Angiotensin Receptor Blockers Suppress Antigen-Specific T Cell Responses and Ameliorate Collagen-Induced Arthritis in Mice

Kayo Sagawa, Katsuya Nagatani, Yoshinori Komagata, and Kazuhiko Yamamoto

Objective. The renin–angiotensin system plays an important role in the regulation of cardiovascular, renal, and endocrine functions. Recent studies have demonstrated that angiotensin II has proinflammatory effects that may contribute to the pathogenesis of immune-mediated diseases. We used the collagen-induced arthritis (CIA) model to investigate the influence of angiotensin II receptor blockers (ARBs) on antigen-specific immune responses and determine whether ARBs have preventive or therapeutic effects on the development of arthritis.

Methods. We administered ARBs (olmesartan, candesartan, and telmisartan) to mice and evaluated antigen-specific T cell proliferation and cytokine production following immunization with ovalbumin (OVA) or type II collagen in Freund's complete adjuvant (CFA) or aluminum hydroxide (alum). Next, we induced CIA in DBA/1 mice and administered olmesartan. The severity and incidence of arthritis were scored according to clinical manifestations, and joint tissue sections were examined histopathologically.

Results. ARBs severely suppressed lymphocyte proliferation and interferon-γ production in mice immunized with OVA or type II collagen in CFA. Olmesartan also suppressed lymphocyte proliferation in mice immunized with ovalbumin in alum. In the CIA model, olmesartan reduced the mean arthritis score and the incidence of severe arthritis, even when it was administered only after disease onset. Histopathologic findings for joint destruction were improved in olmesartan-treated mice.

Conclusion. ARBs suppressed antigen-specific immune responses for Th1 and Th2 in vivo. Furthermore, olmesartan suppressed the development of severe arthritis and joint destruction in the CIA model. These findings suggest that ARBs may have therapeutic potential in rheumatoid arthritis.

The renin–angiotensin system (RAS) plays an important role in the regulation of blood pressure and fluid homeostasis. Two distinct subclasses of the angiotensin II (Ang II) receptors, AT_1 and AT_2, have been described (1,2). Ang II, the major biologically active peptide produced by the RAS, causes cell proliferation and fibrosis via the AT_1 receptor and is a factor in various diseases such as hypertension, glomerular disease, and congestive heart failure (3,4).

Emerging evidence suggests that the RAS, in addition to promoting cell growth and proliferation, may also have potent proinflammatory effects that contribute to disease pathogenesis. For example, Shao et al (5) showed that levels of the Th1 cytokine interferon-γ (IFNγ) increased and those of the Th2 cytokine interleukin-4 (IL-4) decreased in Ang II–infused hypertensive rats with kidney injury, and that the administration of olmesartan, an Ang II receptor blocker (ARB), corrected this imbalance of Th subsets. Ruiz-Ortega et al (6–8) showed that Ang II activated NF-κB and up-regulated NF-κB–related genes both in vivo and in vitro.

Moreover, several recent studies demonstrated the protective effect of RAS antagonists in immunologically mediated diseases. For example, some groups of investigators demonstrated that ARBs significantly ameliorated kidney injury in rat models of chronic renal allograft rejection (9–11). In a model of chronic rejection of cardiac allografts, ARBs significantly amelio-

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rated intimal proliferation of coronary arteries, which is a pathologic finding in the setting of chronic rejection (12). Furthermore, it was reported that captopril, an angiotensin-converting enzyme (ACE) inhibitor, improved arthritis symptoms, clinical scores, plasma viscosity, and the C-reactive protein level in patients with active rheumatoid arthritis (RA) (13). In addition, Godsel et al (14) recently reported that captopril ameliorated experimental autoimmune myocarditis. These studies identified potent effects of the RAS in modulating the immune system.

Nataraj et al (15) reported that the actions of Ang II in stimulating lymphocyte proliferation played a role in modulating immune responses, and that the stimulation of AT_1 receptors on lymphocytes led to an increase in the intracellular calcium concentration. Furthermore, those investigators observed that this AT_1-mediated calcium signal triggered the activation of calcineurin and nuclear factor of activated T cells, and that cyclosporine, a specific inhibitor of calcineurin phosphatase, completely blocked the ability of Ang II to induce proliferation of cultured splenic lymphocytes. However, the mechanism underlying the beneficial actions of RAS inhibitors in preventing immune system injury has not been completely elucidated.

ARBs have been approved for use in treating hypertension, and this clinical practice has spread to many countries. In the present study, we demonstrate that ARBs have additional properties of suppressing antigen-specific Th1 responses in vivo. We evaluated olmesartan for its ability to ameliorate arthritis in the murine collagen-induced arthritis (CIA) model, which is an experimental animal model for human RA. To our knowledge, this is the first study to show antigen-specific immunosuppressive effects of the Th1 response of ARBs in vivo and to demonstrate the protective effects of ARBs in an arthritis model. Our findings suggest that ARBs may be a beneficial treatment for patients with RA.

