral aspect of the knee joint on day 35 of interleukin-1-accelerated collagen-induced arthritis in mice. The knee joints were fixed in formalin-saline for at least 2 weeks, then decalcified, paraffin-sectioned (10 μm) and stained with haematoxylin and eosin. Bar = 500 μm, except for (C); 25 μm. (A) There was no histological evidence of arthritis in the joints obtained from normal mice. P: patella, F: femor, T: tibia. (B) In the vehicle-treated group, there was a massive influx of inflammatory cells and pannus formation into subsynovial connective tissues (arrows), cartilage degradation and bone erosion (arrowheads). (C) The high magnification of (B), clearly showing the massive influx of neutrophils. In contrast, treatment with 100 mg kg^{-1} day^{-1} LY293111Na p.o. (D) or 10 mg kg^{-1} day^{-1} CGS25019C p.o. (E) prevented these changes.

distilled water, acidified to pH 3.0 and immediately applied to Amprep C18 columns (Amersham, Little Chalfont, UK). After washing successively with 20 ml of 10% ethanol and 20 ml of petroleum ether (Wako), leukotriene B_4 and prostaglandin E_2 were finally extracted with 8 ml of ethyl acetate (HPLC grade; Wako). The leukotriene B_4 and prostaglandin E_2 in the eluate were quantified by radioimmunoassay, using [^{3}H]labeled leukotriene B_4 and [^{125}I]labeled prostaglandin E_2 kits (NEK-037 and NEK-020-10, respectively; New England Nuclear, Boston, MA, USA). Preliminary experiments revealed that the recovery rate of [^{3}H]leukotriene B_4 and [^{3}H]prostaglandin E_2 (ca. 8000 dpm, NET-852 and NET-428, respectively; New England Nuclear), added to the samples immediately after homogenization, was approximately 75% and 85%, respectively. Therefore, [^{3}H]prostaglandin E_2 was added to the samples to estimate the recovery rate of both leukotriene B_4 and prostaglandin E_2. Threshold amounts, detectable with these assay systems, were 12.5 pg per tube for leukotriene B_4 and 0.5 pg per tube for prostaglandin E_2.

2.7. Statistical analysis

The results were expressed as means or means ± S.E.M. For the arthritis index and radiographic index, the mean values were compared between groups by Wilcoxon *U*-test and two-way analysis of variance (ANOVA) was applied when appropriate. For the other data, the mean values were compared by Student's *t*-test or Dunnett's test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Leukotriene B_4-induced leukocyte infiltration into the air pouch and the effect of LY293111Na

To determine the amount of leukotriene B_4 suitable for inducing leukocyte infiltration into the 6-day air pouch, leukotriene B_4 was injected at 10–1000 ng per pouch and infiltrating cells were harvested 3 h later. A bell-shaped curve of increasing total cell number was observed with significant increases between 100 and 300 ng (from $0.86 \pm 0.13 \times 10^6$ cells with PBS to between 2.84 ± 0.26 and $3.68 \pm 0.13 \times 10^6$ cells, respectively; Fig. 1A).

Fig. 1B shows the time course of cellular infiltration into the pouch after the injection of 100 ng leukotriene B_4. The increase in cell number was evident at 1 h and continued for at least 6 h. Morphological analysis revealed that neutrophils were the most abundant and were accounted for about 60% of total leukocytes, followed by eosinophils and mononuclear cells, together representing about 20% of total leukocytes. Significant increases in neutrophils were observed from 1 to 12 h after the injection of leukotriene B_4, while an increase in eosinophils was noted between 1 and 6 h, and in mononuclear cells, only at 3 h.

Fig. 2 presents the effects of LY293111Na and CGS25019C on cellular infiltration 3 h after injection of 100 ng leukotriene B_4. LY293111Na (1–100 mg/kg) inhibited the increase in total cell number (by 58% at 100 mg/kg) and neutrophil infiltration (by 69% at 100 mg/kg) in a dose-dependent manner. In contrast, CGS25019C (1–10 mg/kg) was at least 100-fold more potent than LY293111Na to reduce the total cell number (by 73% at 1 mg/kg) and neutrophil (by 87% at 1 mg/kg) infiltration. Both compounds showed only slight inhibition of eosinophils (by 44% at LY293111Na 100 mg/kg, and 46% at CGS25019C 1 mg/kg, both non-significant) and mononuclear cell infiltration (by 37% at LY293111Na 100 mg/kg, and 40% at CGS25019C 1 mg/kg).

