Tetrathiomolybdate Therapy Protects Against Concanavalin A and Carbon Tetrachloride Hepatic Damage in Mice

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Tetrathiomolybdate, an anticopper drug, has been shown to protect mice against pulmonary fibrosis from bleomycin. Our hypothesis is that it does so by inhibiting fibrosis-inducing cytokines. Indeed, we have good evidence, not yet published, that tetrathiomolybdate inhibits pulmonary levels of transforming growth factor–β and tumor necrosis factor-α expression in these bleomycin experiments. Herein, we evaluate tetrathiomolybdate's effectiveness in mitigating hepatitis and fibrosis in mice from the hepatotoxins, concanavalin A and carbon tetrachloride, and its inhibition of cytokines as a possible mechanism. In short-term experiments, concanavalin A elevated serum amino leucine transferase levels several fold, and tetrathiomolybdate completely prevented this increase. In additional experiments, tetrathiomolybdate therapy reversed the elevated serum transaminase levels despite continued concanavalin A injections, with nearly significant serum interleukin-1β inhibition. Concanavalin A given for 12 weeks produced mild fibrosis, whereas concomitant tetrathiomolybdate treatment resulted in normal histology. Carbon tetrachloride given for 12 weeks resulted in very high serum amino leucine transferase levels, high serum transforming growth factor–β levels, cirrhosis as seen histologically, and increase in liver hydroxyproline, a measure of fibrosis. Concomitant tetrathiomolybdate partially and significantly protected against increases in amino leucine transferase and transforming growth factor–β, fully protected against the increase in hydroxyproline, and resulted in normal histology. In conclusion, tetrathiomolybdate protects against the hepatitis and fibrosis produced by these hepatotoxins, probably by inhibiting the excessive increase in inflammatory and fibrotic cytokines. Exp Biol Med 229:857–863, 2004.

Key words: copper; hepatitis; cirrhosis; transforming growth factor–β; interleukin-1β

Tetrathiomolybdate (TM) was first developed as an anticopper drug for the initial treatment of patients with Wilson disease (1, 2). Tetrathiomolybdate is fast acting and relatively nontoxic. Subsequently, TM was shown to have anticancer activity in five rodent tumor models (3–6), in a study of spontaneous cancer in pet dogs (7), and in a phase 1/2 clinical study of 42 patients with metastatic and advanced cancer (8 and unpublished). The mechanism of the anticancer effect is through inhibition of angiogenesis (3, 9–11). Many cytokines that promote angiogenesis are copper dependent, such that if body copper levels are lowered with TM, angiogenic cytokine signaling is inhibited (3, 9–11). As long as copper levels are not lowered excessively, cellular copper requirements are met, avoiding toxic effects from copper deficiency (3–8). The level of ceruloplasmin (Cp), a serum protein that contains copper, is used as a surrogate marker of body copper status (3–8). The liver secretes Cp into the blood in an amount that depends on copper availability (12). The Cp level is maintained at midrange in both preclinical and clinical studies with TM therapy to provide an "antiangiogenic window."

Examination of the fibrotic pathway involving transforming growth factor–β (TGFβ) and connective tissue growth factor suggested to us that these cytokines might also be copper dependent. Overactivity and dysregulation of this pathway lead to fibrotic diseases in many organs (13, 14). Examples are pulmonary fibrosis, cirrhosis, renal fibrosis, and scleroderma (13, 15, 16). As a first test of the hypothesis that the fibrotic pathway was copper dependent, we used the bleomycin mouse model of pulmonary fibrosis (15). Therapy with TM completely prevented the inflammatory and fibrotic sequelae of intratracheal bleomycin instillation at the time of sacrifice 21 days after bleomycin treatment (17, 18). In work being prepared for publication, we have subsequently shown that TGFβ levels, which are

The University of Michigan has recently licensed the antiangiogenic uses of tetrathiomolybdate to Attenuon LLC, and Dr. Brewer and Mr. Dick have equity in Attenuon LLC.