MATERIALS AND METHODS

Mice. Female BALB/c mice (7 weeks of age) and male DBA/1 mice (6–7 weeks of age) were purchased from Japan SLC (Shizuoka, Japan). All of the animal experiments performed in this study were approved by the Animal Research Ethics Board of the Department of Allergy and Rheumatology at the University of Tokyo. The animals were maintained under specific pathogen–free conditions.

**Immunization with ovalbumin (OVA) or bovine type II collagen (CII).** OVA (grade V; Sigma, St. Louis, MO) or bovine CII (Chondrex, Seattle, WA) was solubilized to a concentration of 2 mg/ml in 0.05M acetic acid at 4°C, with constant overnight mixing. Mice were immunized in the footpads by subcutaneous injection of OVA or CII in Freund's complete adjuvant (CFA) emulsion (1 mg/ml; 0.1 ml/mouse). In some experiments, mice were immunized intraperitoneally with 2 μg/ml of OVA in 2 mg of aluminum hydroxide (alum). Immunizations were performed on day 0 and day 10.

Administration of ARBs. Olmesartan medoxomil (the prodrug of olmesartan), candesartan cilexetil, and telmisartan were kindly provided by Sankyo (Tokyo, Japan), Takeda Chemical Industries (Osaka, Japan), and Boehringer Ingelheim (Ingelheim, Germany), respectively. Olmesartan (10 or 15 mg/kg body weight), candesartan (10 mg/kg body weight), or telmisartan (10 mg/kg body weight) was administered orally in 0.5-ml suspensions every day or every other day, depending on the experiment, using a 2.25-mm feeding needle. In order to make uniform suspensions, olmesartan was suspended in carboxylmethyl cellulose sodium (CMC; Sigma), candesartan was suspended in methyl cellulose (Wako, Osaka, Japan), and telmisartan was suspended in hydroxyethyl cellulose (Roche Laboratories, Basel, Switzerland).

Cytokine analysis. Popliteal lymph node cells or splenocytes were isolated from the mice that had received olmesartan, candesartan, telmisartan, or vehicle only. After preparation of a single-cell suspension and red blood cell lysis, the cells were washed in Hanks' balanced salt solution (Sigma) and resuspended in X-VIVO 20 serum-free medium (Cambrex, Walkersville, MD). The cells were cultured in 96-well culture plates (Becton Dickinson, Franklin Lakes, NJ) at a concentration of 4×10^6 cells/ml with 3, 10, 30, 100, or 300 μg/ml of OVA or CII and medium (X-VIVO 20) alone. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 48 hours of incubation, the culture supernatants were collected, and the levels of IL-4, IL-10, and IFNγ were measured. These cytokines were determined by enzyme-linked immunosorbent assay (ELISA) using paired antibodies (PharMingen, San Diego, CA) for the corresponding cytokines, according to the manufacturer's protocol.

Proliferation assays. For the lymphocyte proliferation assay, popliteal lymph node cells or splenocytes were cultured in 96-well culture plates at a concentration of $3–4 \times 10^6$ cells/ml with 3, 10, 30, 100, or 300 μg/ml of OVA, 10 or 100 μg/ml of denatured CII, or medium (X-VIVO 20) alone. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 72 hours of culture, 1 μCi of ^{3}H-thymidine was added to each well, and the cells were incubated for an additional 16 hours at 37°C. After culturing, ^{3}H-thymidine uptake was detected using a microplate scintillation counter. Results are expressed as the mean $\pm$ SEM results of triplicate assays.

ELISA. For the measurement of OVA-specific IgG2a, IgG1, and IgE, blood samples were obtained from the inferior vena cava with a 25-gauge needle on day 7 and day 18 after the OVA/CFA immunization. After the samples had fully coagulated, they were centrifuged, and the sera were collected and stored at -80°C until used. Levels of OVA-specific IgG2a, IgG1, and IgE were determined by ELISA using biotinylated anti-mouse IgG2a, IgG1, and IgE antibodies for capture and biotinylated goat anti-mouse IgG2a, IgG1, and IgE antibodies for detection. For the measurement of CII-specific IgG1 and IgG2a, serum was collected on day 88, as described above.
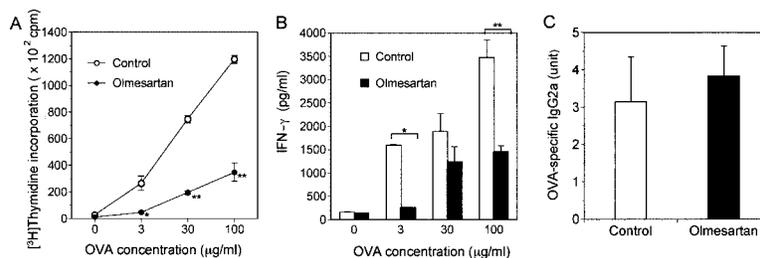
Figure 1. Olmesartan suppresses antigen-specific Th1 responses in BALB/c mice. BALB/c mice were immunized with ovalbumin (OVA) in Freund's complete adjuvant (CFA). Olmesartan (15 mg/kg body weight) or vehicle only (control) was administered every other day, beginning 5 days before immunization. Seven days after immunization, popliteal lymph node cells were obtained and cultured with OVA. **A,** After 72 hours of culture, ^{3}H-thymidine was added, and ^{3}H-thymidine incorporation was measured 16 hours later. $* = P < 0.05$ versus control; $** = P < 0.0005$ versus control. **B,** After 48 hours of culture, supernatants were tested for interferon-γ (IFNγ) concentration by enzyme-linked immunosorbent assay (ELISA). $* = P < 0.05$; $** = P < 0.001$. **C,** Seven days after mice were immunized with OVA in CFA, blood samples were obtained from the inferior vena cava. The levels of OVA-specific IgG2a were determined by ELISA. Values are the mean ± SEM.

