Tetrathiomolybdate Is Effective in a Mouse Model of Arthritis

MARK D. McCUBBIN, GUOQING HOU, GERALD D. ABRAMS, ROBERT DICK, ZHAN ZHANG, and GEORGE J. BREWER

ABSTRACT. ***Objective***. To test for protective effects of therapy with tetrathiomolybdate, a copper-lowering drug, against collagen-induced arthritis in mice.

Methods. Mice were injected with bovine collagen II, and limb joint swelling and erythema were scored. Tetrathiomolybdate treated mice received drug by oral gavage or in drinking water. Plasma ceruloplasmin was followed as a measure of body copper status, and maintained between 20 and 60% of baseline. Urine for isoprostane studies was collected in metabolic cages. At sacrifice, blood was collected for cytokine assays, and hind limbs fixed in formalin.

Results. Tetrathiomolybdate strongly protected against the collagen-induced arthritis as reflected in scores of swelling and erythema, and as seen histologically. Further, tetrathiomolybdate strongly protected against the increase in urine isoprostanes (a marker of oxidant damage) seen in collagen treated controls. The drug also protected against the increase in interleukin 2, interleukin 1ß, and tumor necrosis factor-α levels seen in collagen treated controls.

Conclusion. Based on the positive results reported here, and the good safety profile of tetrathiomolybdate in human studies so far, a trial of tetrathiomolybdate in arthritis syndromes seems warranted. (First Release Oct 15 2006; J Rheumatol 2006;33:2501–6)

Key Indexing Terms:
TETRATHIOMOLYBDATE COPPER ARTHRITIS INTERLEUKIN 2
TUMOR NECROSIS FACTOR-α INTERLEUKIN 1ß ISOPROSTANE

Tetrathiomolybdate (TM) is an anticopper drug under development for the initial treatment of Wilson's disease[1]. The mechanism of action of TM involves forming a tripartite complex with copper and protein[2-5]. Given with food, TM binds food copper and endogenously secreted copper with protein in the alimentary tract, and prevents copper absorption. Given away from food, TM is well absorbed and complexes available plasma copper (often called "free copper") with plasma albumin and renders it unavailable for cellular uptake. This complex is primarily degraded in the liver with copper excretion in the bile[6]. Because of its fast action and low toxicity, TM is proving to be a very useful drug for the initial treatment of Wilson's disease.

From the Departments of Human Genetics, Pathology, and Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan, USA.

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M.D. McCubbin, BS, Research Assistant; G. Hou, PhD, Research Scholar; R. Dick, BS, Research Associate; Z. Zhang, PhD, Research Assistant, Department of Human Genetics; G.D. Abrams, PhD, Emeritus Professor, Department of Pathology; G.J. Brewer, MD, Emeritus Professor, Departments of Human Genetics and Internal Medicine, University of Michigan Medical School.

Address reprint requests to Dr. G.J. Brewer, University of Michigan Medical School, 5024 Kresge Bldg. II, Ann Arbor, MI 48109-0534, USA. E-mail: brewergj@umich.edu

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Work over the past 2 decades has shown that lowering copper levels to a midrange has strong antiangiogenic effects[7-11]. We have subsequently shown that TM, through antiangiogenic properties, has excellent anticancer effects in preclinical models[12-18]. Clinical studies have also been encouraging and are ongoing[19,20]. The mechanism of action appears to be inhibition of multiple angiogenesis-promoting cytokines.

Investigating inhibition of multiple cytokines by TM further, we postulated that TM would also inhibit key cytokines in the transforming growth factor-ß (TGF-ß) pathway, producing fibrosis in fibrotic diseases. To test this we studied the mouse models of bleomycin-induced pulmonary fibrosis[21,22] and carbon tetrachloride-induced cirrhosis[23]. Both studies showed TM to be strongly protective against development of fibrosis and organ injury, and documented inhibition of TGF-ß levels by TM. In other studies we showed TM protection against concanavalin A[23] and acetaminophen[24] liver injury in mice, and doxorubicin heart injury in mice[25]. In the course of these studies we showed that TM inhibited levels of the inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin 1ß (IL-1ß).

