Vanadate, an Inhibitor of Stromelysin and Collagenase Expression, Suppresses Collagen Induced Arthritis

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ABSTRACT. ***Objective.*** Collagen induced arthritis (CIA) is a model of chronic inflammatory synovitis with pannus, neovascularization, and joint destruction similar to rheumatoid arthritis (RA). Matrix metalloproteinases (MMP) are involved in degradation of the extracellular matrix and joint destruction in RA. *c-fos* and *c-jun* are protooncogenes whose products combine to form activating protein (AP-1), a regulatory protein that is required for cell proliferation and the transcription of a variety of genes, including MMP such as collagenase and stromelysin. Administration of vanadium compounds suppresses *c-fos*/*c-jun* expression and AP-1 activity, resulting in inhibition of MMP expression in response to factors such as interleukin 1 (IL-1). We evaluated whether a vanadium AP-1 inhibitor could reduce MMP expression and subsequent joint damage in CIA.

Methods. Vanadate [bis (maltolato) oxovanadium (IV) (BMOV; 10 mg/kg/day)] and the reducing agent N-acetyl cysteine (NAC; 100 mg/kg/day) were given subcutaneously daily in an attempt to suppress established CIA in rats. NAC in combination with vanadate appeared to increase the efficacy of *c-fos*/*c-jun* inhibition, while decreasing toxicity. Controls were given NAC alone. Clinical, radiographic, and histologic measures were evaluated as well as synovial MMP and IL-1α expression.

Results. BMOV therapy, initiated on the day of onset of clinical arthritis, significantly reduced clinical arthritis within 2 days ($p < 0.05$) compared to controls. Significance was maintained to the termination of the study on Day 18 post-arthritis onset ($p < 0.005$), with a maximum difference seen on Day 5 ($p < 0.00001$). Blinded radiographic scores at the completion of the protocols indicated less joint destruction in the experimental group compared to the control group ($p < 0.005$). Scanning and transmission electron microscopy confirmed the preservation of articular cartilage with therapy. In BMOV-treated rats, synovial mRNA expression of collagenase, stromelysin, and IL-lα were reduced by 78%, 58%, and 85%, respectively, compared to controls.

Conclusion. This is the first study of vanadate as a potential antirheumatic agent. Further study of this AP-1 and MMP inhibitor may lead to new treatment options in RA. (First Release August 1 2007; J Rheumatol 2007;34:1802–9)

Key Indexing Terms:
ANIMAL DISEASE MODELS | MATRIX METALLOPROTEINASES
VANADATE | COLLAGEN-INDUCED ARTHRITIS | AP-1

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Matrix metalloproteinases (MMP) initiate degradation of the extracellular matrix, leading to angiogenesis, bone resorption, and wound healing[1-3]. Aberrant MMP activity can contribute to pathologic processes such as joint destruction in rheumatoid arthritis (RA) and tumor metastatic spread. The MMP collagenase and stromelysin play an important role in arthritic disease[4-9]. In cartilage from patients with RA and osteoarthritis, increased levels of collagenase and stromelysin are detected, and their enzymatic activity is correlated with the severity of the lesion. Elevated levels of these enzymes have also been detected in arthritic synovial fluid. Further, *in situ* hybridization techniques have localized collagenase and stromelysin mRNA in arthritis synovial tissue. Promoter-region analysis of human collagenase and stromelysin genes reveals that they contain a 9 base-pair sequence (tumor promoter responsive element, TRE) that is recognized by the activating protein (AP-1), a heterodimeric

complex of transcription factors encoded by the *fos*/*jun* family of protooncogenes.

Expression of *fos*/*jun* can be regulated by several mechanisms. Induction can be stimulated by a variety of growth factors, tumor promoters, cytokines, and ultraviolet irradiation, while *fos*/*jun* downregulation can be indirectly mediated by glucocorticoids and retinoic acid, which interfere with AP-1 binding to the TRE site. The phosphotyrosine phosphatase inhibitor orthovanadate[10] has been shown to selectively inhibit constitutive collagenase production by chondrocytes at the level of transcription[11,12]. Further, orthovanadate completely inhibits interleukin 1 (IL-1) and phorbol ester (PMA) induced *c-fos* and *c-jun* expression in chondrocytes[11]. Because of the important role played by MMP in inflammatory arthritis, bis (maltolato) oxovanadium (IV) (BMOV; Figure 1), a less toxic analog of orthovanadate currently undergoing evaluation as an antineoplastic agent, was chosen for this study to assess its effect on inflammation and joint destruction in the collagen induced arthritis (CIA) rat model of RA.