3.2. Interleukin-1 acceleration of collagen-induced arthritis in DBA/1J mice

Injection of interleukin-1α (0.1–0.3 μg per animal) to type II collagen-immunized mice hastened the onset and

Table 1
Effects of LY293111Na and CGS25019C on antibody levels and the weights of thymus and spleen in interleukin-1-accelerated collagen-induced arthritis of DBA/1J mice (day 35). Drugs were administered p.o. twice a day from day 25 to day 35 (doses in a day were presented). All parameters were measured on day 35 and each value represents the mean ± S.E.M. (CII, type II collagen; DTH, delayed-type hypersensitivity; OD, optical density at 492 nm)

Treatment	Dose (mg/kg)	*N*	Body weight (g)	Spleen weight (g per 100 g body weight)	Thymus weight	Anti-CII antibody		DTH to CII swelling (μm)
						IgG (OD)	IgM (OD)	
Normal		5	25.3 ± 0.8[a]	2.49 ± 0.79[a]	0.82 ± 0.06[b]	0.09 ± 0.06[a]	0.15 ± 0.05[a]	20 ± 4[a]
Vehicle		7	19.7 ± 0.4	7.78 ± 0.40	0.53 ± 0.12	0.31 ± 0.05	0.41 ± 0.08	360 ± 14
LY293111Na	10	7	19.9 ± 0.4	6.12 ± 0.22[c]	0.59 ± 0.08	0.27 ± 0.07	0.46 ± 0.15	358 ± 16
	100	6	20.9 ± 0.6	6.86 ± 0.16	0.40 ± 0.02	0.26 ± 0.05	0.34 ± 0.09	309 ± 22[d]
CGS25019C	1	7	21.0 ± 0.4	5.88 ± 0.40[c]	0.52 ± 0.05	0.22 ± 0.04	0.44 ± 0.09	361 ± 19
	10	7	21.8 ± 0.5[c]	6.66 ± 0.15[c]	0.50 ± 0.02	0.27 ± 0.03	0.48 ± 0.08	371 ± 14

[a] $P < 0.01$ vs. vehicle control (Student's *t*-test).
[b] $P < 0.05$ (Student's *t*-test).
[c] $P < 0.01$ vs. vehicle control (Dunnett's test).
[d] $P < 0.05$ (Dunnett's test).

enhanced the severity of arthritis. The onset of arthritis was quite sudden and severe in mice with 0.3 μg interleukin-1 in comparison to control mice, as shown by a significant increase in the arthritic index from day 27 to day 43 (Fig. 3A). Moreover, radiographic study revealed that bone destruction was also significantly enhanced by 0.3 μg interleukin-1 (from 7.7 ± 0.5 to 11.2 ± 0.3 on day 43, $P < 0.05$; Fig. 3B).

3.3. Effect of LY293111Na on interleukin-1-accelerated collagen-induced arthritis

Fig. 4A shows the effects of LY293111Na and CGS25019C on the arthritis index of interleukin-1-accelerated collagen-induced arthritis. After injection of interleukin-1, the arthritis index of the vehicle-treated group increased gradually and reached a maximum on day 33. LY293111Na (10–100 mg/kg) inhibited the progression of arthritis dose-dependently with significance at 100 mg kg^{-1} day^{-1} (by 35% on day 35) all through the experiment (Wilcoxon *U*-test). Significant differences were also found at both 10 and 100 mg/kg with *F* values being 5.32 ($P = 0.0397$) and 30.53 ($P = 0.0001$), respectively, vs. vehicle control (two-way ANOVA). CGS25019C (1–10 mg kg^{-1} day^{-1}) also dose-dependently and significantly inhibited the increase of the arthritis index (by 38% at 10 mg kg^{-1} day^{-1} on day 35; Wilcoxon *U*-test), with a potency of about 10 times that of LY293111Na. A significant difference was found at 10 mg kg^{-1} day^{-1} with an *F* value of 17.79 ($P = 0.012$) vs. vehicle control (two-way ANOVA). The effects of both compounds appeared to be proportionally greater at the earlier time points, especially day 27 (Fig. 4A). Fig. 4B shows the effects of LY293111Na and CGS25019C on cartilage and bone destruction of joints. Significant inhibition of this destruction, assessed by radiography on day 35, was observed after treatment with LY293111Na (by 26% at 10 mg/kg, 21% at 100 mg/kg) or CGS25019C (by 20% at 1 mg/kg, 28% at 10 mg/kg).