Concanavalin A binds to liver cells, and because it is a T cell antigen, stimulates an immune attack on hepatocytes. Since TM therapy offers strong protection against liver injury in the concanavalin A model, we hypothesized that TM therapy would also have efficacy in other immune-mediated diseases. We evaluated that hypothesis in the collagen-induced arthritis model.

MATERIALS AND METHODS

Bovine type II collagen (CII) was purchased from Chondrex Inc., Redmond, WA, USA. Plasma levels of IL-1ß, IL-2, and TNF-α were obtained using ELISA kits purchased from R&D Systems Inc., Minneapolis, MN, according to their methods. Ceruloplasmin (Cp) was measured by an oxidase method[26]. Isoprostane was measured using an ELISA kit from Oxford Biomedical Research, Oxford, MI, according to their method.

Mice, weight 20-25 g, 6-8 weeks old, were obtained from Jackson Laboratories, Bar Harbor, ME, and were housed at 21 ± 2°C on a 12 h light/dark cycle in polycarbonate cages containing corncob bedding. Experimental animals were housed in the University of Michigan Unit for Laboratory Animal Medicine facility and treated in accord with a protocol approved by the University of Michigan Institutional Animal Care and Use Committee. Animals were provided animal food from Harlan/Teklad, Madison, WI, containing about 2 mg/kg copper and 10 mg/kg zinc and tap water ad libitum.

Experiment 1. Pilot Study in Collagen II (CII) Arthritis Model. Twenty male DBA1/J mice were separated into 4 groups, each with 5 mice. Group 1 received only water, Group 2 received TM and CII, Group 3 received only CII, and Group 4 received only TM. Groups 2 and 4 were given 0.2 mg of TM per mouse per day by oral gavage, beginning 2 weeks before CII injection. At time zero (the time when CII was given), Groups 2 and 3 were treated with 100 mg of CII in Freund's complete adjuvant by intraperitoneal injection, following the method of Palmer, *et al*[27]. Groups 1 and 4 were given an injection of Freund's complete adjuvant in lieu of CII. The daily dosage of TM did not change after injection. Once every week, mice were bled by tail vein to measure plasma Cp levels, which were maintained at 20–60% of baseline values in TM treated animals. Animals were examined daily to monitor weight changes and the swelling and erythema of the footpads. Each of these criteria was scored separately on a scale from 0 to 2 for each paw of each mouse. A score of 0 represented no swelling or erythema, a score of 1 meant mild swelling or erythema, and a score of 2 was reserved for mice who had swelling or erythema that was judged to be severe. After 3 weeks, Groups 2 and 3 were boosted with 100 mg of CII in Freund's incomplete adjuvant, and Groups 1 and 4 were given Freund's incomplete adjuvant. At days 29 and 42, animals from Groups 2 and 3 who were negative for swelling and erythema were boosted again with CII and Freund's incomplete adjuvant. The animals were sacrificed after 10 weeks of treatment, and the final plasma levels of Cp were measured. At sacrifice a hind limb from each animal was fixed in 10% neutral buffered formalin, and the paw was subsequently removed, decalcified en bloc, then paraffin embedded. Each paw was sectioned in a vertical plane (i.e., from dorsal skin to plantar surface) through the midportion of the foot, and the sections were stained with hematoxylin and eosin. Sections prepared in this fashion allowed microscopic evaluation of several of the small joints of the foot in each instance.