MATERIALS AND METHODS

Animals. Syngeneic 8-week-old female Louvain (LOU) rats were fed with standard laboratory chow and housed in the vivarium at the University of California, Los Angeles.

Arthritis induction. Rats were immunized intradermally under ether anesthesia on Day 0 with 0.5 mg native chick collagen II (CII; Genzyme, Boston, MA, USA) solubilized in 0.1 M acetic acid and emulsified in incomplete Freund's adjuvant (IFA; Difco, Detroit, MI, USA)[13]. Onset of clinical arthritis, characterized by erythema and swelling in the hind paws, typically develops in 90–100% of control rats 10–12 days post-CII immunization.

Experimental design. A protocol evaluating the suppression of established CIA with BMOV was carried out since this is more relevant to clinical management of human chronic inflammatory synovitic diseases such as RA. Rats (n = 17) with definite arthritis on Day 10 post-immunization were randomized into 2 groups. Eight control rats received N-acetyl cysteine (NAC) (Sigma, St. Louis, MO, USA) alone at a dose of 100 mg/kg/day subcutaneously (SC). NAC was prepared as a 3% sterile phosphate buffered saline solution. Prior studies (data not shown) demonstrated that NAC in combination with vanadate resulted in increased inhibition of *c-fos*/*c-jun* expression and decreased toxicity. Nine experimental rats received NAC 100 mg/kg/day SC, as well as BMOV 10 mg/kg/day SC. BMOV was prepared fresh daily to a dilution of 1 mg/ml in 5% dextrose at 50°C followed by 0.22 µm millipore filtration (Corning Costar, Kennebunk, ME, USA). On Day 11 post-arthritis onset, the dose of BMOV was increased to 15 mg/kg/day, and maintained throughout the remainder of the study period.

Arthritis assessments. Clinical arthritis severity of each limb was scored daily based on an integer scale of 0–4[13,14]. A score of 0 indicated an unaffected limb, while a score of 4 represented maximal swelling and erythema involving the ankle and distal digits. The arthritic index of a rat is 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.

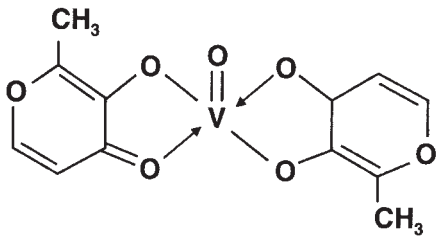

Figure 1. The chemical structure of BMOV.

Radiographs of the hind limbs were obtained at the end of the experiment on Day 18 post-arthritis onset. An investigator blinded to the treatment protocol assigned a score to each limb, based on the degree of soft tissue swelling, joint space narrowing, periosteal new bone formation, and the presence of erosions and/or ankylosis (0 = normal; 3 = maximal joint destruction)[15]. Each rat had a maximal possible radiographic index of 6.

Humoral immunity. Rat serum was collected on Day 18 post-arthritis onset to measure anti-CII IgG antibodies using an enzyme linked immunosorbent assay[16,17]. Antibody titers determined in quadruplicates were normalized against a previously standardized curve and were expressed as the absorbance at 490 nm at a serum dilution of 1:250.

Collagenase, stromelysin, and IL-1 expression. Three rats from each group were randomly chosen on Day 18 post-arthritis onset to evaluate collagenase, stromelysin, and IL-1 expression by Northern blot[18]. Synovial biopsies in each group were pooled and homogenized in the presence of RNAStat-60 (Tel Test). Total RNA was isolated following the manufacturer's instructions, washed in 70% ethanol, and dissolved in 30 µl RNA loading buffer (Sigma) containing ethidium bromide. The RNA was electrophoresed on a 1% agarose formaldehyde gel and transferred to 0.45 µm nylon filter membrane (Magna NT, MSI). The blot was prehybridized in 50% formamide, 5× SSPE, 5× Denhardt's solution, 1% sodium dodecyl sulfate, 200 µg/ml ssDNA, and 50 µg/ml tRNA. The rat collagenase cDNA (bp 1–550 of locus RATCOL; Genbank accession no. M60616), rat stromelysin (American Type Culture Collection, Rockville, MD, USA), or rat IL-1α (Genbank accession no. D00403) were labeled by random-primed incorporation of ^{32}P-dATP (Random Primed Labeling Kit, Boehringer-Mannheim). After overnight hybridization at 42°C, the blot was washed in 1× SSPE at 37°C and exposed to Kodak X-Omat AP film for 24 h at –70°C with an intensifying screen. The blots were stripped with 50% formamide in 2× SSPE, checked for residual counts, and reprobed. The resulting autoradiographs were digitized and analyzed with National Institutes of Health image software, and normalized for ribosomal RNA. Mild RNA degradation and differences in RNA transfer based on size of the RNA species can occur in Northern blot analysis when evaluating RNA isolated from complex tissues. Potential issues related to these concerns should be corrected by normalizing to RNA content because the samples were processed at the same time.