Fig. 5 shows the effects of LY293111Na and CGS25019C on histological changes of knee joints. In comparison to normal mice (Fig. 5A), the vehicle-treated mice had not only the influx of inflammatory cells, mostly neutrophils, and pannus formation into the joint space and synovium, but also cartilage degradation and bone erosion (Fig. 5B,C). On the other hand, treatment with 100 mg kg^{-1} day^{-1} LY293111Na (Fig. 5D) or 10 mg kg^{-1} day^{-1} CGS25019C (Fig. 5E) markedly prevented these changes. Both compounds significantly reduced the increase in spleen weight (by 31% with LY293111Na 10 mg/kg, 36% with CGS25019C 1 mg/kg) but had no effect against the decrease in thymus weight (Table 1). With respect to the humoral and cellular immune responses, only a slight decrease of anti-type II collagen antibody and a slight but significant decrease of the delayed-type hypersensitivity reaction (by 15%) were observed with 100 mg/kg of LY293111Na (Table 1).

3.4. Effect of LY293111Na and reference anti-inflammatory compounds on interleukin-1-accelerated collagen-induced arthritis

Fig. 6A shows the effects of LY293111Na and several anti-inflammatory drugs on interleukin-1-accelerated collagen-induced arthritis. At 100 mg kg^{-1} day^{-1}, LY293111Na significantly reduced the arthritic score at the earlier time point (Wilcoxon *U*-test) and moderately prevented the increase of the arthritis index throughout the experiment (*F* value = 3.32, $P = 0.0935$ vs. vehicle con-

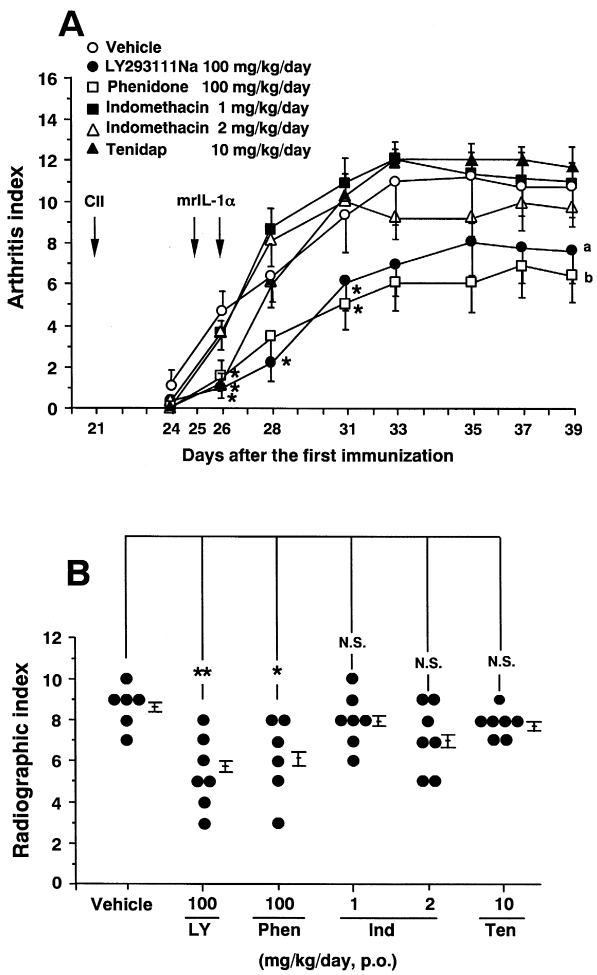

Fig. 6. Effects of LY293111Na and anti-arthritic drugs on interleukin-1-accelerated collagen-induced arthritis in mice. Arthritis was induced as described in Fig. 3. LY293111Na (LY) was administered p.o. twice a day (the dose given per day is shown), while phenidone (Phen), indomethacin (Ind) and tenidap (Ten) were administered p.o. once a day from day 25 to day 39. (A) Changes of arthritis index. Each value represents the mean ± S.E.M. for six to seven animals. (B) Radiographs of mice were taken on day 39 and radiographic scoring was assigned as in Fig. 3. N.S., not significant, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. vehicle control (Wilcoxon *U*-test). [a]Not significant ($P = 0.0935$), [b]$P < 0.05$ vs. vehicle control (two-way ANOVA).