Experiment 2. Definitive Study in Collagen II Arthritis Model. Given the positive collagen arthritis pilot study, we replicated Experiment 1 in a definitive study using blinded observers. The differences between Experiments 1 and 2 are as follows. The sample sizes for the CII and CII/TM groups were increased and there were fewer controls. A second difference was that TM was administered in the drinking water, rather than by gavage. Third, a blinded scoring system with two blinded scorers was used for assessing swelling and erythema. Last, near the end of the study, urine was collected from all individual mice by using metabolic cages, and isoprostane levels in the urine determined. Twenty-two mice were separated into 3 groups. Groups 1 and 2 had 10 mice each and Group 3 had 2 mice. Group 1 received TM and CII in Freund's complete adjuvant, Group 2 received only CII in Freund's complete adjuvant, and Group 3 received only Freund's complete adjuvant and water. Mice from Groups 1 and 2 were boosted at 3 weeks with CII in Freund's incomplete adjuvant, and Group 3 received Freund's incomplete adjuvant. At 31 and 40 days, animals from Groups 1 and 2 who were negative for erythema and swelling were boosted again with CII in Freund's incomplete adjuvant. Mice from Group 1 were started on 0.03 mg of TM/ml of drinking water during TM pretreatment. The concentration of TM in the drinking water was then adjusted periodically to try to maintain ceruloplasmin in the target range (20–60% of baseline). After the initial CII injection, the dose of TM was lowered to 0.02 mg/ml and then to 0.015 mg/ml after day 31. The doses were then raised to 0.0225 mg/ml for one cage and 0.0175 mg/ml for the other Group 1 cage. Mice were moved into the proper cage such that the higher dose would help to lower the Cp values of some mice while the lower dose would keep those with low Cp values stable. Mice were sacrificed after 8 weeks of treatment, the final plasma levels of Cp, TNF-α, IL-1ß, and IL-2 obtained, and one hind limb from each animal removed, and prepared for histologic analysis as in Experiment 1.

Statistical analysis. For comparison of means, ANOVA was used, followed by Scheffe's test for multiple comparisons. In the case of the isoprostane data, a t test was used.

RESULTS

Figure 1 shows the swelling and erythema scores in the mice of Experiment 1, the pilot collagen II (CII) experiment. Four of the 5 CII control mice had swelling and erythema, and the lines show the average scores for all 5. None of the CII plus TM, adjuvant plus TM, or adjuvant mice showed detectable swelling or erythema. Cp levels were maintained at 20–60% of baseline in TM treated animals (data not shown).

Figure 2 shows the average scores for swelling (part A) and erythema (part B) of the 3 groups of mice in Experiment 2 (definitive study), as evaluated by 2 blinded scorers. Beginning on about day 33 the CII control animals began to develop elevated swelling and erythema scores, which persisted over the duration of the experiment. Nine of the 10 mice in the CII group developed swelling and erythema and the lines in Figures 2A and 2B are the averages of all 10 mice. These mean elevations in the CII animals were very significantly different, statistically (p 0.01–0.0001), than the means of CII TM treated animals.

Figure 3 shows the mean and standard error (SE) for serum IL-2, IL-1ß, and TNF-α for the mice of Experiment 2. TM treated CII exposed animals had significantly lower values in all cases than did CII controls.

Figure 4 shows the mean and SE of the urine isoprostane values from the mice of Experiment 2. The CII animals had markedly elevated values compared to controls. TM treated CII animals had values similar to controls', and very significantly less than CII animals' (p = 0.004).

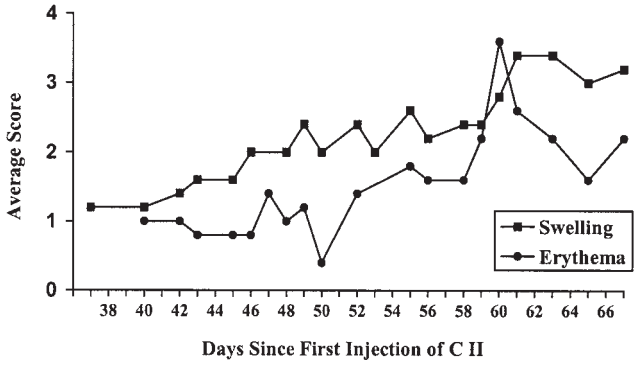


Figure 1. Mean swelling and erythema scores of CII injected mice of Experiment 1 (pilot study). None of the CII injected mice that were treated with TM showed detectable swelling or erythema.