Electron microscopy of synovium. Rats in each group were selected on Day 18 post-arthritis onset to study joint morphology. Scanning and transmission electron microscopy was performed on glutaraldehyde-fixed joints. Ankle joints from arthritic control and BMOV-treated rats were removed, critical-point dried, and gold sputter-coated for scanning electron microscopy (Philips 501) to examine the trochlear surfaces. Transmission electron microscopy, using a Jeol 1200EX, was also performed on the articular cartilage of the trochlear surfaces of naive, arthritic control, and BMOV-treated animals.

Statistics. Student's t-test was used to analyze group means of continuous variables. Means (± SEM) are shown. A p value < 0.05 was considered significant.

RESULTS

Rats given BMOV and NAC demonstrated significant regression of established arthritis compared to controls within 2 days post-arthritis onset ($p < 0.05$; Figure 2). Control rats, receiving NAC alone, developed severe arthritis, indicating that the reducing agent did not modify arthritis development[14,16]. The mean daily arthritis scores of the control and experimental groups remained statistically different throughout the rest of the study ($p < 0.005$ on Day 18 post-arthritis onset). The blinded radiographic scores of the experimental group were significantly lower than the control group ($p <$

0.005; Figures 3 and 4). All experimental rats tolerated the combination of BMOV and NAC without weight loss (Table 1), although slight skin thickening at the vanadate injection site was noted. Diarrhea was not observed when BMOV was given at a dose of 10 mg/kg/day. However, when the dose was increased to 15 mg/kg/day on Day 11 post-arthritis onset, a few experimental rats manifested minor diarrhea.

The mean anti-CII IgG titer of the control group was significantly higher than that of the experimental group ($p < 0.04$; Table 1). The biologic significance of this difference remains unclear, however, since the magnitude of the difference was minimal and previous experiments have shown that arthritic rats often produce marginally higher titers of anti-CII IgG than nonarthritic rats.

Scanning and transmission electron micrographs showed dramatic cartilage destruction in the control joints (Figures 5 and 6), with exposed or absent chondrocytes in the denuded cartilage. Joints from BMOV-treated rats showed little cartilage damage and intact cartilage.

Synovial expressions of collagenase, stromelysin, and IL-1α mRNA were readily detected in the vehicle control group (Figure 7). When normalized for RNA loading, expression of all 3 genes was decreased in the BMOV-treated group compared to the control group. The inhibition of collagenase, stromelysin, and IL-1α gene expression was 78%, 58%, and 85%, respectively.

DISCUSSION

Therapy using BMOV in combination with NAC resulted in a significant reduction in arthritis severity by both clinical and radiologic criteria. The control group, receiving only NAC, experienced a progressive, severe arthritis course typical of untreated CIA. Among animals in the experimental group, the antiarthritic effects of BMOV were evident early, with a significant difference in daily arthritis scores noted by Day 2 of treatment. The experimental group initially received a relatively low dose (10 mg/kg/day), but an increased daily dose of BMOV (15 mg/kg/day) was then instituted and was even more effective. Northern blot analysis revealed a 78% and 58% reduction in collagenase and stromelysin mRNA expression, respectively, in synovial tissues of rats treated with BMOV compared to control rats. In addition, the gene expression of