Induction of CIA. CII (Chondrex) was solubilized to a concentration of 2 mg/ml in 0.05M acetic acid at 4°C, with constant overnight mixing. For the induction of CIA, CII was emulsified with an equal volume (1:1) of CFA (4 mg/ml; Chondrex). Mice were injected subcutaneously ~1–2 cm from the base of the tail with 100 μl of the emulsion (day 0). On day 21, the mice received a booster injection, for which the collagen was emulsified with Freund's incomplete adjuvant (IFA; Difco, Detroit, MI) instead of CFA; the mice were injected with 100 μl of the emulsion near the base of the tail at a location different from that used for the first injection. Development of arthritis was assessed by inspection 3 times weekly. The clinical severity of arthritis in each paw was quantified according to a graded scale from 0 to 4, as follows: 0 = no swelling, 1 = swelling in one digit or mild edema, 2 = moderate swelling affecting several digits, 3 = severe swelling affecting most digits, and 4 = the most severe swelling and/or ankylosis. A mean arthritis score was determined by summing the scores of all joints of all mice and dividing the result by the total number of mice in the group. The mean ± SEM values were determined.

Histopathology. All mice were killed on day 74, and the joints of the left hind paw were fixed in 10% phosphate buffered formaldehyde solution and decalcified in Parengy decalcification solution overnight. The tissue was then processed and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E), using standard methodology. The joints were studied by 2 blinded examiners from the Sapporo General Pathology Institute (Sapporo, Japan). The pathologic condition was scored in 5 categories, as follows: cartilage, cellularity, pannus, bone erosion, and ankylosis. Each category was graded from 0 to 4 as follows: 0 = normal, 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked.

Statistical analysis. Results are expressed as the mean ± SEM. The Mann-Whitney U test was used to analyze the clinical scores, the incidence of severe arthritis, and histologic findings. The unpaired t-test was used to analyze the results of cytokine and proliferation assays and serum antibody levels. P values less than 0.05 were considered significant.

RESULTS

Suppression of OVA-specific Th1 response by ARBs. To examine the immunomodulatory effects of ARBs, we administered olmesartan in vivo and checked OVA-specific T cell proliferation and cytokine production following immunization with OVA. BALB/c mice received either olmesartan (15 mg/kg) suspended in CMC or CMC only, every day beginning 5 days before immunization until the day on which the mice were killed. Seven days after immunization, we obtained blood samples and popliteal lymph nodes from the mice and performed cytokine analyses and proliferation assays. As shown in Figure 1A, in the mice that received olmesartan, OVA-specific proliferation was significantly suppressed compared with that in the control group. IFNγ production (Figure 1B) was also reduced in the olmesartan-treated mice. In contrast, no production of either IL-4 or IL-10 was detected in either group (results not shown). Furthermore, there were no significant differences between groups in the serum levels of OVA-specific IgG2a (Figure 1C).

To examine whether the immunosuppressive effect of Th1 is olmesartan-specific, we examined the effects of the 2 other ARBs, candesartan and telmisartan, using the same method. In the candesartan-treated
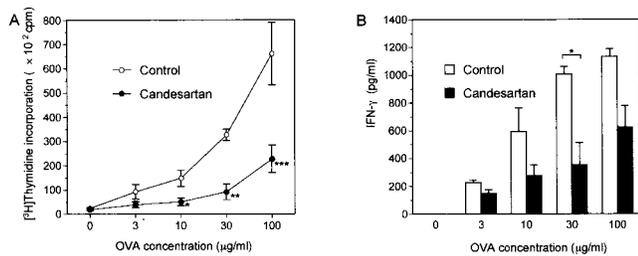
Figure 2. Candesartan reduces OVA-specific Th1 responses in vivo. BALB/c mice were immunized with OVA in CFA. Either candesartan (10 mg/kg) suspended in methylcellulose or methylcellulose only (control) was administered every day, beginning 5 days before immunization. The mice were killed, and the popliteal lymph node cells were cultured as described in Figure 1. **A,** OVA-specific proliferation of the lymphocytes was measured by ^{3}H-thymidine incorporation. $* = P < 0.05$ versus control; $** = P < 0.005$ versus control; $*** = P < 0.01$ versus control. **B,** Production of IFNγ was measured by ELISA. $* = P < 0.05$. Values are the mean $\pm$ SEM. See Figure 1 for definitions.

group, proliferation and IFNγ production (Figures 2A and B) were suppressed significantly, to the same extent as in the olmesartan-treated group ($P < 0.005$ to $P < 0.05$). In the telmisartan-treated group, proliferation and IFNγ production were also reduced compared with that in the control group, but the immune suppression of the Th1 response was milder than that observed with the other ARBs (results not shown). Serum levels of OVA-specific IgG2a also were not significantly different between the control and the telmisartan-treated groups (results not shown). These results suggested that ARBs suppress OVA-specific Th1 responses in vivo.