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very high in the lungs of bleomycin controls at 21 days, are maintained at near-normal levels in the lungs of bleomycin animals treated with TM. In this work, we also found that the RNA expression levels of tumor necrosis factor-α (TNF-α), the major inflammatory cytokine involved in the inflammatory response to bleomycin, which is very elevated in the lungs of bleomycin controls at 7 days, was kept at near-normal levels at 7 days by TM treatment of bleomycin animals. By starting TM treatment after the inflammatory peak and then finding fibrosis inhibition by TM at the 21-day point, we were able to show that the fibrosis inhibition is an independent effect of TM not simply due to prior suppression of inflammation (18).

In this current work, we are extending our observations to the liver, using both the concanavalin A (Con A) and carbon tetrachloride (CT) models of liver injury in the mouse. Concanavalin A injected intravenously in the mouse produces acute hepatitis, marked by the release of the transaminase enzyme, amino leucine transferase (ALT), into the serum (19). Continued injection of Con A for weeks can produce some fibrosis, but a better model for this is CT treatment. Carbon tetrachloride injected intraperitoneally or given by oral gavage also produces acute hepatitis marked by an elevation of serum ALT level. However, continued injection for 12 weeks produces a well-established cirrhosis (20). Our objectives in the present work were to determine if TM therapy protects against these types of liver injury and, if so, whether inhibition of inflammatory and/or fibrotic cytokines in plasma could be detected.

Materials and Methods

Mice. The BALB/c (Experiments 1, 2, and 3) and CBA/J (Experiment 4) mice were 20-g females purchased from The Jackson Laboratory, Bar Harbor, ME. Experimental animals were housed in the University of Michigan Unit for Laboratory Animal Medicine facility and treated in accordance with a protocol approved by the University of Michigan Institutional Animal Care and Use Committee in accordance with the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (21). Animals were assigned to groups randomly, were provided food and water *ad libitum*, and kept on a 12:12-hr light:dark cycle.

Con A and CT Treatment. Concanavalin A and CT were purchased from Sigma Chemical Co., St. Louis MO. In Experiments 1, 2, and 3, Con A–treated mice received a weekly intravenous (tail vein) injection of 15 mg/kg of body wt of Con A dissolved in 0.3 ml of 0.9% sodium chloride. Control mice received an intravenous injection of 0.3 ml of 0.9% sodium chloride alone. In Experiment 4, CT-treated mice received intraperitoneal injections of 1 ml/kg of body wt of CT in 200 μl of olive oil twice per week. Control animals received 200 μl of olive oil alone by intraperitoneal injection twice per week.

TM Treatment. Tetrathiomolybdate was given in 0.25 ml of water by intragastric gavage once daily in the doses and times indicated in the various studies.

Con A Experiments. Three types of experiments were performed. In Experiment 1, mice were divided into four groups: six to receive Con A only, four to receive Con A plus TM, six to receive saline instead of Con A, and three to receive TM only. In the TM-treated animals, TM was given in a daily dose of 0.9 mg, beginning 5 days before the first Con A injection. Concanavalin A was administered for 4 weeks. Twenty-four hours after the first three injections, blood was taken for ALT assay. Twenty-four hours after the fourth injection, all the animals were sacrificed and ALT and Cp measured on all.

In Experiment 2, mice were divided into three groups: six to receive Con A only, six to receive Con A plus TM, and four to receive saline instead of Con A. In the TM-treated animals, TM was withheld until after the fourth Con A injection and then initiated at a dose of 0.9 mg/day. Twenty-four hours after the fourth through 12th Con A injections, blood was taken for ALT assay from individual animals of the Con A and Con A plus TM groups and occasionally from a control animal. Different animals were used for these blood draws to avoid overbleeding. Concanavalin A injections were continued for 12 weeks and the animals sacrificed for histologic analysis of the livers.