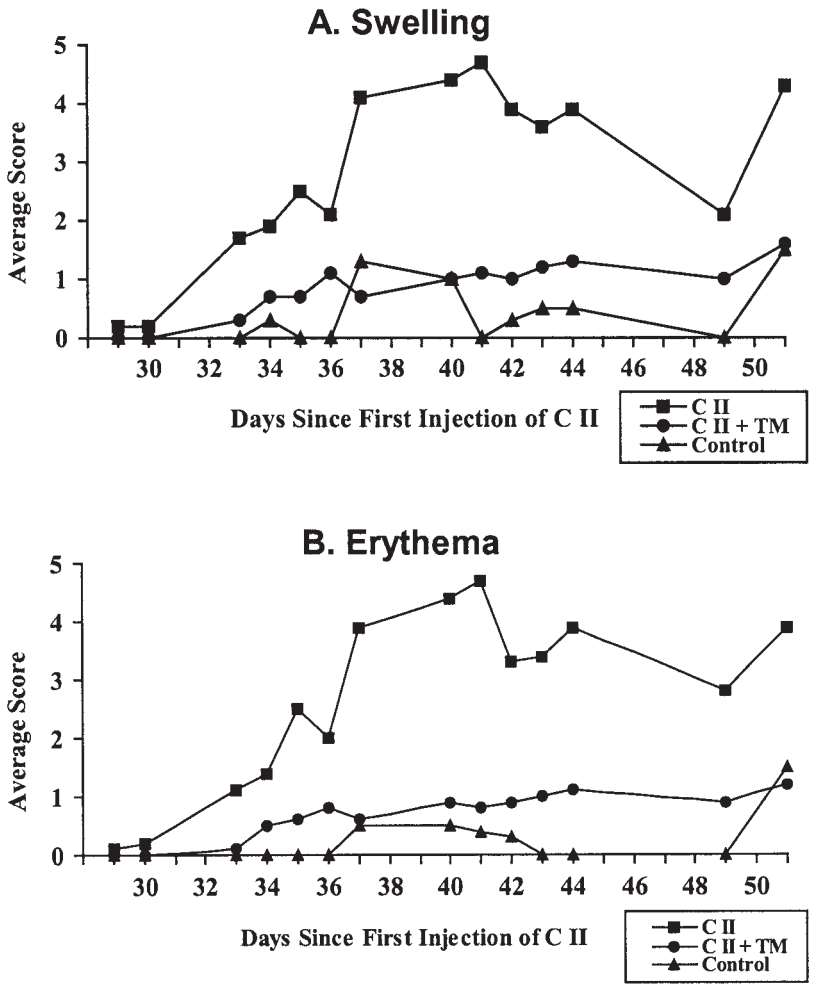


Figure 2. Average of scores from 2 blinded scorers for swelling (A) and erythema (B) in the 3 groups of mice of Experiment 2 (definitive study). The means of the CII data for swelling after day 36 were significantly different than the means of the CII/TM data (p 0.0001–0.0003). The means of the CII data for erythema after day 36 were significantly different than the means of the CII/TM data (p 0.0001–0.01).

Regarding histology, in the collagen treated animals of Experiment 1 and 2 (with one exception out of 15 animals), there was, within the periarticular soft tissues, a prominent, mixed inflammatory cellular infiltrate, including macrophages, lymphocytes, and variable numbers of polymorphonuclear leukocytes. Cartilaginous surfaces were variably eroded or destroyed, and reactive changes were evident in subjacent bone. The severity of the arthritis induced by collagen treatment was graded on a scale of 0 (normal) to 3, taking into account the number of joints involved in the particular paw, and the extent of articular destruction and distortion. All specimens were examined and scored in a "blind" fashion. The results, shown in Table 1, indicate that treatment with TM completely prevented the arthritis in Experiment 1, and significantly inhibited it in Experiment 2. The histology of a representative joint from the control and treatment groups is shown in Figure 5.

