An Angiogenesis Inhibitor, 2-Methoxyestradiol, Involutes Rat Collagen-Induced Arthritis and Suppresses Gene Expression of Synovial Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor

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ABSTRACT. ***Objective.*** Rheumatoid arthritis (RA) pannus may be dependent on angiogenesis and several critical growth factors including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). 2-Methoxyestradiol (2ME2), an endogenous metabolite with low estrogen receptor affinity, has both antiangiogenic and antiproliferative activity. 2ME2 was assessed in the rat collagen-induced arthritis (CIA) model to determine if it could prevent or involute established synovitis.

Methods. Rats were immunized on Day 0 with collagen and randomized to a vehicle control or two 2ME2 prevention arms. In additional studies, multiple parallel treatment arms were initiated at Day 10 after arthritis onset.

Results. 2ME2 in preventive protocols at 30 or 100 mg/kg significantly delayed the onset and reduced the severity of clinical and radiographic CIA. In established CIA, oral 2ME2 at 50 mg/kg/bid, 100 mg/kg/day, and 300 mg/kg/day reduced severity compared to vehicle controls. Efficacy of 2ME2 delivery by osmotic pumps at 60 mg/kg/day was equivalent to 300 mg/kg/day by daily gavage. The 3 oral treatment protocols all significantly reduced radiographic scores in a dose-dependent fashion, with the greatest benefit at 300 mg/kg. 2ME2 showed marked suppression of synovial gene expression of proangiogenic bFGF and VEGF, with parallel reduction of synovial blood vessels. Serum antibody levels to native type II collagen were not reduced, suggesting that 2ME2 did not influence humoral immunity.

Conclusion. Our results indicate that 2ME2 may represent a novel agent for the treatment of inflammatory autoimmune diseases such as RA. (First Release Sept 15 2008; J Rheumatol 2008;35: 2119–28; doi:10.3899/jrheum.080302)

Key Indexing Terms:
ANIMAL DISEASE MODELS ENDOTHELIAL CELLS 2-METHOXYESTRADIOL
VASCULAR ENDOTHELIAL GROWTH FACTOR ANGIOGENESIS INHIBITION

Angiogenesis is crucial for organ development and repair, and contributes to the pathogenesis of numerous disorders including proliferative retinopathies, malignancies, and inflammatory arthritic conditions. Inhibition of angiogenesis has been extensively studied for the treatment of a variety of cancers, and in recent years, potential as an antirheumatic therapeutic option has been increasingly recognized. New blood-vessel formation appears to be one of the many processes required during the pathogenesis of proliferative synovitis, of which rheumatoid arthritis (RA) is the prototype. Under normal conditions, the synovium maintains a balance between pro- and antiangiogenic forces. However, in the inflammatory synovium, this balance is lost and there is an increase in proangiogenic factors, leading to endothelial cell proliferation and pannus growth. Studies have revealed elevated levels of multiple proangiogenic molecules, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), in RA synovium with no significant change in antiangiogenic molecules, such as endostatin[1]. The prospect of regaining balance between pro- and antiangiogenic factors in the synovium has led researchers to study inhibition of angiogenesis for the treatment of inflammatory arthritis.

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Typically, the subsynovial tissue is replete with blood vessels and lymphatics, but these do not invade the synovial layer[2]. During the development of RA, however, vascular endothelial cells increasingly recruit inflammatory cells to the synovium. To support rapid pannus proliferation, an additional blood-vessel network must develop. RA synovium has been shown to have an altered density of sublining vasculature and increased expression of proangiogenic mediators[3]. Within the pannus, endothelial cells proliferate and migrate to the site of inflammation and hypoxia[4]. Even with this heightened growth of new vessels, the synovium remains an area of relative hypoperfusion and hypoxia. There are many parallels between the growth of RA pannus and the growth of tumors. Both proliferate beyond the reach of their supplying vasculatures, making formation of new blood vessels critical to their progression.

2-Methoxyestradiol (2ME2), shown structurally in Figure 1, is a naturally-occurring endogenous estrogen metabolite with a low affinity for the estrogen receptor (0.05%). It has antiproliferative, antiangiogenic, and proapoptotic activity[5-10]. Mechanistically, 2ME2 attaches to the colchicine-binding site of tubulin, leading to microtubule depolymerization and downregulation of transcription factors, including hypoxia inducible factor-1α (HIF-1α), nuclear factor-κB, and Stat-3. In addition, 2ME2 has been reported to induce apoptosis through the intrinsic and extrinsic pathways and reactive oxygen species. 2ME2 inhibits tumor-associated angiogenesis[5,11,12] and malignant progression in multiple tumor models in the absence of dose-limiting toxicities. It is being evaluated in several oncology Phase II clinical trials at doses as high as 6000 mg/day. Manageable changes in liver function tests and hypophosphatemia have been described in some patients. Our study evaluates the potential therapeutic effects of 2ME2 in rat collagen-induced arthritis (CIA), an animal model of RA.

MATERIALS AND METHODS

The rat CIA model. Syngeneic 8 to 10-week-old LOU rats, immunized intradermally with native type II collagen in incomplete Freund's adjuvant (IFA) on Day 0, will typically develop arthritis on Day 10 in 90% of the animals, with progression to joint destruction by Day 28. The severity of disease can be reproducibly scored clinically, radiographically, and histologically. As in RA, the hallmark of arthritis is pannus formation with subsequent cartilage and bone erosions. Although mice are susceptible to CIA, the arthritis is milder, with onset that can be spread over days to weeks. Murine arthritis is typically induced with complete Freund's adjuvant, whereas rat CIA requires only IFA.

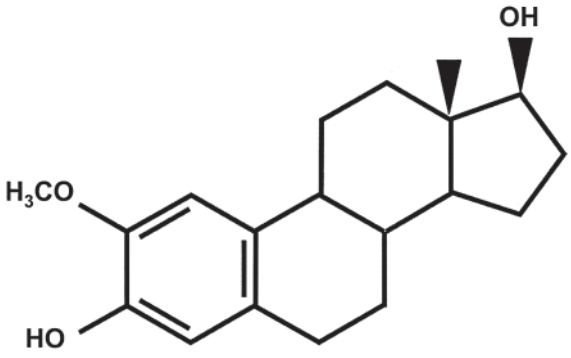

Figure 1. The structure of 2-methoxyestradiol (2ME2).