In Experiment 3, mice were divided into two groups: 9 to receive Con A only and 10 to receive Con A plus TM. In TM-treated animals, TM treatment was begun 5 days before the first Con A injection at a daily dose of 0.9 mg. Concanavalin A was injected twice at weekly intervals. Two hours after the second injection (Week 2), the animals were sacrificed and cytokine measurements made in the blood.

CT Experiments. In Experiment 4, mice were divided into four groups: five to receive CT only, five to receive CT plus TM, three to receive the vehicle for CT (olive oil), and three to receive TM only. Carbon tetrachloride was given in twice-weekly doses for 12 weeks. Tetrathiomolybdate treatment was begun in TM-treated animals after 4 weeks of CT injections at a dose of 0.9 mg once daily. After 12 weeks, the animals were sacrificed for histopathology studies of the liver, hydroxyproline assays of the liver, and ALT and TGFβ assays in the serum.

Copper Status. In the presence of TM therapy, serum copper cannot be used to assess copper status because of the accumulation in the blood of a tripartite complex of TM, copper, and albumin. We use serum Cp as a surrogate measure. Ceruloplasmin was assayed by its oxidase activity as previously described (5).

Hydroxyproline Assay in the Liver. This assay was performed as previously described (18).

Serum ALT Assay. This assay was performed using a quantitative colorimetric method commercially available from Sigma Diagnostics (Procedure 505), St. Louis, MO.

Cytokine Assays. Interleukin-1β (IL-1β), TNF-α,

Table 1. Serum ALT Results in Mice 24 hrs After Each of Four Weekly Serial Injections of Con A in Experiment 1[a]

	Week of injection					
				Fourth		
Animal type	First	Second	Third	*n*	Mean[b]	SD
Saline control	35	44	57	6	41	5.2
TM only control	85	39	49	3	38	6.2
Con A only	179	265	361	6	168	47.9
Con A plus TM	52	50	63	4	74	10.6

[a] ALT, amino leucine transferase; Con A, concanavalin A; TM, tetrathiomolybdate. ALT levels are given in Sigma-Frankel units per liter. Each Sigma-Frankel unit equals 0.48 of an international unit.
[b] Statistical evaluation of the group data after the fourth injection involved use of the Student's *t* test with the Scheffé correction. The mean of the Con A group was statistically significantly different from the means of all three other groups ($P < 0.001$). The Con A plus TM group mean was not statistically significantly different than the mean of either control group.

and TGFβ serum levels were determined by a quantitative sandwich enzyme immunoassay method using commercially available kits purchased from R & D Systems, Minneapolis, MN.

Statistics. For comparisons of means, analysis of variance was used followed by the Scheffé test for multiple comparisons when appropriate.

Results

Con A Studies. In Experiment 1, where Con A was given for 4 weeks, serum ALT levels in single animals from each group (to avoid overbleeding) were measured 24 hrs after the first three injections (Table 1). Then all of the animals in each group were sacrificed and ALT assays performed on all the animals (Table 1; under fourth week of injection). The data are consistent in that Con A elevated ALT levels 4- to 6-fold, and TM strongly, and significantly at Week 4, protected against those elevations. The Cp levels at the time of sacrifice in the Con A plus TM groups averaged 47% in the Con A group, and the means were highly statistically significantly different ($P = 0.0008$, with the Scheffé correction) (data not shown).

In Experiment 2, Con A was given for 4 weeks before TM treatment was started in TM-treated animals. At Week 4, before any TM treatment, an individual animal from each group was bled 24 hrs after Con A injection for ALT assay. At 4 weeks, Con A–treated animals had an approximately 4-fold elevation of ALT levels compared with the saline control (Fig. 1). After that, at weekly intervals, different individual animals from the Con A and Con A plus TM groups were bled 24 hrs after Con A injection. Data for Weeks 5–7 are shown in Figure 1. Subsequent to TM therapy, ALT levels decreased to nearly normal during a 3-week period despite continued Con A injections. The Con A injections in these animals were continued for 12 weeks. The ALT levels in the six Con A animals bled during this period were statistically significantly higher than the six Con A plus TM animals bled during this same period ($P = 0.03$) and also significantly higher than the four control animals bled during this period ($P = 0.04$). The Con A plus TM animals were not different than controls. At 12 weeks, the animals were sacrificed and their livers examined histologically. The Con A animals had some inflammatory changes and minimal fibrosis, whereas the Con A plus TM animals had histologically normal livers (data not shown). The Cp levels at the time of sacrifice in the TM group averaged 18% of the Con A group, and the means were highly statistically significantly different ($P = 0.0006$) (data not shown).