Suppression of CII-specific Th1 response by ARBs. To confirm that the immunosuppressive effect of ARBs is antigen-specific, we examined whether olmesartan suppressed the response to CII or mitogen after immunization with CII in CFA. DBA/1 mice received olmesartan, 10 mg/kg, every day beginning 5 days before being immunized with CII in CFA. Nine days after immunization, we obtained blood samples and popliteal lymph nodes from the mice. The lymphocytes were cultured with CII or concanavalin A in vitro, and cytokine analyses and proliferation assays were carried out. As shown in Figures 3A and B, CII-specific proliferation was significantly suppressed in the olmesartan-treated group, in which IFNγ production was also suppressed. Th2 cytokines, such as IL-4 and IL-10, were not detected (results not shown). Moreover, there were no statistically significant differences between the serum levels of CII-specific IgG1 and IgG2a (results not shown). These results suggested that olmesartan influenced only the antigen-specific response in vivo, because

concanavalin A–induced proliferation and production of IFNγ were not affected (Figures 3A and B).

Suppression of OVA-specific Th2 cell proliferation by ARBs. We also studied the influence of olmesartan on Th2 responses. BALB/c mice received intraperitoneal injections of OVA/alum on day 0 and day 10. Beginning on day -9 until the day on which the mice were killed, the mice received either olmesartan (10 mg/kg) suspended in CMC or CMC only (control) every other day. On day 18, splenocytes were obtained, and cytokine production and proliferation were analyzed. At the same time, OVA-specific IgG1 and IgE levels in sera were measured. As shown in Figure 4, proliferative responses of spleen cells isolated from olmesartan-
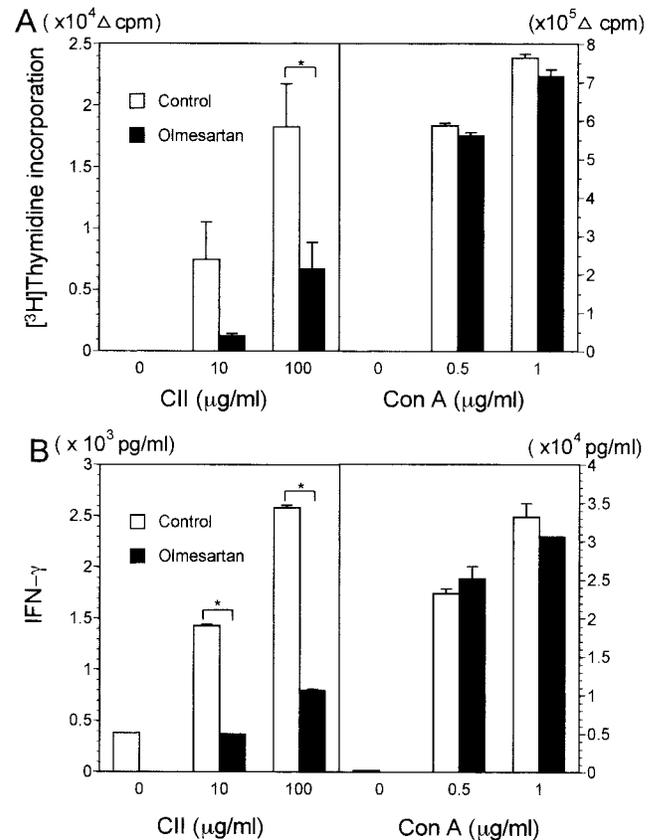
Figure 3. Administration of olmesartan to DBA/1 mice inhibits the Th1 response induced by immunization with type II collagen (CII) in CFA. Beginning 5 days before immunization, olmesartan (10 mg/kg) or vehicle only (control) was administered every day until the mice were killed. On day 9, popliteal lymph node cells were obtained and cultured with CII (10 μg/ml or 100 μg/ml), concanavalin A (Con A; 0.5 μg/ml or 1.0 μg/ml), or medium alone. **A,** Proliferation of lymphocytes was measured by ^{3}H-thymidine incorporation. $* = P < 0.05$. **B,** Production of IFNγ was measured by ELISA. $* = P < 0.0005$. Values are the mean and SEM. See Figure 1 for other definitions.
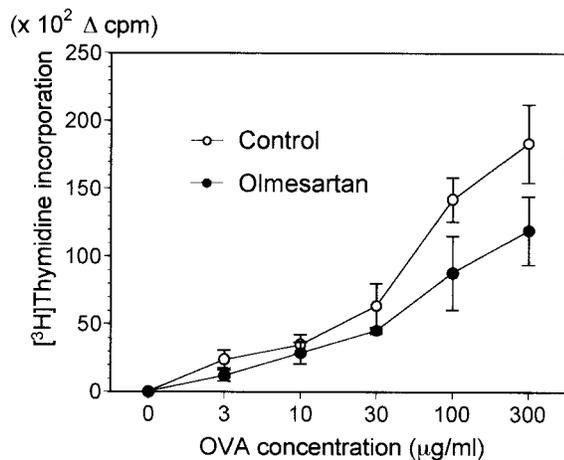
Figure 4. Olmesartan suppresses ovalbumin (OVA)–specific Th2 proliferation. BALB/c mice were immunized intraperitoneally with 2 μg of OVA in 2 mg of aluminum hydroxide on days 0 and 10. Beginning 9 days before immunization, olmesartan (10 mg/kg) suspended in carboxylmethyl cellulose sodium (CMC) or CMC only (control) was administered every day until the mice were killed. On day 18, spleen cells and blood samples were obtained. Spleen cells were cultured with OVA or medium alone. Proliferation of the cells was measured by the method described in Figure 1. Values are the mean ± SEM.