Induction of CIA. Animal experiments were performed in accord with the Institutional Animal Care and Use Committee at UCLA. All research involving laboratory animals and recombinant DNA techniques is in compliance with NIH guidelines. All vertebrate research was in compliance with regulations promulgated by the US Department of Agriculture under amendments of the Animal Welfare Act, public law 99-198. Syngeneic LOU rats were maintained and bred in an in-house colony. Rats were housed in barrier cages with no more than 4 adults per cage, provided with *ad libitum* food and water, and weighed at the beginning and end of the study. To induce arthritis, anesthetized rats (100–150 g, 8-10 weeks old) were injected intradermally on Day 0 with 0.5 mg of native chick type II collagen (Elastin Products Co., Owensville, MO, USA) solubilized in 0.1 M acetic acid and emulsified in equal volume of IFA (Difco, Detroit, MI, USA).

Protocols with 2ME2. Syngeneic rats were immunized on Day 0 with type II collagen and randomized to a vehicle control group (n = 12) or 2 prevention arms, 30 mg/kg/day (n = 11) or 100 mg/kg/day (n = 11), starting on Day 0. The 2ME2 was provided in a NanoCrystal® colloidal dispersion (Elan Drug Delivery, Inc., King of Prussia, PA, USA) and administered by oral gavage. Parallel treatment arms were initiated at Day 10, at the time of arthritis onset, with 10 mg/kg/day (n = 10), 30 mg/kg/day (n = 11), or 100 mg/kg/day (n = 11). In a subsequent study, additional rats (n = 52) were enrolled in one of 6 treatment arms beginning at arthritis onset (day 10). These included (1) oral vehicle control; (2) 2ME2 50 mg/kg/day bid; (3) 100 mg/kg/day; (4) 300 mg/kg/day; (5) subcutaneous osmotic pumps (Alzet, 2ML4; Cupertino, CA, USA) with vehicle control; and (6) subcutaneous osmotic pumps delivering 2ME2 60 mg/kg/day (Figure 2). Osmotic pumps were loaded 24 hours prior to surgery and incubated in sterile saline to achieve the steady-state pumping rate by the time of implantation, as per manufacturer's instructions. Rats were scored daily for clinical arthritis signs and radiographs were obtained at the conclusion of the study (Day 28).

Assessment of clinical arthritis. The severity of arthritis was evaluated by standard methods as described[13]. Clinical severity is quantified by blinded daily scoring of each hind paw from 0 to 4 (0 = normal, 4 = maximum) based on increasing levels of swelling and periarticular erythema (0, no erythema or swelling; 1+, isolated ankle swelling; 2+, swelling/erythema of ankle and proximal half of tarsal joints; 3+, swelling/erythema of ankle and all tarsal joints up to metatarsophalangeal joints; 4+, swelling/erythema of entire paw including digits[14-17]. The arthritic index of a rat was defined as the sum of its 4 limb scores. Since CIA typically involves only the hind limbs, an arthritic index of 6 to 8 is considered severe arthritis.

Radiographic severity. High-resolution blinded digital radiographs of both hind limbs were performed for rats sacrificed at Day 28. Each limb was scored 0–3, with a summed maximum score of 6 based on the extent of soft-tissue swelling, joint space narrowing, bone destruction, and periosteal new bone formation, (0, normal; 1+, soft-tissue swelling only; 2+, soft-tissue swelling and early erosions; 3+, severe erosions)[15-17]. 3D Micro-computed tomography was performed on a few selected rats for qualitative assessment only[18].

Light microscopy. Hind limbs were harvested, fixed in phosphate-buffered 10% formaldehyde, decalcified, and stained. Paraffin blocks were sectioned at approximately 5 microns. The tarsus and digits were cut in a sagittal plane and sections stained with hematoxylin and eosin or safranin O. Scoring (0–17 scale) was performed by a single blinded observer using a modified Mankin method[19]. Digital images were recorded with an Olympus BX51 light and fluorescent microscope equipped with an Olympus DP70 digital camera and MicroSuite™ Five imaging software. Final images were prepared using the Adobe Photoshop™ program.

Immunohistochemical staining for von Willebrand factor (vWF). Rear hind limbs were resected and fixed in 10% buffered formalin. Limbs were decal-