In Experiment 3, in blood taken 2 hrs after the second Con A injection, there were trends toward serum TNF-α being lower in the Con A plus TM samples than in the Con A samples, but the means were not statistically significantly different. Mean serum IL-1β levels 2 hrs after the second Con A injection were 37% of the Con A mean in the Con A plus TM samples, and this difference was close to being statistically significant ($P = 0.08$) (data not shown).

CT Studies. In Experiment 4, CT was given for 12 weeks in CT-treated animals, and TM treatment was started at the beginning of the fifth week in TM-treated animals. All animals were sacrificed at 12 weeks. Figure 2 shows the serum ALT data at the 12-week point. (The two control groups were pooled, since their means were similar.) The

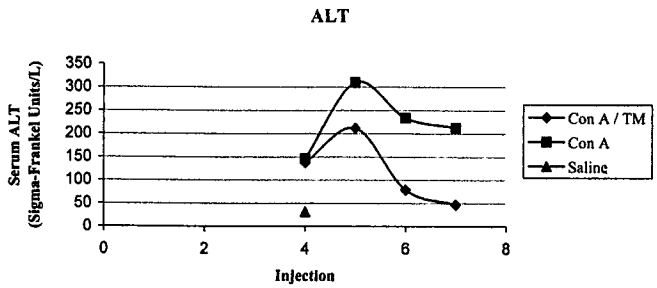

Figure 1. Concanavalin A (Con A) was given for 4 weeks before initiation of tetrathiomolybdate (TM) in TM-treated animals in Experiment 2. An individual animal from each of the three groups was bled 24 hrs after Con A injection at Week 4 for amino leucine transferase (ALT) assay. Different individual animals were bled at Weeks 5, 6, and 7 from the Con A and Con A plus TM groups for ALT assay.

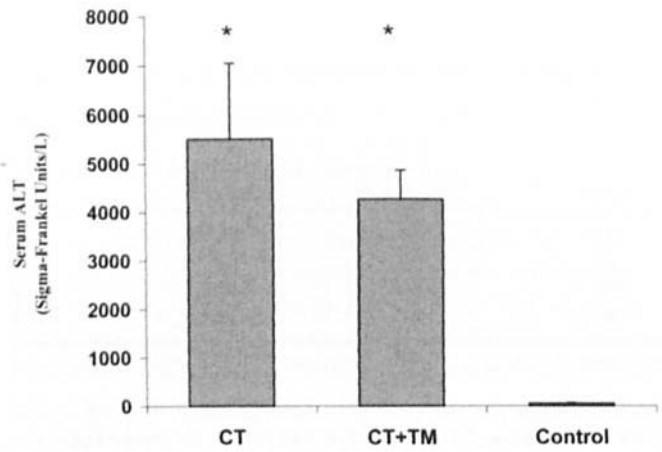

Figure 2. Mean and SD of serum amino leucine transferase data at 12 weeks in the carbon tetrachloride study (Experiment 4). Asterisk indicates means are statistically significantly different at $P < 0.05$.