The Cp values of the TM treated mice of Experiment 2 averaged 20–60% of controls, except during weeks 7 and 9, when the Cp values of the first cage of TM treated mice approached those of controls. The three TM treated mice with scores of 2 or 3 (Table 1) were from this first cage.

DISCUSSION

We have shown strong protection against joint inflammation and swelling in the bovine collagen arthritis mouse model. Experiment 1 (pilot study), the first of the collagen II injection arthritis experiments, was "cleaner" than the second experiment (Experiment 2, definitive study). That is, 4 of 5 CII control mice in Experiment 1 had relatively severe arthritis, both by erythema and swelling, and by histopathology. The 5 TM treated mice were completely normal with no erythema and no swelling, and normal histology (the histology was examined by a blinded observer, GDA). In Experiment 2, while overall

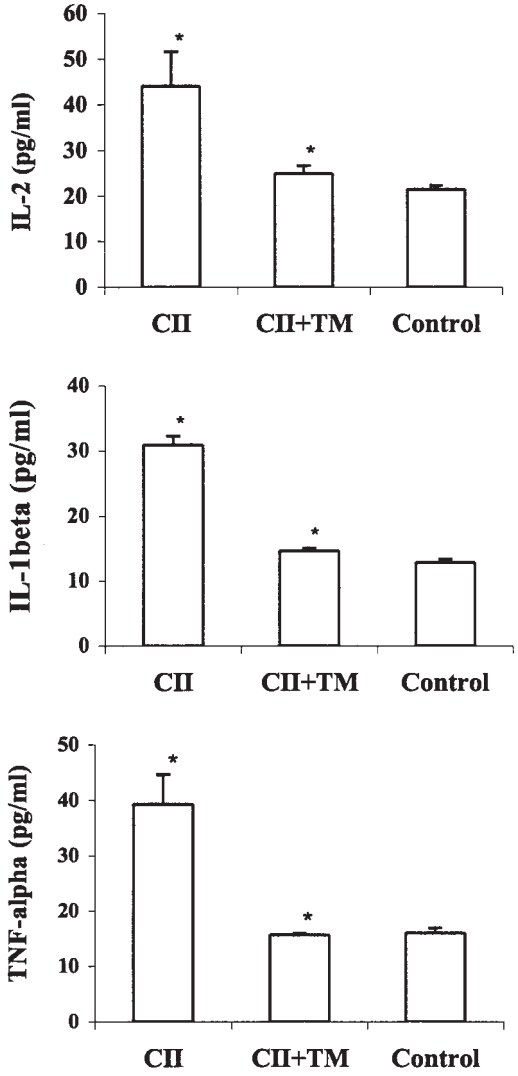


Figure 3. Serum values (mean and SE) for IL-2, IL-1ß, and TNF-α at sacrifice in mice of Experiment 2. The means for IL-2 of CII and CII + TM were significantly different ($p < 0.03$). The means for IL-1ß of CII and CII + TM mice were significantly different ($p < 0.01$). The means for TNF-α of CII and CII + TM mice were significantly different ($p < 0.01$). * Significantly different from each other.

the differences between CII and CII/TM mice were very significant, there were low-grade abnormalities in TM treated mice, and histopathology was graded 2 in 2 mice and 3 in another by the blinded observer. We believe the differences in the 2 experiments are primarily due to differences in copper control. The second experiment used TM in the drinking water, while the first used TM by oral gavage. The Cp control was poorer in the second experiment, particularly in the first cage of 5 mice. These 5 mice contributed both the two 2 and one 3 histopathology scores (Table 1).