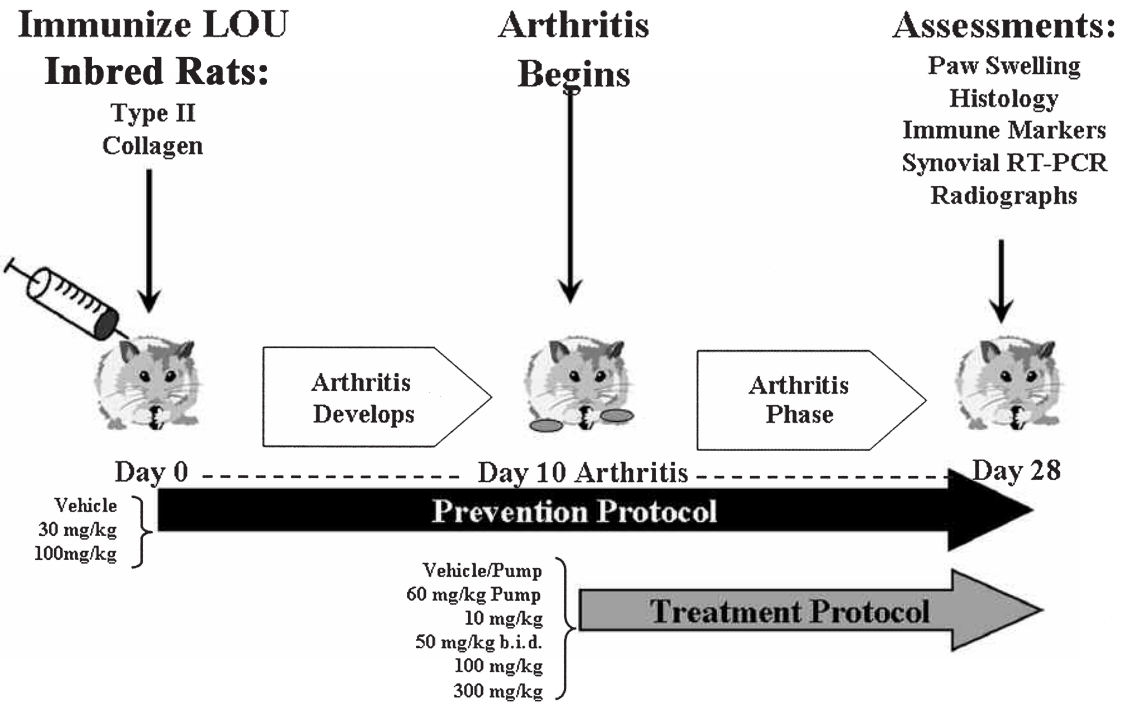

Figure 2. An overview of the protocol. Rats were immunized with type II collagen on Day 0, developed clinical arthritis on Day 10, and were sacrificed on Day 28. Prevention protocols began on Day 0 with vehicle control or 2ME2 30 mg/kg or 100 mg/kg. Treatment protocols began on Day 10 with vehicle, 60 mg/kg via osmotic pump, 10 mg/kg, 50 mg/kg bid, 100 mg/kg, or 300 mg/kg. Clinical arthritis was scored from Days 10 to 28 and at sacrifice, samples were analyzed for histologic and radiographic changes as well as synovial gene expression and blood-vessel density.

cified with 5% formic acid, dehydrated through a series of ascending ethanol solutions, and embedded in paraffin. Sagittal sections through the middle of the hind paw were obtained using a rotary microtome (Model RM2165, Leica Microsystems, Bannockburn, IL, USA). Four-micron sections were stained for vWF to visualize neovascularization. Briefly, sections were incubated with primary antibody, rabbit anti-vWF antibody (1:250; Dako, Carpinteria, CA, USA), followed by incubation with biotinylated anti-rabbit IgG (1:100; Sigma-Aldrich, St. Louis, MO, USA). Blood vessels were then visualized following sequential incubation with streptavidin horseradish peroxidase and 3'3'-diaminobenzadine (DAB; Dako).

Reverse transcription-polymerase chain reaction (RT-PCR) blot. Synovium samples from rat controls given vehicle were compared with synovium harvested from rats administered 60 mg/kg/day 2ME2 via osmotic pumps. Samples were micro-dissected from hind limbs on Day 28, homogenized, and RNA was extracted using the RNeasy mini-kit (Qiagen, Valencia, CA, USA) according to the protocol of the manufacturer. Total RNA was reverse-transcribed into cDNA using Taqman® Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA). Each sample was analyzed using 180 ng of cDNA in the reaction. Primer sequences were as follows; VEGF, forward: cgt cta cca gcg cag cta tt, reverse: gca ttc aca tct gct atg ctg cag g; bFGF, forward: ctc tac tgc aag aac ggc ggc ttc tt, reverse: cag ccg tcc atc ttc ctt cat agc; interleukin 6 (IL-6), forward: gac ttc aca gag gat acc ac, reverse: ctt agc cac tcc ttc tgt gac tc; IL-1ß, forward: cca gat gag agc atc cag ctt caa, reverse: agt gca gct gtc taa tgg gaa c; and tumor necrosis factor-α (TNF-α), forward: cta ctg aac ttc ggg gtg atc, reverse: ctt gtc cct tga aga gaa cct g. Thermal cycling was initiated at 94°C for 2 min (94°C for 30 s, 56°C for 30 s, 72°C for 30 s] × 35 cycles, 72°C for 5 min. Electrophoresis was performed with the E-gel system (1.2% agarose, 20 min running time; Invitrogen E-gel system). Density of bands on the electrophoresis gels was quantified using a Bio-Rad ChemiDoc XRS Imager with QuantityOne software (Bio-Rad, Hercules, CA, USA). Percentage reduction was calculated by the following formula: 100 – (target band/naive band) × 100.

Antibodies to collagen. Serum antibody titers to type II collagen were assayed by ELISA using peroxidase-conjugated goat anti-rat IgG antibodies according to the manufacturer's instructions (Accurate Chemical and Scientific, Westbury, NY, USA)[15-17,20]. In brief, type II collagen was solubilized at a concentration of 2.5 mg/ml in 0.1 M acetic acid overnight and then dialyzed with 0.2 M NaCl/0.05 M Tris, pH 7.2. Plates were coated with collagen solution at concentration 12.5 µg/ml and incubated at least 72 h at 4°C. After incubation, plates were washed 3 times with buffer and 200 µl of test serum along with appropriate controls were added at dilution 1:2500 based on previous titrations. Plates were incubated 18 h at 4°C, washed, and 200 µl of peroxidase-conjugated goat anti-rat IgG antibody, at dilution 1:20,000, were added to each well. Plates were incubated in the dark for 30 min at room temperature, washed, and 200 µl of O-phenylenediamine were added and incubated 1 h at room temperature in the dark. The reaction was stopped with 25 µl of 0.25 N sulfuric acid and the plates were read at 490 nm.

Statistics. Differences in clinical disease activity between control and treatment groups were compared using Student's t-test for parametric continuous variables. The control group was compared to each treatment group using repeated measures regression modeling techniques for differences in clinical joint counts over Days 10 through 28. Several covariance structures were tested across the treatment groups, including autoregressive, autoregressive heterogeneous, and autoregressive moving average. In previous experiments, the heterogeneous autoregressive covariance structures provided the best fit and were used in the regression models to evaluate differences in clinical joint counts between groups over time. SAS Proc Mixed (version 8.2) was used for regression modeling. The radiographic and cytokine groups were compared using Student's t-test for parametric continuous variables and the Wilcoxon rank-sum test for nonparametric continuous variables. The chi-square test for association was used for categorical variables; Fisher's exact test was used for small sample sizes. The significance level was prespecified at $p < 0.05$.