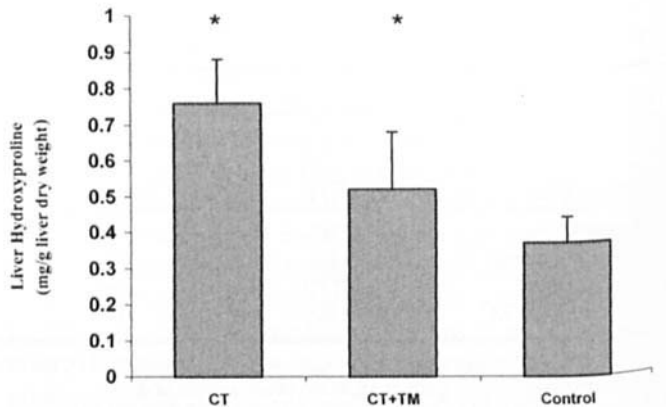

Figure 3. Mean and SD of hydroxyproline content of the liver at 12 weeks in the carbon tetrachloride study (Experiment 4). Asterisk indicates means are statistically significantly different at $P < 0.03$.

CT treatment strongly elevates ALT levels over control levels, and TM partially and significantly ($P = 0.05$) protects against the CT-induced increase. At lower doses of CT and shorter time frames, this effect was even more pronounced (data not shown).

Hydroxyproline levels at the 12-week point in Experiment 4 were elevated by CT treatment, and TM therapy almost completely and significantly ($P = 0.03$ with the Scheffé correction) prevented this CT-induced increase (Fig. 3). (The two control groups were pooled, since their means were similar.) The CT plus TM and control means were not significantly different.

In keeping with this biochemical evidence of TM protection against fibrosis, histopathology studies clearly documented TM's protection against CT-induced cirrhosis (Fig. 4). The CT control animals had a well-developed cirrhosis, whereas the CT plus TM therapy animals were histologically normal (Fig. 4).

Also in Experiment 4, TGFβ levels were measured in the plasma at the 12-week point (Fig. 5). (The two control groups were pooled, since their means were similar.) The data show that CT treatment significantly elevates plasma TGFβ levels ($P < 0.02$ with the Scheffé correction) and that TM therapy almost completely and statistically significantly ($P = 0.02$ with the Scheffé correction) prevents TGFβ elevation in response to CT.

The plasma Cp data from Experiment 4 are shown in Figure 6. The mean Cp level in CT-treated animals was markedly and statistically significantly lower in the TM-treated group. The Cp levels are statistically significantly elevated in CT animals vs controls ($P = 0.0003$), because Cp is an acute-phase reactant.

Discussion

As was the case in the bleomycin lung injury study reported earlier (17, 18), TM strongly protects against liver injury from the two hepatotoxins studied herein. Tetrathiomolybdate protected against serum ALT level elevations from Con A (Table 1), even when TM treatment was started after several injections of Con A (Fig. 1). In the 12-week study with CT, TM treatment started 4 weeks after CT partially protected against CT-induced ALT level elevations (Fig. 2) and completely protected against fibrosis induced by CT as measured by hydroxyproline levels (Fig. 3) and as seen histologically (Fig. 4).

In our prior bleomycin study, the TM protection against lung injury appeared to be mediated by cytokine inhibition. In work being prepared for publication, we have shown TM inhibition of TGFβ and TNF-α in the lung. Therefore, we postulate that the mechanism of TM protection against liver injury is also mediated by inhibition of these inflammatory and fibrotic cytokines. In the current study, our cytokine studies were limited to plasma and protein assays. (In the bleomycin study, we included assays in lung and also performed RNA assays.) In the Con A study, we saw trends toward a lower TNF-α protein level in the plasma of TM-treated Con A animals than in Con A animals, but the results were not statistically significant. Concanavalin A increased serum levels of IL-1β protein, TM appeared to decrease these levels, and this effect approached statistical significance ($P = 0.08$). In the CT studies, CT elevated serum levels of the profibrotic cytokine, TGFβ, and TM therapy clearly caused a significant decrease in these levels at the 12-week point (Fig. 5), when well-developed cirrhosis was occurring in CT controls.