treated mice were lower than those of cells isolated from controls, but the differences between groups were not statistically significant. Serum OVA-specific IgG1 and IgE levels were not statistically significantly different between the olmesartan-treated group and the control group (results not shown). Concentrations of IL-4, IL-10, and IFNγ in the culture supernatants were below the detection limit of the ELISA (data not shown). These results suggested that although the suppression level of the Th2 response was considerably weaker than that of the Th1 response, ARBs reduced OVA-specific proliferation of Th2 cells without shifting from the Th1 response to the Th2 response.

Blockade of the development and progression of CIA by ARBs. CIA is a commonly used mouse model of human RA. Because CII-specific immune responses by draining lymph node cells were suppressed in vitro (Figure 3), we next administered olmesartan to mice with CIA in order to examine immunosuppression of Th1 responses by ARBs in this disease model. Mice received immunizations with CII in CFA on day 0 and with CII in IFA on day 21. Beginning on day −9, each mouse received olmesartan (10 mg/kg) suspended in CMC or CMC only (control); administration continued every other day until day 70. The severity of arthritis in the mice was scored on a scale of 0–4 for each limb. The

mean arthritis score was determined by summing the scores of all joints of the mice and dividing the resulting value by the total number of mice in the group. The incidence of severe arthritis was determined by the percentage of mice that had at least 1 joint with a score of 4. Progression of arthritis was evaluated until day 70 after immunization, and the number of paws affected and the mean clinical scores were recorded.

In the control group, severe arthritis began to appear beginning ~35 days after immunization and peaked on day 70 after immunization (Figure 5A). Olmesartan-treated mice had milder arthritis compared with control mice (mean ± SEM arthritis score 10.9 ± 0.57 versus 13.9 ± 1.0), and their scores were statistically significantly lower than those of controls on days 51, 56, 66, and 70 as well as at the end of the experiment (Figure 5A). Thirty-nine days after immunization, the incidence of arthritis was 100% in both the control and olmesartan-treated groups, and this incidence remained unchanged for the rest of the experiment (Figure 5B). The incidence of severe arthritis (defined as a score of 4) was lower in the olmesartan-treated group than in the control group treated with CMC alone (Figure 5C), but there was no statistically significant difference between these groups.

To determine whether olmesartan administration prevented articular destruction, histologic sections obtained from the hind paws of the mice were examined. The left hind paws of all mice in each group (n = 10 per group) were analyzed grossly and histopathologically by staining with H&E on day 74 after immunization. The histopathologic arthritis score was assessed according to findings of cartilage destruction, synovial hypertrophy, pannus formation, bone erosion, and ankylosis. Results of the histopathologic examinations are summarized in Table 1. Histopathology revealed statistically significant reductions in cartilage loss, cellular infiltrates, pannus formation, bone erosion, and ankylosis. Thus, suppression of the clinical scores correlated with the reduction in histopathologic findings. These results suggest that ARBs blocked the development and progression of CIA by suppressing Th1 responses to CII and local inflammation.