RESULTS

Clinical and radiographic effects of 2ME2 in prevention and initial low to medium-dose treatment protocols. Oral administration of 2ME2 in preventive protocols at 30 mg/kg/day or 100 mg/kg/day, beginning on Day 0, significantly reduced the severity of CIA compared to vehicle controls (6.1 ± 0.7, $p < 0.03$; 5.4 ± 0.7, $p < 0.004$, respectively, compared to vehicle controls 7.6 ± 0.3). The onset of arthritis was also

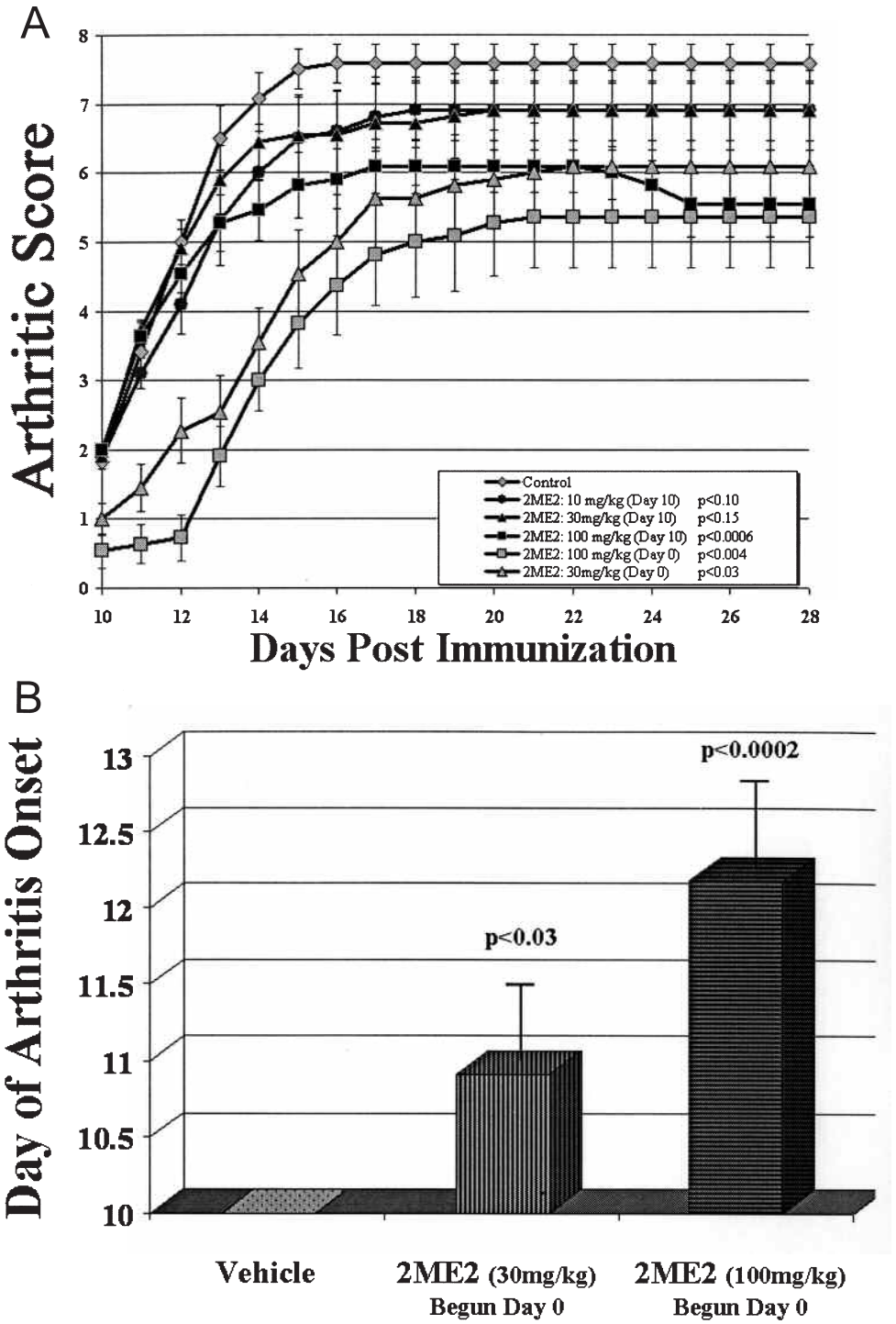

Figure 3. A. Arthritis scores in the prevention protocol (30 mg/kg or 100 mg/kg) beginning on the day of immunization, Day 0, inhibited arthritis ($p < 0.03$, $p < 0.004$, respectively, compared to vehicle control). Concurrent studies with treatment protocols (10 mg/kg, 30 mg/kg, and 100 mg/kg) beginning at clinical disease onset, Day 10, suppressed arthritis at the 100 mg/kg dose ($p < 0.10$, $p < 0.15$, $p < 0.0006$ compared to vehicle control). B. Prevention protocols with 2ME2 at 30 mg/kg and 100 mg/kg delayed the onset of clinical arthritis ($p < 0.03$, $p < 0.0002$ compared to vehicle controls).

delayed by treatment with 30 mg/kg/day ($p < 0.03$) and 100 mg/kg/day ($p < 0.0002$) (Figure 3). Concurrent treatment protocols with 2ME2, beginning at clinical disease onset on Day 10 with 10 mg/kg/day (Day 28 arthritis score 6.9 ± 0.4, $p < 0.10$), 30 mg/kg/day (Day 28 arthritis score 6.9 ± 0.6, $p < 0.15$), and 100 mg/kg/day (Day 28 arthritis score 5.5 ± 0.5, $p < 0.0006$), suppressed arthritis only at the 100 mg/kg/day dosing level compared to vehicle control-treated rats.

Prevention protocols evaluating oral administration of 2ME2 at 30 mg/kg/day (Day 28 radiographic score 3.1 ± 0.6) and 100 mg/kg/day (Day 28 radiographic score 4.3 ± 0.7) significantly reduced radiographic damage ($p < 0.0004$ and $p < 0.00004$, respectively) compared to vehicle controls (Day 28 radiographic score 5.5 ± 0.3) (Figure 4). Treatment protocols had a dose-dependent radiographic benefit at 10 mg/kg/day (Day 28 radiographic score 4.2 ± 0.5), 30 mg/kg/day (Day 28 radiographic score 3.6 ± 0.6), and 100 mg/kg/day (Day 28 radiographic score 2.4 ± 0.4) ($p < 0.02$, $p < 0.008$, $p < 0.000004$) compared to vehicle controls.