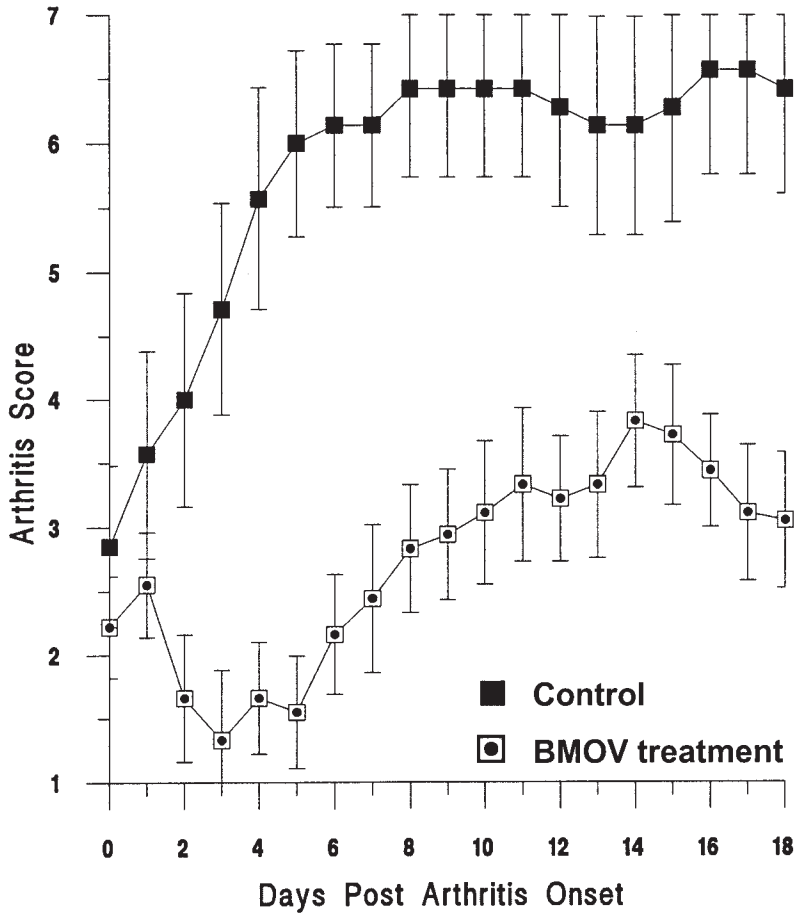

Figure 2. Daily mean arthritis scores. The difference between the control and the BMOV-treated group was statistically significant on Day 2 post-arthritis onset ($p < 0.05$). The difference remained significant throughout the remainder of the study period, with $p < 0.005$ at the completion of the protocol on Day 18 post-arthritis onset.

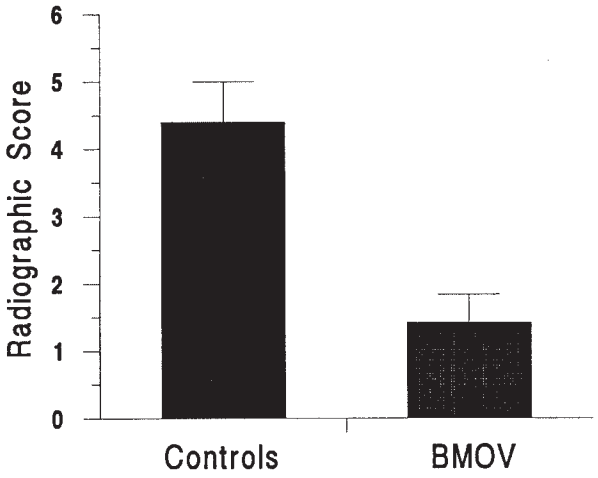

Figure 3. Radiographic severity scores on Day 18 post-arthritis onset were significantly lower in BMOV-treated rats ($p < 0.002$) compared to controls.

IL-1α, a cytokine important in rheumatoid synovial inflammation, cartilage degradation, and proteoglycan synthesis regulation[19], was suppressed by 85% compared to control samples.

The AP-1, formed by the protooncogenes *c-fos* and *c-jun*, modulates a variety of cellular functions involved in cell proliferation and differentiation at the transcriptional level[4-6]. In many cells, *c-fos* and *c-jun* are expressed at low basal levels. They can undergo rapid and transient induction in response to growth factor and cytokine stimuli including platelet-derived growth factor, basic fibroblast growth factor, endothelial growth factor, nerve growth factor, tumor necrosis factor-α (TNF-a) and TNF-ß, heat shock protein, cAMP, calcium ionophores, and UV irradiation. In RA synovium, intense nuclear staining for the *c-fos* protein has been demonstrated in RA synovial stromal and inflammatory cells, chondrocytes, and synovial lining cells. Transcriptional activation of the collagenase gene is dependent on the formation and activation of AP-1, the heterodimer consisting of *c-fos* and *c-jun*. MMP-1 (fibroblast-type) collagenase is one of the few enzymes capable of cleaving the triple helix of interstitial collagen, a major factor in RA joint matrix degradation. Collagenase, found at the junction between eroded cartilage and overlying RA pannus, has been shown by *in situ* hybridization methods to be produced primarily in the synovial lining-layer cells[20]. The promoter region of the stromelysin gene also contains an AP-1 binding site, and evidence suggests that the stromelysin expression is *c-fos*-dependent[21] and is regulated by c-Jun amino terminal kinase[22].