It was important to determine whether similar effects can be obtained by administering olmesartan after the onset of CIA. Therefore, we next administered olmesartan to DBA/1 mice before and after CIA became clinically detectable. For this experiment, olmesartan was administered every day. According to the prophylactic protocol, olmesartan (10 mg/kg) or vehicle only was administered, beginning 5 days before immunization
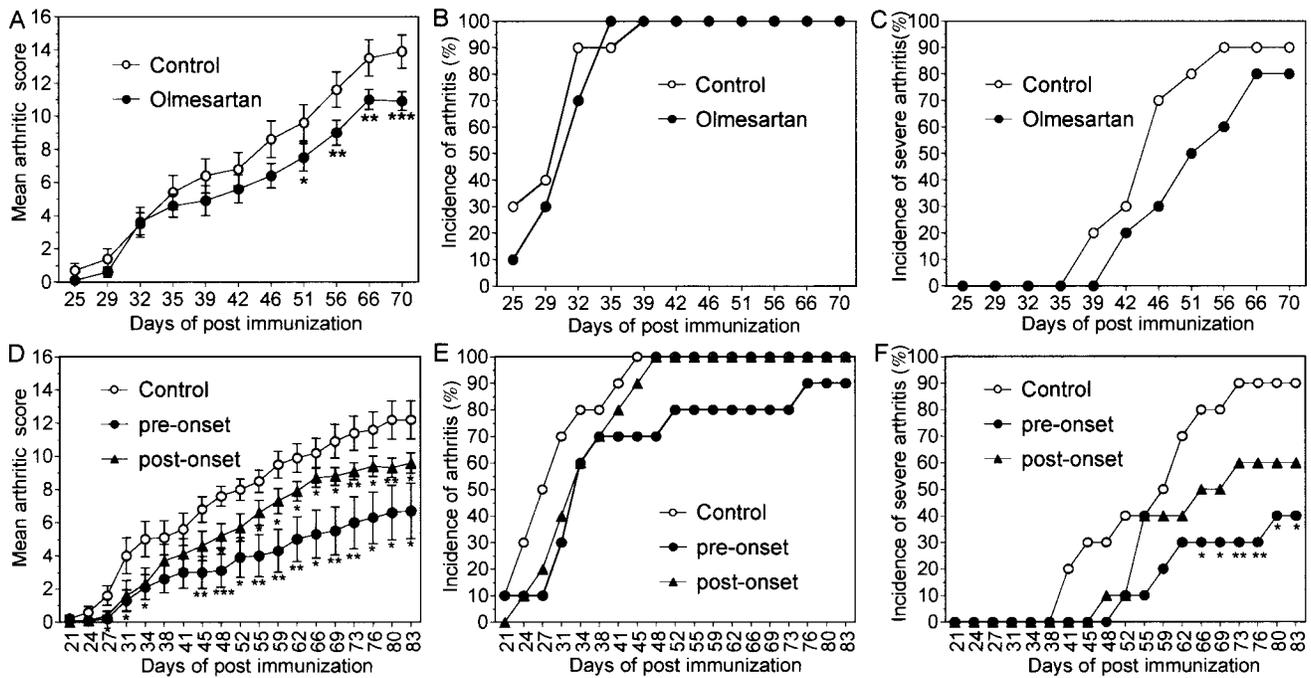
Figure 5. Administration of olmesartan blocks development of collagen-induced arthritis (CIA). Arthritis was induced in DBA/1 mice by immunization with type II collagen (CII) in Freund's complete adjuvant on day 0. On day 21, mice were injected subcutaneously with CII in Freund's incomplete adjuvant. **A–C,** Administration of olmesartan before immunization. Beginning 9 days before immunization and continuing until day 70, mice received olmesartan (10 mg/kg) suspended in carboxylmethyl cellulose sodium (CMC) or CMC only (control) every other day. Clinical scores were determined as described in Materials and Methods. **A,** Mean $\pm$ SEM arthritis scores in the 2 groups. $*$ = $P < 0.05$ versus control; $**$ = $P < 0.01$ versus control; $***$ = $P < 0.005$ versus control. **B,** Incidence of arthritis in the 2 groups. **C,** Percentage of mice with severe arthritis (arthritis score = 4). Representative results of 2 independent experiments are shown (n = 10 mice/group). **D–F,** Administration of olmesartan after disease onset. Mice received olmesartan (10 mg/kg) every day, beginning on the day after clinically evident onset of arthritis and continuing until day 87 after onset. **D,** Mean $\pm$ SEM arthritis scores in mice that received CMC alone (control; n = 10), mice that received olmesartan 5 days before immunization (pre-onset; n = 10), and mice that received olmesartan beginning on the day after onset of clinically evident arthritis (post-onset; n = 10). $*$ = $P < 0.05$ versus control; $**$ = $P < 0.01$ versus control; $***$ = $P < 0.005$ versus control. **E,** Incidence of arthritis. **F,** Percentage of mice with severe arthritis (arthritis score = 4). $*$ = $P < 0.05$ versus control; $**$ = $P < 0.01$ versus control.

and continuing until day 87; according to the therapeutic protocol, olmesartan (10 mg/kg) or vehicle only was administered, beginning on day 25 and continuing until day 87 (Figures 5D–F).