Clinical and radiographic effects of 2ME2 in medium to high-dose treatment protocols by gavage or osmotic pump delivery. Oral 2ME2 in established CIA at 50 mg/kg bid (Day 28 arthritis score 4.3 ± 0.7) and 100 mg/kg qd (Day 28 arthritis score 4.7 ± 0.5) were similar in clinical efficacy ($p < 0.02$ for both) compared to vehicle control-treated rats (Day 28 arthritis score 6.6 ± 0.7) (Figure 5). At 300 mg/kg/day, clinical severity (Day 28 arthritis score 3.2 ± 0.5) was markedly suppressed ($p < 0.0004$) compared to vehicle controls. Continuous infusion of 2ME2, by implanted osmotic pumps that delivered 60 mg/kg/day (Day 28 arthritis score 3.3 ± 1.2), was equivalent to 300 mg/kg/day by daily gavage.

Radiographic scores (Figure 6) paralleled the clinical findings. 2ME2 at 50 mg/kg/bid (Day 28 radiographic score 2.1 ± 0.7) and 100 mg/kg/day (Day 28 radiographic score 2.9 ± 0.4) had comparable structural efficacy ($p < 0.02$ and $p < 0.05$, respectively) compared to vehicle control-treated rats. 2ME2 at 300 mg/kg/day by gavage (Day 28 radiographic score 1.3 ± 0.4) was the most efficacious ($p < 0.0002$ compared to vehicle controls) and was similar to continuous subcutaneous infusion at 60 mg/kg/day via osmotic pump (Day 28 radiographic score 1.5 ± 0.7).

Light microscopy and immunohistochemical staining for vWF. The influence on histological joint destruction was evident on blinded paraffin sections. 2ME2 reduced cellular infiltrates, pannus, cartilage loss, proteoglycan loss, bone resorption, and osteoclast number (data available in supplemental file). Immunostaining for vWF showed marked angiogenesis in vehicle control sections and virtually no angiogenesis in sections from 2ME2 treated rats (100 mg/kg/day), which were devoid of pannus (Figure 7).

Synovial gene expression by RT-PCR. 2ME2 (60 mg/kg/day, continuous infusion) had selective effects on synovial gene expression. Compared to controls, the major promoters of angiogenesis, bFGF and VEGF, were suppressed 83% and 47%, respectively, by Day 28 (Figure 8A). Proinflammatory cytokines IL-6 and IL-1ß were reduced 52% and 31%, but TNF-α was minimally inhibited — by 5%.

Antibodies to collagen. Serum antibody titers to type II col-

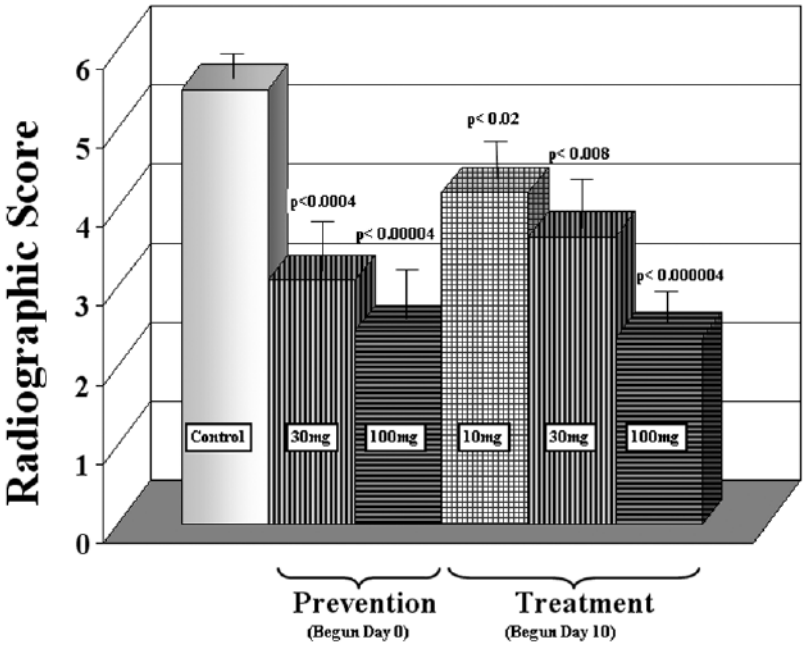

Figure 4. Radiographic scores. Prevention protocols at 30 mg/kg/day and 100 mg/kg/day significantly reduced radiographic damage ($p < 0.0004$, $p < 0.00004$, respectively, compared to vehicle controls). Treatment protocols had a dose-dependent benefit at 10 mg/kg/day, 30 mg/kg/day, and 100 mg/kg/day ($p < 0.02$, $p < 0.008$, $p < 0.000004$, compared to controls).

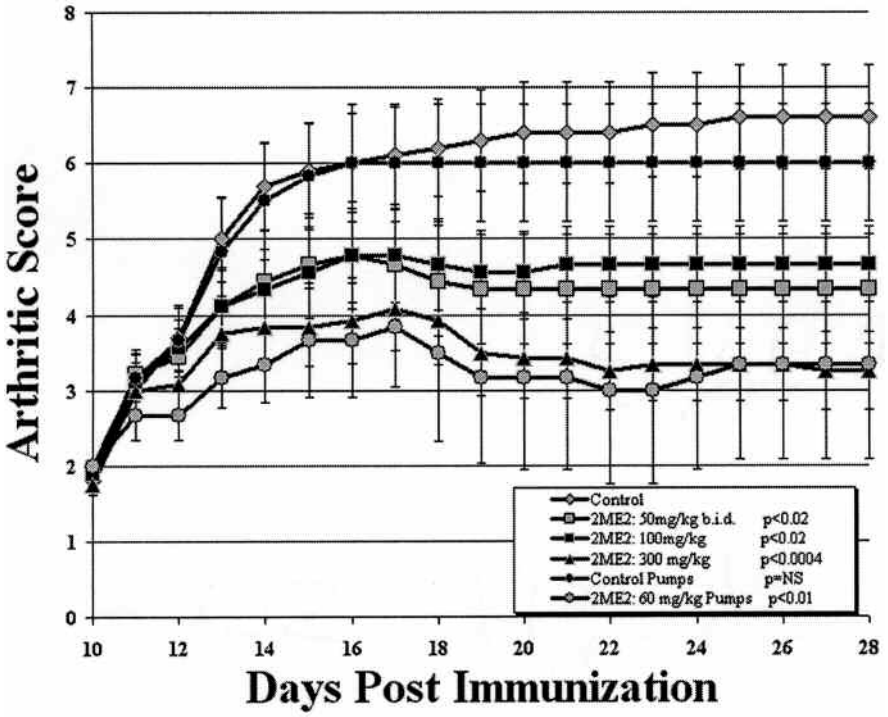

Figure 5. Arthritis scores of treatment arms at higher doses or via osmotic pumps. 2ME2 at 50 mg/kg/day bid was similar in efficacy to 100 mg/kg/day ($p < 0.02$ for both compared to vehicle controls), although 300 mg/kg/day was the most efficacious ($p < 0.0004$ compared to controls). Continuous infusion of 60 mg/kg/day via osmotic pump was comparable to the gavage dose of 300 mg/kg/day.