trol, two-way ANOVA). Phenidone also showed a greater effect at the earlier time point and significantly inhibited the increase of the arthritis index (F value = 4.77, P = 0.0495 vs. vehicle control, two-way ANOVA). Indomethacin at 2 mg kg^{-1} day^{-1} partially reduced this index (by 17% on day 33), but 1 mg kg^{-1} day^{-1} of indomethacin and 10 mg kg^{-1} day^{-1} of tenidap had no inhibitory effect. Significant inhibition of cartilage and bone destruction, assessed by radiography on day 39, was observed for the treatment with 100 mg/kg of LY293111Na (37% inhibition) and phenidone (29%). In contrast, indomethacin (1 and 2 mg/kg) and tenidap (10 mg/kg) had no significant inhibitory effect (Fig. 6B). The doses for indomethacin and tenidap were maximal, because a higher dose of either one caused lethal gastrointestinal toxicity (unpublished data).

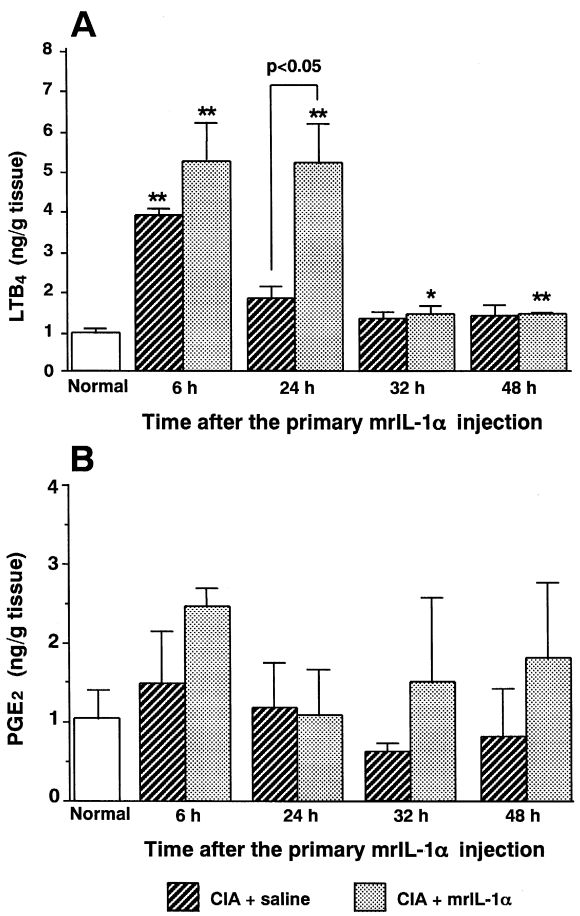

Fig. 7. Changes of leukotriene B_4 and prostaglandin E_2 levels in paws with interleukin-1-accelerated collagen-induced arthritis in DBA/1J mice. Mice were immunized with 100 μg of type II collagen on days 0 and 21, then 0.3 μg per animal of interleukin-1α was injected s.c. on day 25 (0 h) and day 26 (24 h) as described in Fig. 3. Mice were killed 6, 24, 32 and 48 h after the primary interleukin-1α injection, and (A) leukotriene B_4 and (B) prostaglandin E_2 levels in paws were measured. Paws from normal DBA/1J mice were collected on day 25. Each value represents the mean ± S.E.M. for four to five animals. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. normal control (Student's t-test).

3.5. Changes of leukotriene B_4 and prostaglandin E_2 levels in paws

As shown in Fig. 7A, marked increases of the leukotriene B_4 levels in arthritic paws in comparison to those of normal animals were observed 6 and 24 h after the interleukin-1 injection (from 1.0 ± 0.1 in normal paws to 5.2 ± 1.0 and 5.2 ± 1.0 ng/g tissue, respectively, both $P < 0.01$). In contrast, a significant increase of the leukotriene B_4 levels was observed only 6 h after the saline injection in the collagen-induced arthritis group. Furthermore, leukotriene B_4 levels in the interleukin-1-accelerated collagen-induced arthritis groups were significantly higher than those in the collagen-induced arthritis groups 24 h after the injection of interleukin-1 (5.2 ± 1.0 vs. 1.8 ± 0.3 ng/g tissue, $P < 0.05$).

The tendency to an increase of prostaglandin E_2 levels was observed in interleukin-1-accelerated collagen-induced arthritis at 6, 32 and 48 h after the interleukin-1 injection in comparison to the collagen-induced arthritis group, although it was not statistically significant (Fig. 7B).