It appears that TM is a somewhat general protectant against much of the organ injury produced by toxic agents. So far we have shown protection against bleomycin injury in the lung and, in this article, Con A and CT in the liver. Our working hypothesis is that these protections by TM do not involve inhibiting the initial toxic effect of the agent but rather inhibiting the subsequent excessive inflammatory response and the subsequent excessive harmful fibrosis. The best evidence for the lack of a TM effect on initial toxicity of these agents is our bleomycin work (18). In this situation, bleomycin is given only once and sets off the inflammatory

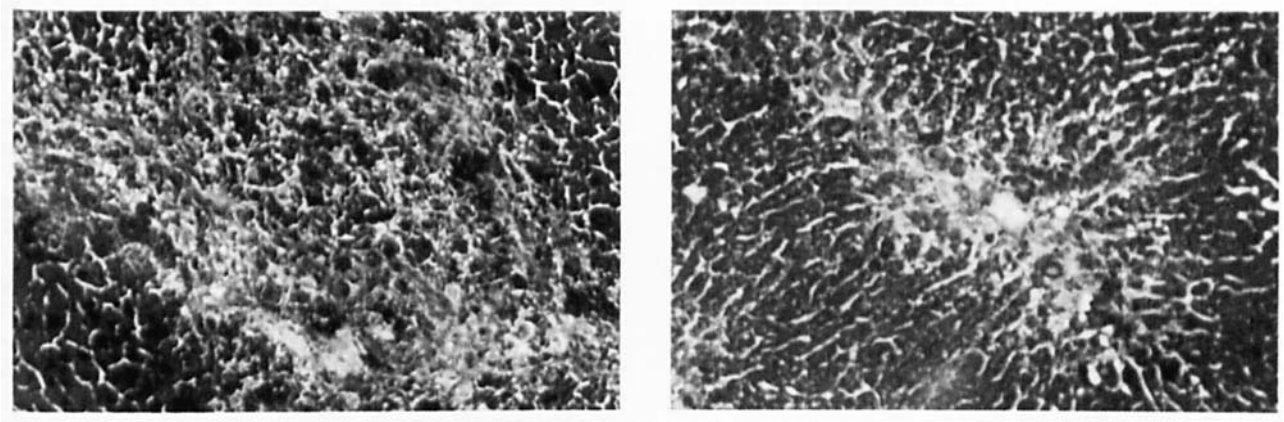

Figure 4. Masson trichrome stains of the liver of two mice treated with carbon tetrachloride (CT) for 12 weeks from Experiment 4. The control CT-treated animal is on the left and shows extensive fibrosis (blue staining). The CT-treated animal that received tetrathiomolybdate is on the right and shows normal histology.

and fibrotic cascade. Treatment with TM started on Day 7 after bleomycin, which brings the copper level down by approximately Day 10 or 11, still has a strong antifibrotic effect by Day 21 (18). Presumably, the bleomycin has been disposed of and is no longer producing new damage during the last 10 to 11 days in this type of study. In the present article, TM treatment started after damage from Con A or CT is also effective in inhibiting subsequent damage. However, these studies are less compelling as evidence against protection from initial damage, since the damaging agents continue to be administered in these liver models. The likely mechanism of the protective effect against subsequent excessive inflammation and fibrosis has already been discussed, namely, inhibition of excessive activity of inflammatory and fibrotic cytokines.

If this hypothesis is correct, it suggests that TM has the potential to be useful in preventing further damage from dysregulated inflammatory and fibrotic responses irrespective of the injury. This would mean that autoimmune diseases, which often have high levels of inflammatory and fibrotic cytokines, might be effectively treated with TM. These liver models, in which the injurious agent is repeatedly administered and TM protects against much of the injury, are likely very relevant to TM protection against the responses to autoimmune injury, which is also continuous.

The protection by TM against cirrhosis induced by CT and against pulmonary fibrosis induced by bleomycin suggests that TM deserves to be tested for efficacy in human disease of cirrhosis, pulmonary fibrosis, renal fibrosis, scleroderma, and other fibrotic diseases associated with excessive TGFβ production (13, 14, 16). A clinical trial in idiopathic pulmonary fibrosis is under way, and trials are planned in scleroderma and primary biliary cirrhosis. Since these types of diseases are generally not treated effectively with current approaches, efficacy of TM therapy would be welcome.