lagen were assayed at the end of the study on Day 28. Vehicle control and all orally treated 2ME2 groups had high IgG antibody levels at a dilution of 1:2500 on a standardized ELISA curve with optical density (OD) read at 490 nm (vehicle, 0.300 OD; prevention 30 mg/kg/day, 0.307 OD; prevention 100 mg/kg/day, 0.302 OD; treatment 10 mg/kg/day, 0.307 OD; treatment 30 mg/kg/day, 0.305 OD; and treatment 100 mg/kg/day, 0.283 OD) (Figure 8B). Even in the prevention arms, which had the maximum exposure to 2ME2 throughout the entire 28-day protocol including the peri-immunization period with native type II collagen, no humoral immunosuppression was evident.

DISCUSSION

RA is the most common chronic inflammatory autoimmune arthritis and, despite major advances in recent biologic interventions, many patients still have active disease. Because the primary pathophysiologic mechanisms operating in RA remain enigmatic, treatment modalities have largely been directed at immunosuppressive and antiinflammatory processes. Targeting angiogenesis does not require altering an already dysregulated immune system. Because angiogenesis is associated primarily with pathologic processes in adults, its inhibition is relatively selective for nonphysiologic processes. The primary exceptions are wound-healing and a small component of the reproductive cycle in women.

In our rat CIA study, 2ME2 significantly regressed clinical and radiographic disease in a dose-dependent manner when administered by gavage. Initiation of 2ME2 treatment prior to onset of arthritis was not necessary to achieve marked regression of synovitis, although early intervention significantly delayed the onset of CIA, especially at 100 mg/kg. Daily oral therapy for established arthritis indicated that in this model doses > 30 mg/kg were needed for effective clinical resolution. This was also evident for structural damage, as determined by blinded radiographic scores. An oral once-daily dose of 100 mg/kg was equivalent to 50 mg/kg bid for both clinical and radiographic scores. Delivery of 60 mg/kg by an osmotic pump, however, was similar in efficacy to 300 mg/kg by oral gavage. This result suggests that a steady-state level of 2ME2 achieved equivalent arthritic inhibition at a lower total dose. Whether this is due to increased bioavailability from the subcutaneous delivery system or to elimination of any trough effects cannot be determined from our data.

Pannus was involuted by 2ME2 therapy and was associated with a marked reduction in synovial blood vessels, as shown by vWF staining of endothelial cells (Figure 7). As a result, subsequent cartilage and bone destruction did not occur. These findings suggest that angiogenesis is still reversible with 2ME2, even in established CIA, where vessel growth has already occurred. Neovascularization in pannus tissue undergoes continuous remodeling and necessitates the presence of growth factors for its maintenance. This is a critical requirement for any potential angiogenesis-inhibitor to be effective in RA. Angiogenesis is controlled