Control mice that were treated with vehicle only according to the prophylactic protocol showed signs of

Table 1. Impact of ARB treatment in the murine CIA model*

Pathology category	Control	ARB-treated	P†
Cartilage	2.9 $\pm$ 1.20	1.0 $\pm$ 1.41	0.008
Cellularity	2.9 $\pm$ 1.20	1.0 $\pm$ 1.33	0.006
Pannus	2.7 $\pm$ 1.25	1.1 $\pm$ 1.52	0.028
Bone erosion	2.9 $\pm$ 1.20	0.9 $\pm$ 1.20	0.003
Ankylosis	2.6 $\pm$ 1.0	1.0 $\pm$ 1.33	0.013

* Values are the mean $\pm$ SEM pathology score (0 = normal, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked). ARB = angiotensin II receptor blocker; CIA = collagen-induced arthritis.
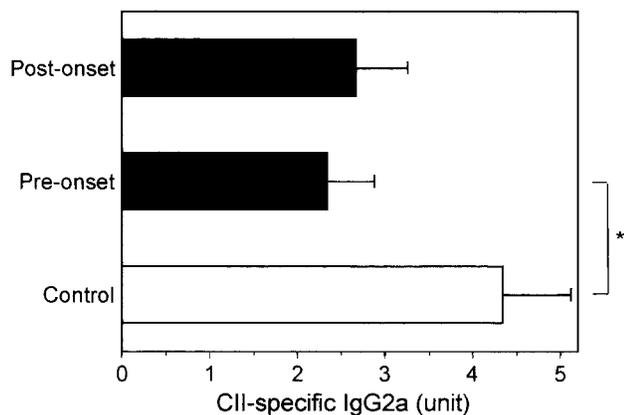† By Mann-Whitney U test.

arthritis beginning ~21 days after immunization and peaking on day 80 after immunization (Figure 5D). Compared with daily administration of CMC only, administration of olmesartan according to the prophylactic protocol effectively suppressed disease. Among mice treated according to the prophylactic protocol, the mean $\pm$ SEM arthritis score at the end of the experiment was 12.2 $\pm$ 1.14 in the control group versus 6.7 $\pm$ 1.69 ($P = 0.029$) in the olmesartan-treated group (Figure 5D). In contrast, among mice treated with olmesartan according to the therapeutic protocol, the mean $\pm$ SEM arthritis score at the end of the experiment was 9.6 $\pm$ 0.62 ($P = 0.014$) (Figure 5D). Among mice treated according to the prophylactic protocol, the mean arthritis score (Figure 5D), incidence of arthritis (Figure 5E), and incidence of severe arthritis (Figure 5F) in the olmesartan-treated group were suppressed compared

Figure 6. Measurement of type II collagen (CII)–specific IgG2a. In the experiment referred to in Figure 5D, blood samples were obtained from the inferior vena cava on day 88. Levels of anti-CII IgG2a in the 3 groups (control, pre-onset, and post-onset [n = 10 per group]) were determined by enzyme-linked immunosorbent assay. Values are the mean and SEM. ∗ = $P < 0.05$.

with the control group. Among mice treated according to the therapeutic protocol, the incidence of severe arthritis was reduced compared with that in the control group (Figure 5F), but 48 days after immunization the incidence of arthritis was 100% in both the control and olmesartan-treated mice and remained unchanged for the duration of the experiment (Figure 5E). Finally, on day 88 after immunization, serum CII-specific levels of IgG1 and IgG2a were reduced in the olmesartan-treated group (Figure 6), and the reduction in CII-specific IgG2a levels was significant ($P = 0.049$). These data indicated that olmesartan suppressed CIA both before and after disease onset.

DISCUSSION

In this study, we examined the influence of ARBs on antigen-specific Th1 and Th2 responses in vivo. Furthermore, we assessed the immunosuppressive effects of ARBs on the development of the murine CIA model, which is a Th1-driven animal model of human RA. Naive CD4+ T cells differentiate into 2 distinct subpopulations, Th1 cells and Th2 cells, each of which produces its own panel of cytokines and mediates separate functions (16). Th1 cells secrete IFNγ, IL-2, and tumor necrosis factor α (16), thereby activating macrophages, inducing delayed-type hypersensitivity responses, and helping in the immunoglobulin isotype switch from IgM to IgG2a (17). In contrast, Th2 cells secrete IL-4, IL-5, and IL-10 in response to extracellular

bacterial pathogens and help in the immunoglobulin isotype switch from IgM to IgG1 and IgE (16,17).

In our study, the proliferation of antigen-specific Th1 cells and the production of IFNγ in vitro were suppressed by ARB administration in vivo (Figures 1 and 2), although the suppressive effect of telmisartan was smaller than that of the other ARBs, olmesartan and candesartan (data not shown). However, production of the Th1-dependent IgG antibody (IgG2a) was not suppressed (Figure 1C). In addition, ARBs also reduced antigen-specific Th2 cell proliferation, although the level of suppression of Th2 responses was lower than that of Th1 responses (Figure 4). As in the case of Th1, production of Th2-dependent IgG antibody (IgG1) was not significantly different between ARB-treated mice and controls (data not shown). Generally, the proliferation of Th1 cells prevents the generation of Th2 cells, whereas the proliferation of Th2 cells prevents the generation of Th1 cells (18). In a continuous Ang II infusion model of rats, Shao et al (5) showed that Ang II polarized CD4+ T cells into Th1 lymphocytes, and that the polarization was normalized by ARBs. Interestingly, in our study ARBs suppressed not only Th1 responses but also Th2 responses in vivo without enhancing the production of Th2 or Th1 cytokines. It is possible that ARBs suppress both Th1 and Th2 responses in cases in which CD4+ T cells are extremely polarized into Th1 or Th2 cells.