The bovine collagen arthritis model is immune-mediated. The results in this model add to the previous work, in which TM protected against immune-mediated concanavalin A hepatitis[23] and offered partial protection against diabetes in the

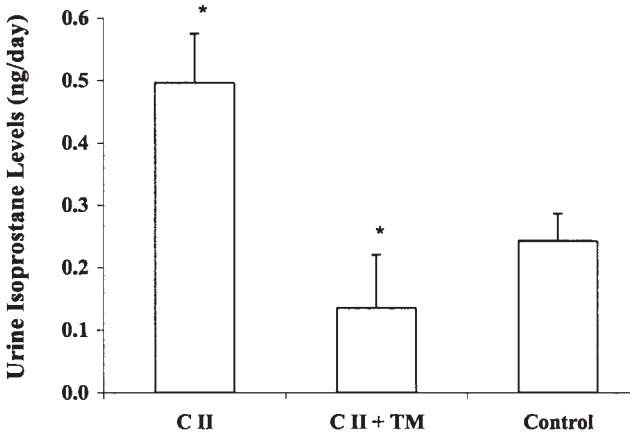


Figure 4. Mean and SE for isoprostane data from mice of Experiment 2 in the week before sacrifice. The mean of the CII animals was significantly different from the mean of the CII/TM animals ($p = 0.004$). * Significantly different from each other.

non-obese diabetic (NOD) autoimmune mouse model[28]. Thus, it is clear that TM is able to mitigate damage from autoimmune and immune-mediated disease in mouse models.

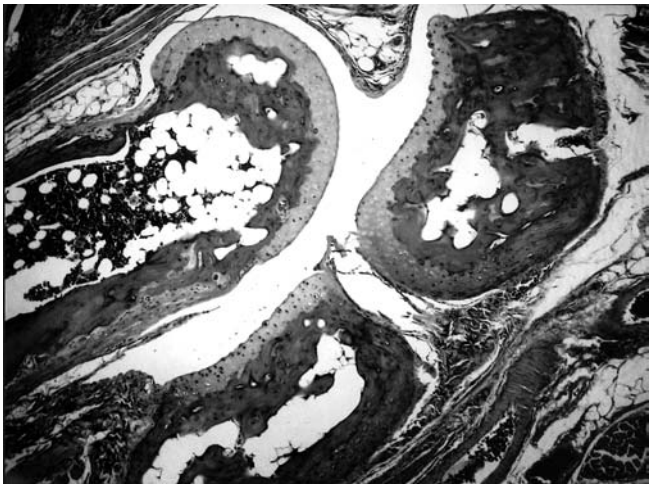
This protection by TM against organ damage extends beyond immune-related events to include protection against xenobiotic agents, including bleomycin lung damage[21,22], carbon tetrachloride[23], and acetaminophen[24] liver damage, and doxorubicin heart damage[25].

The mechanism of these protective effects is of great interest. We presume it has to do with lowered copper availability as a result of TM action, and the copper dependence of one or more steps in injury causation. We have previously shown a lowered amount of the injurious inflammatory cytokines TNF-α and IL-1ß, as well as a lower level of IL-2, in serum of some of these models, as shown here in the arthritis model (Figure 3). IL-2 and interferon-γ (IFN-γ) are important activators of other immune cells and cells such as monocytes and macrophages that may be involved in the release of TNF-α and IL-1ß, so a lowered IL-2 and IFN-γ (not measured in these studies) could account for the reduced amounts of these damaging cytokines. The transcription of IL-2 and IFN-γ requires the activity of nuclear factor-kappa B (NF-κB)[29,30], known to be inhibited by TM action[31]. So it is possible the pathway of injury, both in the immune mediated and xenobiotic agent injuries, involves NF-κB activation by activated T lymphocytes, transcription of IL-2 and IFN-γ, and activation of inflammatory cells and release of their cytotoxic cytokines by IL-2 and IFN-γ. TM may act by simply blocking NF-κB activation.