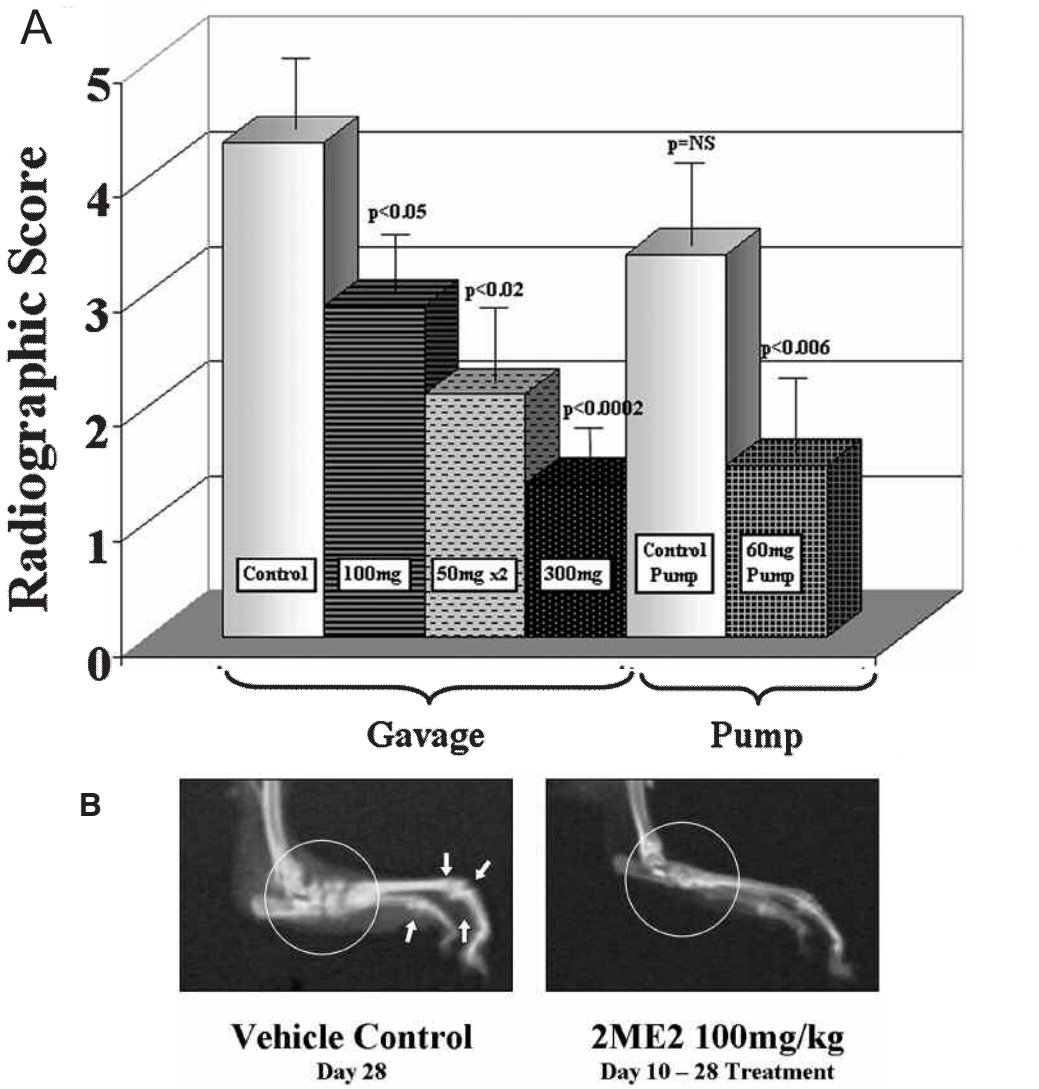

Figure 6. A. Radiographic scores of treatment arms at higher doses or via osmotic pump. 2ME2 at 100 mg/kg/day and 50 mg/kg/day bid were similar in efficacy ($p < 0.05$, $p < 0.02$, respectively, compared to vehicle controls), although 300 mg/kg/day was the most efficacious ($p < 0.0002$ compared to controls). Continuous infusion of 60 mg/kg/day via osmotic pump had comparable radiographic scores to the gavage dose of 300 mg/kg/day. B. Left: Representative radiograph of a vehicle control hind limb on Day 28. Circle shows swelling and structural damage of the ankle and tarsal joints; arrows indicate distal involvement. Right: Representative radiograph from a rat treated with 2ME2 100 mg/kg/day beginning on Day 10 shows minimal soft tissue or structural damage.

by a variety of factors, although VEGF and bFGF are particularly important. VEGF is the most important and actively studied proangiogenic molecule. It promotes blood-vessel formation in early development and plays a central role in the growth of new blood vessels[21]. It is highly endothelial cell-specific and is associated with numerous angiogenesis-related disease states including cancer, endometriosis, macular degeneration, synovitis, and in-stent restenosis. VEGF acts to stimulate endothelial cell proliferation, survival, and chemotaxis by binding high-affinity tyrosine kinase receptors (VEGF-R), which are expressed on endothelial cells[22]. In RA, VEGF, a potent inducer of synovial proliferation via its ability to induce angiogenesis, has been shown to be synthesized by various cell types in the synovium, including macrophages, fibroblasts, vascular smooth muscle cells, and synovial lining cells. VEGF and VEGF-R concentrations are elevated in the synovium and peripheral blood of patients with RA[23-29]. Levels of VEGF are also high in the synovium of patients with juvenile RA[30]. Because VEGF plays a central role in blood-vessel growth, it has been a major therapeutic target for inhibition of angiogenesis[31].

Synovial VEGF is regulated by a variety of molecules, including transforming growth factor-ß, IL-1, IL-17, and IL-18[32-35]. In cancer cells, IL-6 has been shown to increase VEGF, possibly via the JAK/STAT pathway[36,37]. The relative hypoxia within pannus[38], due to tissue proliferation that outstrips the blood supply and markedly increased tissue consumption[39], also stimulates HIF-1α to increase tran-

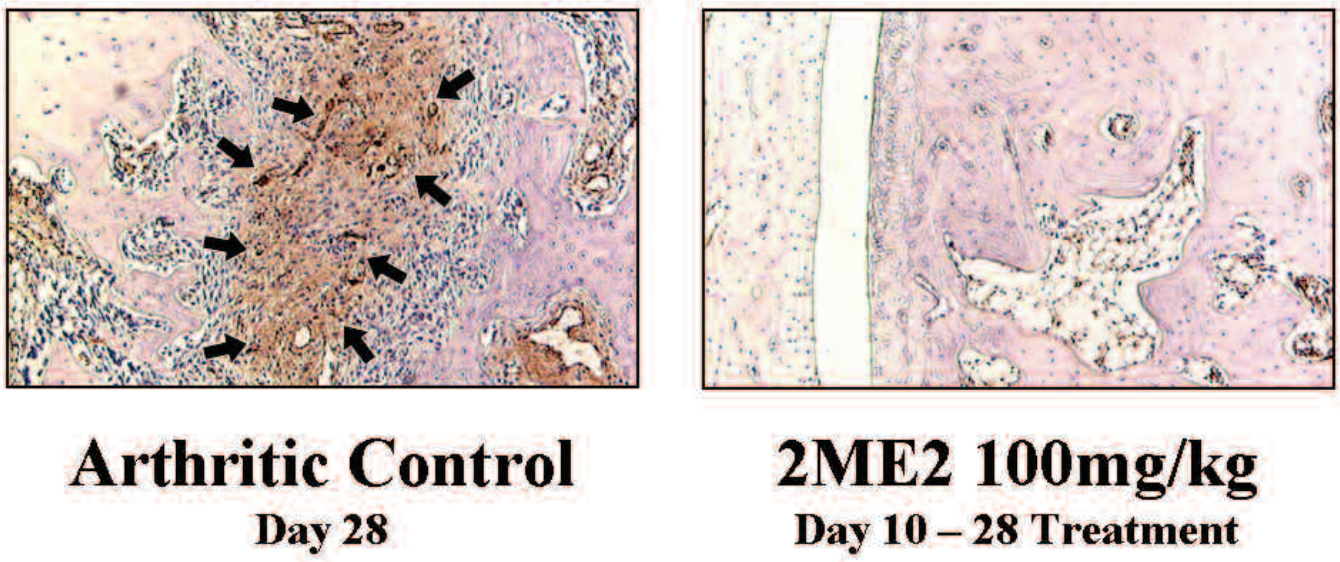

Figure 7. Immunostain with von Willebrand factor (vWF) to identify blood vessels. Left: Day 28 arthritic control shows complete destruction of the ankle and cartilage, with marked pannus and angiogenesis (arrows). Right: The ankle treated with 2ME2 100 mg/kg/day shows intact cartilage and virtually no vWF immunostain of vessel pannus (original magnification ×100).

scription of the VEGF gene[40-42]. Of note, 2ME2 has been shown to decrease HIF-1α in several tumor and nontumor systems[5,6,8,43]. bFGF is also a major proangiogenic synovial factor and, in adjuvant arthritic rats, it can stimulate vessel formation and subsequent joint destruction[44]. As shown in Figure 8A, synovium from CIA rats administered 60 mg/kg 2ME2 by osmotic pump had a marked reduction in bFGF by 83%, in VEGF by 47%, in IL-6 by 52%, and in IL-1ß by 31%, compared to controls. This effect on proangiogenic expression appears to be an important mediator of CIA involution observed in our study.