In our study, daily BMOV therapy resulted in significant suppression of established CIA, along with a corresponding decrease in collagenase, stromelysin, and IL-1α mRNA expression. Previous *in vitro* investigation has shown that vanadium compounds can completely inhibit IL-1 and PMA-induced *c-fos* and *c-jun* expression in chondrocytes[11]. They have also been shown to inhibit collagenase transcription in chondrocytes[11,12]. BMOV may act on *c-fos* and *c-jun* expression through its interaction with reactive oxygen species (ROS) and an intracellular second messenger involving H_2O_2[23]. There is increasing evidence that ROS play an important intermediate role in cellular processes such as signal transduction and the control of gene expression. ROS that mediate DNA damage may cause oncogene activation[24]. Intracellular second messengers H_2O_2 and superoxides can activate *c-fos*/*c-jun* transcription[23,25]. Vanadium compounds react with H_2O_2 and NADH, forming oxo/peroxo-vanadates and hydroxy free radicals[26]. It is possible that alterations in intracellular redox states[27] or activity of a vanadyl intermediate[26] may interfere with protooncogene transcription. Moreover, vanadate's action as a tyrosine phosphatase inhibitor may influence intracellular signal transduction. It is likely that BMOV acts by interfering with AP-1 activation in the arthritis model, although additional evidence demonstrating functional impairment of the pathway *in vivo* is required to support this conclusion. Other mechanisms, including inhibition of tyrosine kinases and decreased ROS production, could also contribute to these observed results.

Aside from skin thickening at the vanadate injection site, BMOV and NAC were well tolerated by the rats. Preliminary work using a different vanadium compound, orthovanadate, in the absence of NAC, revealed that higher dosing of vanadate resulted in rapid and complete resolution of clinical arthritis but with subsequent diarrhea and weight loss. Previous studies by other investigators exploring the use of vanadate analogs for their insulin mimetic actions in animal models of diabetes have been hampered by toxicities[28,29]. Vanadate administered orally produced digestive intolerance and large doses intraperitoneally were also toxic. Studies (data not shown) have demonstrated that the slower absorption of BMOV by subcutaneous injection results in minimal toxicity and that is why this route was chosen for our study.

Vanadate inhibits rapidly dividing cells *in vitro*, while nonproliferating cells are unharmed by exposure to the compound, a characteristic that, along with *c-fos*/*c-jun* and collagenase inhibition, has led to evaluation of vanadate as an antineoplastic agent. *In vivo* studies have shown inhibition of chemically induced malignancies in rats administered oral vanadate[30,31]. Vanadate's effects on rapidly proliferating cells, which are present in the pannus of CIA, may occur via the generation of hydroxyl free radicals, resulting in DNA cleavage[32]. Glutathione (GSH), an important cellular antioxidant that also serves as a cosubstrate for antioxidative enzymes such as GSH peroxidase, has been shown to bind intracellularly with vanadate[33]. The formation of vanadate-GSH complexes may explain the observed reduction in GSH stores noted in control and diabetic rats maintained on a diet supplemented with vanadate[34]. Prior *in vivo* work revealed that the addition of the intracellular precursors of GSH, N-acetylcysteine, potentiates the anti-tumor effects of vanadate while reducing toxicity, suggesting a possible role for the vanadate-