4. Discussion

The present study showed clearly that the 6-day air pouch in mice is a good model for assessing the in vivo potency of LTB_4 receptor antagonists against the neutrophil infiltration induced by leukotriene B_4. It was also shown that leukotriene B_4 plays an important role in the development and progression of interleukin-1-accelerated collagen-induced arthritis in mice. This was evidenced by the inhibition of arthritis with LTB_4 receptor antagonists and a 5-lipoxygenase inhibitor, but not with cyclooxygenase inhibitors, which was further supported by a significant increase of the leukotriene B_4 level, but not of prostaglandin E_2 in arthritic paws.

The 6-day-old air pouch, whose lining structure somewhat resembles synovial tissue, is recognized as a useful model for studying the inflammatory responses and mechanisms of connective tissue degradation (Edwards et al., 1981; Willoughby et al., 1986). Although the in vivo chemoattractant activity of leukotriene B_4 has been well documented in the lungs and skin (Silbaugh et al., 1987; Ford-Hutchinson, 1990), these sites do not represent synovial tissues. In the present study, we clearly showed that leukotriene B_4 was capable of inducing a bell-shaped curve of leukocyte infiltration into the 6-day air pouch with neutrophils being the most abundant leukocytes. These results are consistent with the well-known in vitro characteristics of chemotactic activity of leukotriene B_4 (Ford-Hutchinson, 1990). Orally administered, the LTB_4 receptor antagonist, LY293111Na, inhibited leukotriene B_4-induced leukocyte infiltration in a dose-dependent manner and CGS25019C also strongly inhibited this response.

LY293111Na had been reported to inhibit leukotriene B_4-aerosol-induced pulmonary neutrophilia and the ex vivo expression of CD11b on neutrophils in rhesus monkeys (Allen et al., 1996). Furthermore, it inhibits allergen-induced neutrophilia in asthmatic patients (Evans et al., 1996). These findings suggest that oral LY293111Na might effectively block the neutrophil infiltration if leukotriene B_4 serves as a primary chemoattractant in the synovial cavity of patients with rheumatoid arthritis.

Since interleukin-1 has been found in the synovial fluids and joint space of arthritic patients and is capable of directly stimulating the activities of various cells in the joint (Mizel et al., 1981; Dewhirst et al., 1985; Krakauer et al., 1985), this cytokine is considered to play important roles in the development and/or maintenance of rheumatoid arthritis (Wood et al., 1983; Arend and Dayer, 1995). Hom et al. (1988, 1991) extended these findings by demonstrating the usefulness of the interleukin-1-accelerated model of collagen-induced arthritis, not only as a model for defining the nature of human rheumatoid arthritis, but also for the development of anti-arthritic drugs. Interleukin-1 produces a severe and more reproducible form of the arthritis, with a shorter time course of observation, than in the conventional collagen-induced arthritis.

Thus, we next studied the effect of LY293111Na on interleukin-1-accelerated collagen-induced arthritis in mice to explore its potential as an anti-arthritic agent. LY293111Na significantly suppressed the progression of arthritis, as well as articular bone destruction. Another LTB_4 receptor antagonist, CGS25019C, also inhibited the arthritis and its potency was about 10-fold greater than that of LY293111Na, i.e. a similar difference in potencies was noted for the two compounds in the air pouch model, as well as in interleukin-1-accelerated collagen-induced arthritis, indicating that the inhibition of the arthritis is due to the antagonism against LTB_4 receptors by these compounds. The inhibitory effects of the compounds on the arthritis appeared to be marginal, with only inhibition of the accelerated part of collagen-induced arthritis with interleukin-1, probably as a result of other mediators being operative for collagen-induced arthritis. The inhibition of arthritis was further confirmed histologically: both compounds inhibited the influx of inflammatory cells, most of which were neutrophils, and pannus formation into joint space, as well as cartilage degradation and bone erosion. Since depletion of circulating neutrophils by their antibody was effective in collagen-induced arthritis (Courtenay et al., 1980; Schrier et al., 1984) and since a neutrophil elastase inhibitor also suppressed the incidence of collagen-induced arthritis in both rats and mice (Kakimoto et al., 1995), neutrophils seem to play a critical role in the pathogenesis of this model. In addition, the lack of effect of LY293111Na and CGS25019C on humoral and cellular immunological responses in the present study strongly suggests that reducing the infiltration and activation of neutrophils by suppressing the function of leukotriene B_4 is responsible for the amelioration of interleukin-1-accelerated collagen-induced arthritis. Similar results were also reported for an LTB_4 receptor antagonist, CP-105,696, that ameliorated interleukin-1-accelerated collagen-induced arthritis without interfering with the humoral immune response (Griffiths et al., 1995) that plays a crucial role in pathogenesis of collagen-induced arthritis (Stuart and Dixon, 1983).