Of interest, 2ME2 reduced TNF-α expression by only 5% (Figure 8A). This may indicate that 2ME2 does not directly affect inflammatory pathways or TNF-α-mediated blood-vessel growth. Proinflammatory factors in the synovium, such as TNF-α, can stimulate angiogenesis by increasing synoviocyte angiopoietins that then bind to tyrosine kinase receptors on RA capillaries[45]. TNF-α can also increase VEGF expression. In peripheral blood mononuclear cells, TNF-α from synovial fluid of RA patients upregulated VEGF[23]. Conversely, VEGF levels in the serum and synovium were reduced in patients treated with TNF-α-blocking agents[35,46]. The effect of TNF-α blockers on VEGF has also been found in other inflammatory processes such as psoriatic arthritis[47]. The minimal effect of 2ME2 on TNF-α expression in CIA indicates that this is not its primary mechanism of action.

Antibodies to the relevant antigen, type II collagen, were not suppressed by 2ME2 in the rat CIA model (Figure 8B). Longer exposure, begun as a prevention intervention on the day of immunization with collagen, was similar to shorter exposure, begun 10 days later at onset of arthritis. This indicates that 2ME2 does not preclude humoral immune responses or inhibit established antibody production. All experimental 2ME2 rats had high-titer antibodies comparable to untreated vehicle control rats. Consequently, humoral immunosuppression does not explain the benefits observed with active therapy, and is consistent with an angiogenesis-inhibition pathway. This conclusion is also supported by data from murine arthritis studies that found no inhibition of IgG antibodies, no effects on CD4+ or CD8+ T cells, no reduction of delayed-type hypersensitivity, and no effect on adhesion molecules, but significant suppression of endothelial cell proliferation[48]. In rat adjuvant arthritis[49], however, data suggest that 2ME2 may have effects beyond inhibition ofangiogenesis. These include diminished *in vitro* proliferation response to purified protein derivative and inhibited neutrophil migration after cutaneous TNF-α challenge.

Despite major advances in RA therapies, many patients have partial responses or lose efficacy with continued management. Inhibition of angiogenesis is a unique intervention that does not rely on immunomodulation. Consequently, it might be used in patients that are inadequately managed, even with the newest biologic response modifiers, or employed in combination regimens to target several discrete pathways. 2ME2 treatment in prevention protocols delayed the onset and decreased the severity of CIA. It also suppressed clinical and radiographic progression of established disease. These benefits were dose-dependent, although continuous infusion of 2ME2 appeared to suppress synovitis at lower doses than the oral route. Involution of pannus was associated with reduced synovial VEGF and bFGF expression as well as decreased angiogenesis. The results indicate that 2ME2 may represent a novel agent for the treatment of inflammatory autoimmune diseases such as RA.

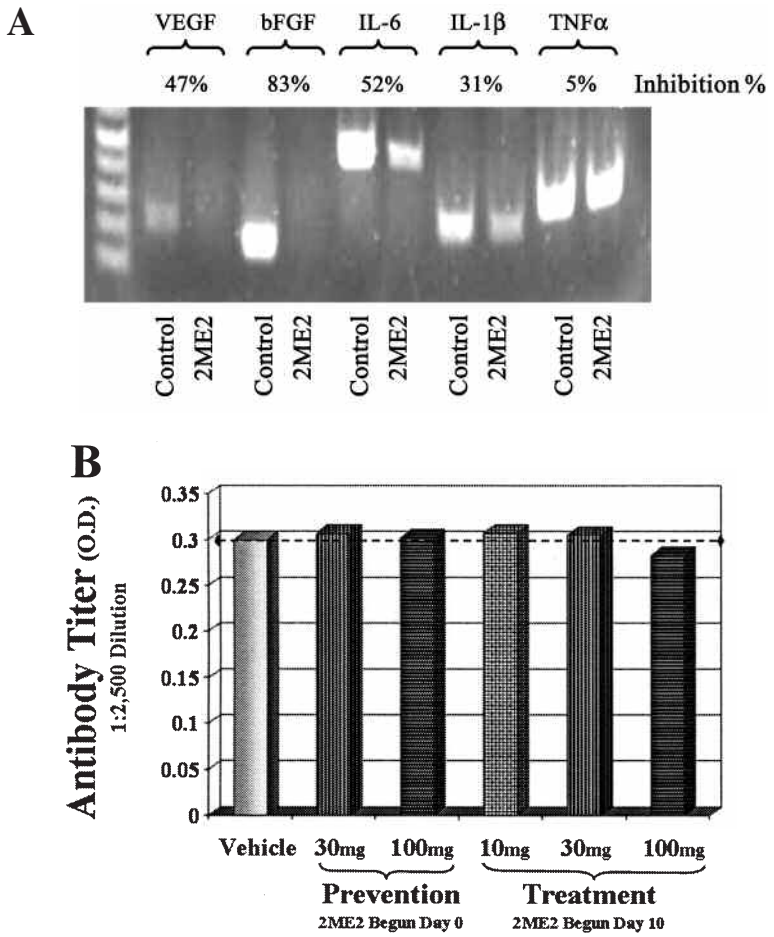

Figure 8. A. RT-PCR blot for synovial Day 28 expression of VEGF, bFGF, IL-6, IL-1ß, and TNF-α. The first of each pair of lanes represents vehicle control synovium on Day 28 compared to 2ME2 60 mg/kg/day via osmotic pump synovium. Compared to controls, 2ME2 reduced VEGF by 47%, bFGF by 83%, IL-6 by 52%, IL-1ß by 31%, and TNF-α by 5%. B. Serum antibody titers to type II collagen assayed on day 28. All groups had excellent immune responses at a dilution of 1:2500 on a standardized ELISA curve. Even prevention arms, with 28 days of 2ME2 exposure beginning on Day 0 of immunization, were not altered.

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