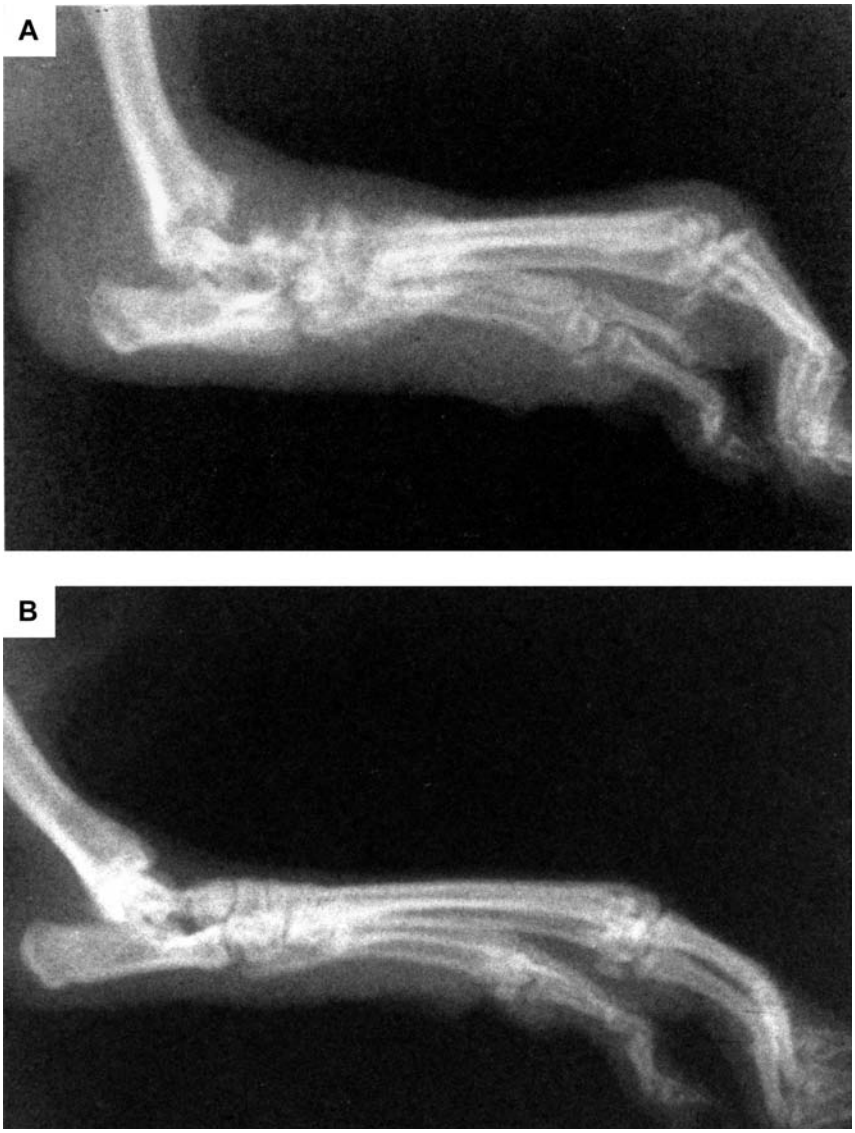

Figure 4. Radiographs representative of control and experimental hind limbs. A typical arthritic control limb (A) shows soft tissue swelling and bone erosions. These features are absent in the BMOV-treated experimental limb (B).

Table 1. Clinical arthritis assessments, weight, and humoral immune responses of control and BMOV-treated rats.

	Final Arthritis Score, Day 18	Maximum Arthritis Score[a]	Final Weight, g[b]	Antibody to CII[c]
Arthritic control	6.43 ± 0.81	6.57 ± 0.81	136 ± 4	0.084 ± 0.01
BMOV-treated	3.06 ± 0.53*	3.83 ± 0.53**	133 ± 7[†]	0.067 ± 0.01[††]

[a] Maximum arthritis scores for the control group occurred on Days 16 and 17 post-arthritis onset; maximum arthritis scores for the BMOV-treated group occurred on Day 14 post-arthritis onset. [b] Starting weights (day of immunization) and 10 days later (day of arthritis onset) were not significantly different. [c] Absorbance at 490 nm at serum dilution 1:2500. * $p < 0.003$ compared to control. ** $p < 0.01$ compared to control. [†] Not significant compared to control. [††] $p < 0.04$ compared to control.