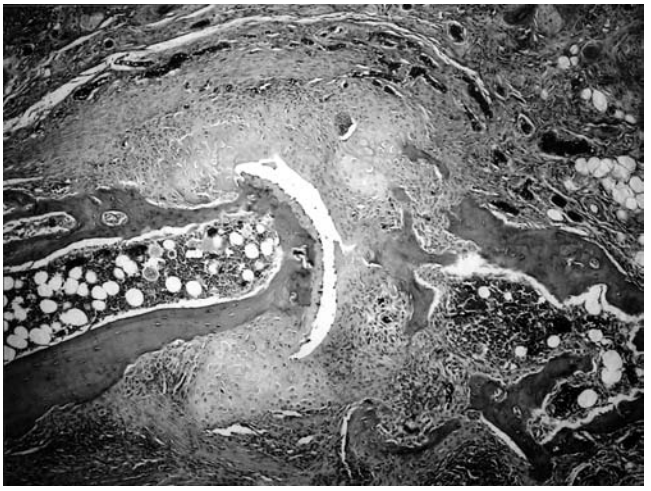
Isoprostanes are a stable marker of reactive oxygen species production and oxidant damage[32], and their elevation in the urine of CII treated animals suggests that part of the mechanism of damage from the immune-mediated collagen arthritis is oxidant in nature. This would not be surprising since the resulting inflammatory reaction in the joints attracts inflammatory cells, including neutrophils, that release reactive oxy-

Table 1. Histologic results of collagen II arthritis experiments.

Treatment	Collagen	Collagen + TM	TM Alone	No Treatment
		Experiment 1		
Scores	0,3,2,1,1	0,0,0,0,0	0,0,0	0,0,0
		p (collagen vs collagen + TM) = 0.04		
		Experiment 2		
Scores	3,3,2,2,1	0,2,2,3,0		0,0
	2,2,3,3,2	0,1,0,1,0		0,0
Mean ± SE	2.3 ± 0.2	0.9 ± 0.3		0
		p (collagen vs collagen + TM) < 0.02		



A



B

Figure 5. A. Paw from an animal in Experiment 2, given TM along with collagen. The cartilaginous joint surfaces are intact, and there is no appreciable inflammatory reaction in the adjacent synovial tissues. (Of 10 animals treated in this fashion, 5 were essentially negative, 2 showed focal inflammation, and 3 had significant arthritis.) *B.* Paw from an animal in Experiment 2 given collagen but no TM. There is obvious and frank destruction of joint surfaces with reactive changes in bone (right) and surrounding soft tissues. (Of 10 animals treated with collagen alone, one had only focal inflammation, while 9 had significant arthritis.)

gen species. The inhibition of isoprostane production by TM could indicate that inhibition of oxidant damage is one mechanism of TM protection against organ injury. Many of the organ-damaging molecules, such as doxorubicin and carbon tetrachloride, are known to cause damage through oxidant mechanisms, and TM strongly protects against damage from these molecules.

However, if TM inhibits NF-κB, IL-2, and IFN-γ production, and thereby inhibits production of the inflammatory cytokines such as TNF-α and IL-1ß, it would be expected that reactive oxygen species and oxidation would also be inhibited. TNF-α and IL-1ß contribute to the inflammatory reaction by mobilizing and activating inflammatory cells that, in turn, produce reactive oxygen species. It would thus be expected that TM treated animals would have less isoprostane. According to this scenario, lowered oxidant damage would be secondary to prevention of the inflammatory cascade. However, we also cannot rule out a more direct effect on reduction of oxidant radical production as a mechanism of TM protection against organ damage.

While this manuscript was in preparation, a report was published describing similar findings in terms of TM suppression of arthritis in rats induced by injection of complete Freund's adjuvant[33]. Our study is somewhat different, in that it used mice and bovine collagen–induced arthritis, and adds cytokine and isoprostane data. Thus, there are now 2 studies reporting a beneficial effect of TM in arthritis animal models.

TM has already seen fairly extensive use in humans, in Wilson's disease[1], in cancer[19,20], and in macular degeneration[34]. It is relatively well tolerated, with overtreatment anemia and leukopenia the only significant toxicity. This is easily overcome by drug holiday or dose reduction. Based on the favorable results found in the animal models that have been studied, trials of TM in arthritis syndromes seem warranted.

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