Several recent studies have demonstrated the protective effects of RAS antagonists in immunologically mediated conditions such as myocarditis, chronic allograft rejection, and antiglomerular basement membrane nephritis (9–12,14,19–21). However, the mechanism underlying the beneficial actions of RAS inhibitors in preventing immunologic injury in these models is still unclear. To analyze the immunosuppressive effect of ARBs on Th1 responses in a disease model, we administered olmesartan orally in a murine CIA model. We chose olmesartan from among the ARBs because it suppressed Th1 responses in vivo more potently than did the other ARBs tested. There were no signs that blood pressure was reduced in any of the mice throughout this study. In our study, the development and progression of CIA appeared to be blocked in the olmesartan-treated group (Figure 5). Furthermore, not only the clinical scores but also results of the histologic analysis of olmesartan-treated mice revealed that their joints had much milder inflammation compared with control mice (Table 1). Importantly, olmesartan was effective even when it was introduced after the onset of arthritis (Figures 5D–F). These data suggest that ARBs may be

useful therapeutically in RA, and that Ang II may be involved in the development of CIA.

CIA is associated with a Th1-polarized immune response, rendering it an excellent model in which to explore the effect of olmesartan in vivo. To confirm the relationship between the CII-specific immune responses in vitro and CIA in vivo, we examined CII-specific proliferation and cytokine production by draining lymph node cells obtained from mice belonging to the same strain, DBA/1 (Figure 3). According to our data, CII-specific proliferation and IFNγ production were suppressed in vitro (Figures 3A and B). Moreover, in order to make sure that the suppressive effects of olmesartan were antigen-specific, we examined the response of lymphocytes to a mitogen (Figures 3A and B). Concanavalin A–induced proliferation and IFNγ production were similar between the olmesartan-treated and control groups, indicating that olmesartan suppresses only antigen-specific responses. During the acute phase (day 9), the levels of CII-specific IgG2a were also similar between the olmesartan and control groups, but during a later phase (day 88) the levels in the olmesartan group were significantly suppressed (Figure 6). These data suggest that olmesartan can effectively suppress anti-collagen B cell responses during a later phase of CIA.

It has been reported that immunocompetent cells, including T cells, macrophages, and dendritic cells, are equipped with components of the RAS, and that they can participate in the production of Ang II (22–24). It has also been reported that AT$_1$ receptors are expressed in human synovium (25), and that ACE activity in synovial fluid is increased in patients with arthritis (26–28). It has been demonstrated that both AT$_1$ and AT$_2$ receptors activate the NF-κB pathway and upregulate the NF-κB gene (6–8,29–32). The constitutive activation of the NF-κB pathway is often associated with inflammatory diseases such as RA, inflammatory bowel diseases, multiple sclerosis, and asthma (33). In our study, ARB administration attenuated the development of CIA clinically and pathologically, suggesting that Ang II, which in the CIA model is locally generated in the synovium, exacerbates inflammation of the synovium in articular muscle via the up-regulation of NF-κB. Alternatively, it has been speculated that another mechanism allows ARBs to directly suppress Th1 responses, because the AT$_1$ receptor is present on T cells (34–36).

Ang II acts via AT$_1$ and/or AT$_2$ receptors. AT$_1$ receptors are involved in cell proliferation as well as in the production of cytokines and extracellular matrix proteins by cultured cells (4,32,37,38). AT$_2$ receptors regulate blood pressure control and renal natriuresis,

and, after vascular injury, inhibit both cell proliferation and neointimal formation. Because Ang II activates NF-κB via both AT$_1$ and AT$_2$ receptors, and because Esteban et al (31) showed that only combined treatment with AT$_1$ and AT$_2$ antagonists completely blocked renal inflammatory infiltration and NF-κB activation in Ang II–infused mice, therapy combining AT$_1$ and AT$_2$ antagonists may be more effective than therapy using AT$_1$ antagonist alone in reducing the inflammation of arthritis. In this study, we administered a relatively high dose of olmesartan to mice. This approach was used because Shao et al demonstrated an increase in the level of IFNγ and a decrease in the level of IL-4 in Ang II–infused rats and showed that this imbalance in T cell subsets was reversed by olmesartan, in a dose-dependent manner (5). Furthermore, in the CIA model, mean arthritis scores were only slightly improved when olmesartan was administered every other day but were extremely improved when olmesartan was administered daily. Thus, for more effective suppression, the means of administration and the doses of ARB need to be modified.

In conclusion, our findings suggest that ARBs restrain exacerbation of arthritis in the CIA model. It was previously reported that the ACE inhibitor captopril improved arthritis symptoms and laboratory values in patients with active arthritis (13). However, it has never been reported that ARBs may be of therapeutic benefit to patients with arthritis. It has become clear that several serine proteases, including kallikrein, cathepsin G, and chymase, are related to ACE-independent Ang II formation in vivo (39,40); in particular, chymase is responsible for most Ang II formation in humans (41). The ARBs have much greater potential than ACE inhibitors for blocking angiotensin II production, and they may be better drugs for patients with arthritis and hypertension.

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