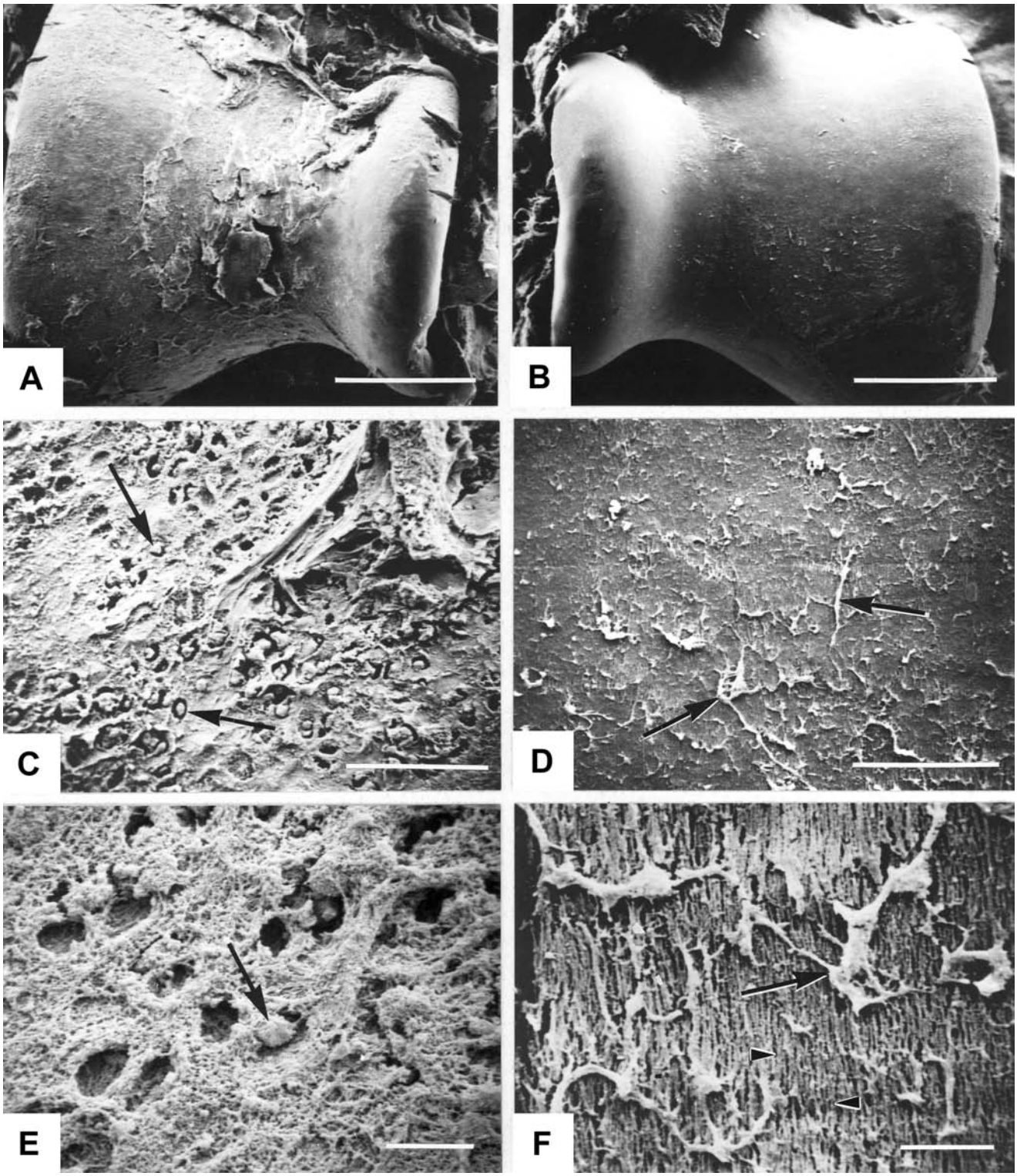

Figure 5. Scanning electron microscopy of the ankle articular surface. In an arthritic control rat (panels A, C, E with increasing magnification) the articular hyaline cartilage of the trochlear surface has been eroded, with exposure of chondrocytes (arrows, panel C). Numerous inflammatory cells are adhering to the destroyed cartilage matrix (E). In contrast, the BMOV-treated rats (panels B, D, F, same magnifications) exhibited a normal articular surface characterized by scant adhering elements (arrows in panels D and F) and a smooth articular surface with orderly arrangement of collagen fibrils (arrowheads, panel F). Original magnifications: panels A and B 25× (bar = 1 mm); panels C and D 260× (bar = 100 μm); panels E and F 1700× (bar = 10 μm).