Similarly, the protection by TM against excessive inflammation, as illustrated by the inhibition of hepatitis from Con A evidenced by the reduced ALT levels, suggests evaluating TM in diseases of excessive inflammation. Such trials are further suggested by TM inhibition of TNF-α and IL-1β. Diseases of this type would include autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, vasculitis of various types, and Crohn disease.

Along these same lines, TNF-α–based antibodies have been shown to be effective in a series of diseases with TNF-

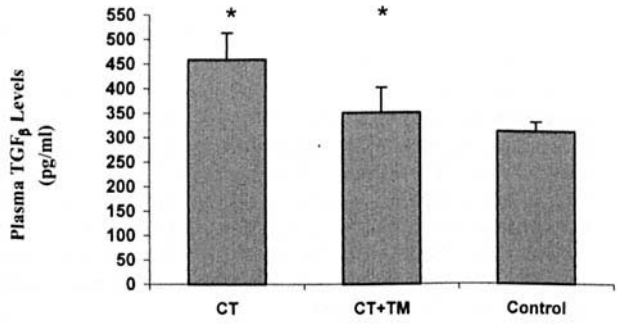

Figure 5. Mean and SD of plasma transforming growth factor–β levels at 12 weeks in the carbon tetrachloride study (Experiment 4). Asterisk indicates means are statistically significantly different at $P < 0.02$.

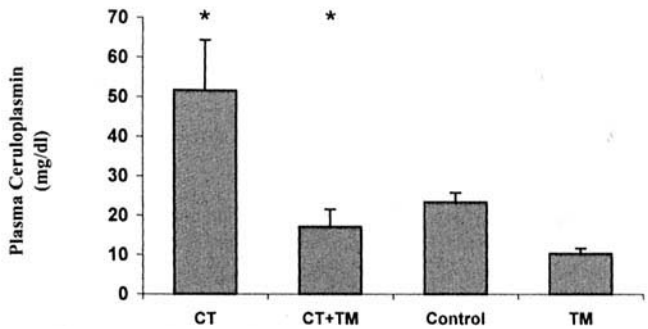

Figure 6. Mean and SD of plasma ceruloplasmin levels at 12 weeks in the carbon tetrachloride study (Experiment 4). Asterisk indicates means are statistically significantly different at $P < 0.0002$.

α elevations, including rheumatoid arthritis (22, 23), Crohn disease (24, 25), psoriasis (26–28), and many others. Tetrathiomolybdate therapy, which is given orally, should be tried in diseases where injection of such antibodies has proven effective. Besides oral administration, a potential advantage of TM is that it does not inhibit cytokines below their constitutive levels (Fig. 5 and unpublished data), while inhibiting much of the increase induced by injury. This may avoid some of the problems caused by antibody inhibition, such as tuberculosis reactivation by TNF-α antibodies (23), or activation of inflammation, shown by gene knockouts of TGFβ in animals (29, 30).

The relative safety of TM therapy becomes important when discussing clinical applications. Obviously, copper levels cannot be pushed too low or copper deficiency problems will emerge. Our strategy has been to lower Cp levels to a midrange, which means that the liver still has enough copper to synthesize approximately half the normal amount of holoceruloplasmin. At these levels, cells in the body have enough copper to synthesize normal amounts of required cuproenzymes, such as lysyl oxidase, cytochrome oxidase, and superoxide dismutase, but cytokine signaling is inhibited in many cases. When Cp levels are pushed very low with TM therapy, the first adverse effect seen is bone marrow depression, because the bone marrow has a relatively high requirement for copper to make cells. The resulting anemia and/or leukopenia is relatively mild and quickly responsive to a dose reduction or drug holiday. If the dose is reduced when bone marrow effects are seen, other adverse effects from deepening copper deficiency are not seen. Tetrathiomolybdate has been safely used in this manner in a number of published (8, 31, 32) and ongoing clinical trials.

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