Hom et al. (1991) have already reported that dexamethasone, cyclophosphamide, azathioprine and methotrexate were active, but non-steroidal antiinflammatory drugs (NSAIDs) were less effective to suppress the interleukin-1-accelerated collagen-induced arthritis in mice. In the present study, cyclooxygenase inhibitors, indomethacin and tenidap, were also less effective than a 5-lipoxygenase inhibitor and LTB_4 receptor antagonists, further supporting the importance of leukotriene B_4 as an inflammatory mediator in this model of arthritis.

To verify the crucial role of leukotriene B_4 in the present study, we next examined the changes in leukotriene B_4 and prostaglandin E_2 levels in the paws of mice with interleukin-1-accelerated collagen-induced arthritis. We found a dramatic increase of leukotriene B_4 levels for at least 24 h in the paws of interleukin-1-treated mice, but only a slight increase of prostaglandin E_2 level. This is the first report of findings clearly indicating a relationship between up-regulation of leukotriene B_4 levels and changes in the severity of arthritis in mice. Saline injection also induced a significant increase of leukotriene B_4 at 6 h in immunized mice. The reason for this is not clear, however, since saline injection induced a noticeable increase of leukocyte number at 0.5–6 h in the air pouch (Fig. 1B), some trauma caused by the subcutaneous injection procedure might account for this phenomenon by stimulating periarticular leukocytes in collagen-induced arthritis mice. In human rheumatoid arthritis, increases of leukotriene B_4 levels in the synovial fluid and infiltration of neutrophils into the joint space were detected, and their levels correlated well with the severity of arthritis (Davidson et al., 1983; Ford-Hutchinson, 1990; Grignani et al., 1996). Ahmadzadeh et al. (1991) also reported that the leukotriene B_4 level in synovial fluid from patients with rheumatoid arthritis is significantly higher than that of patients with osteoarthritis. Furthermore, one study showed that there were no significant differences in the levels of prostaglandins between active and inactive rheumatoid arthritis, while the release of leukotrienes was significantly lower in the inactive phase of rheumatoid arthritis (Wittenberg et al., 1993). Since the interleukin-1-accelerated collagen-induced arthritis in mice corresponds with the acute inflammatory phase of rheumatoid arthritis, the marked increase of leukotriene B_4 levels and apparent efficacy of LTB_4 receptor antagonists in the present study are of interest in terms of rheumatoid arthritis treatments.

In the present study, the inhibitory effect of CGS25019C in both air pouch and interleukin-1-accelerated collagen-in-

duced arthritis models in mice was more than 10-fold stronger than that of LY293111Na. These results do not mirror their affinities for the human neutrophil LTB_4 receptor (Jackson et al., 1999), in which the affinity of CGS25019C (IC_{50} = 4 nM) was about four times stronger than that of LY293111Na (17 nM). In clinical studies, the effective dose of CGS25019C for inhibiting CD11b up-regulation was 300–500 mg/day (Morgan et al., 1995), and more than 500 mg/day was associated with gastrointestinal side-effects (Morgan et al., 1994; Sawyer, 1996). On the other hand, 48 or 200 mg/day of LY293111Na was enough to reduce the up-regulation of CD11b ex vivo and in a clinical study on atopic asthmatics, in which 112 mg/day of LY293111Na was given (Marder et al., 1996; Sawyer et al., 1995). These findings, along with the in vitro strong efficacy on human neutrophil functions (Jackson et al., 1999), indicate that LY293111Na is likely to be equipotent to or more potent than CGS25019C when used in human patients. The reason for these discrepancies is not clear, but it might be explicable by species differences in LTB_4 receptors and/or absorption, metabolism and excretion pathways between mice and humans (Sawyer, 1996; Huang et al., 1998).

In conclusion, leukotriene B_4 appears to be an important inflammatory mediator of the acute phase of interleukin-1-accelerated collagen-induced arthritis in mice, and the clinical relevance of the present results remains to be investigated in clinical trials of LY293111Na for human rheumatoid arthritis.

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