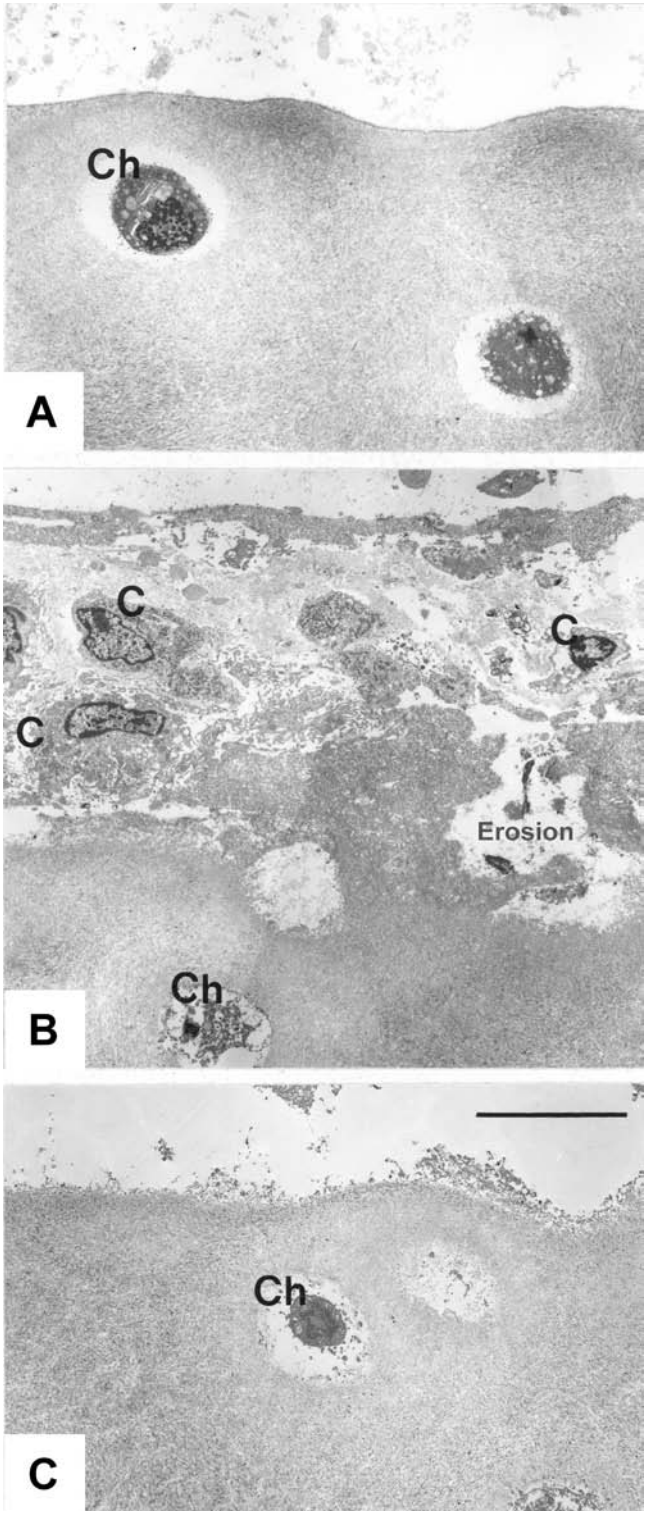

Figure 6. Transmission electron microscopy shows trochlear articular cartilage from naive (A), arthritic control (B), and BMOV-treated (C) rats. The typical ultrastructure of the naive cartilage (panel A; Ch: chondrocytes) is in contrast to the arthritic control cartilage (panel B) with its articular surface invaded by inflammatory cells (C: inflammatory cells) and eroded surface. The articular cartilage of BMOV-treated rats (panel C) is indistinguishable from the naive cartilage (bar = 10 μm).

GSH intermediary. However, single-agent NAC administered to the control rats in our study did not alter the progressive inflammatory arthritis with joint destruction typically seen in untreated CIA.

Daily BMOV in combination with NAC resulted in significant reduction of CIA and subsequent joint destruction in rats with minimal toxicity at the dosages utilized. A concomitant decrease in collagenase, stromelysin, and IL-1α gene expression was also observed. Blocking MMP activity in CIA, and potentially RA, may be a more efficacious approach than interfering with a specific cytokine, since pathologic overproduction of MMP may not be the result of a single cytokine, but due to the actions of many. Alternatively, BMOV may also act as an antiproliferative agent through hydroxyl radical formation and DNA cleavage. Although additional studies of BMOV are necessary to characterize its mechanism of action in CIA, it may represent a promising new class of compounds in the treatment of inflammatory synovitis.

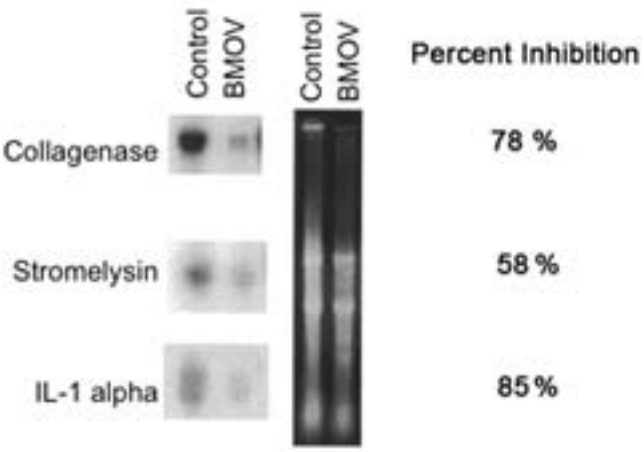

Figure 7. Northern blot of collagenase, stromelysin, and IL-1α expression. Ankle joints from vehicle-treated (control) and vanadate-treated (BMOV) rats were processed for RNA and Northern blot analysis as described. Figure shows the Northern blot that was stripped and sequentially reprobed. Compared to controls, collagenase, stromelysin, and IL-1α were reduced 78%, 58%, and 85%, respectively, in the treated animals. Results were normalized to RNA content as determined in the ethidium stained gel.

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