

Effect of Gold Sodium Thiomalate and Its Thiomalate Component on the In Vitro Expression of Endothelial Cell Adhesion Molecules

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Abstract

Endothelial adhesion molecules play an important role in the tissue recruitment of leukocytes in inflammatory conditions such as rheumatoid arthritis. We have investigated the effect of the antirheumatic drug gold sodium thiomalate on adhesion molecule protein and mRNA expression in cultured human endothelial cells. Gold sodium thiomalate inhibited cytokine (TNF, IL-1, IL-4)-stimulated expression of vascular cell adhesion molecule-1 and E-selectin but not intercellular adhesion molecule-1 on endothelial cells. Gold sodium thiomalate also suppressed TNF-stimulated increases in vascular cell adhesion molecule-1 and E-selectin mRNA levels but had no effect on intercellular adhesion molecule-1 mRNA. Thiomalate (mercaptosuccinate), but not gold thioglucose or D-penicillamine, mimics the effect of gold sodium thiomalate at equimolar concentrations. We propose that the inhibition of vascular cell adhesion molecule-1 and E-selectin expression by gold sodium thiomalate is due to its thiomalate and not its gold component. Gold sodium thiomalate has a direct effect on endothelial adhesion molecule expression, and this may contribute to its antiinflammatory activity. (*J. Clin. Invest.* 1994. 94:1864–1871.) **Key words:** cell adhesion molecules, drug effects • endothelium • intercellular adhesion molecule-1 • vascular cell adhesion molecule-1 • E-selectin

Introduction

Leukocyte infiltration is a key component of chronic inflammation (1). Adhesion molecules on endothelial cells play an important role in targeting and facilitating leukocyte migration out of the blood vessels, in response to an inflammatory stimulus.

Stimulation of endothelial cells in vitro with inflammatory cytokines such as TNF results in increased expression of inter-

cellular adhesion molecule-1 (ICAM-1),¹ vascular cell adhesion molecule-1 (VCAM-1), and E-selectin (2, 3). ICAM-1 is expressed on a wide variety of cell types but only at low levels on unstimulated endothelial cells (4). TNF or IL-1 stimulation induces increased expression of ICAM-1 that reaches a plateau after 24 h and remains elevated for at least 2 d. VCAM-1 is not constitutively expressed and peaks after 10–24 h of TNF stimulation. E-selectin expression is restricted to endothelial cells (4). While there is no basal expression of E-selectin on endothelial cells, continuous TNF or IL-1 stimulation results in a maximum level after 6 h which then declines (2, 4). The transient expression of E-selectin in vitro may not always be relevant in vivo. Sustained in vivo E-selectin expression has been observed in chronically inflamed tissues including psoriatic skin and rheumatoid synovium (5, 6). Moreover, in vitro and in vivo IFN- γ prolongs E-selectin expression. It has been suggested that IFN- γ , released at sites of inflammation, may extend the duration of E-selectin expression (7, 8).

Elevated levels of TNF have been demonstrated in rheumatoid arthritic joints (9, 10). Increased expression of VCAM-1 (11), E-selectin (6), CD31 (PECAM-1), and β_3 integrin subunit (vitronectin receptor) (12) is present on the endothelial cells of rheumatoid synovium compared with normal or osteoarthritic synovium. A number of antiinflammatory drugs have been shown to affect adhesion molecules and/or leukocyte–endothelial interactions (for review see reference 13). Notably, the expression of ICAM-1 on endothelial cells is reduced by colchicine (14) and dexamethasone (15). Furthermore, synovial biopsies from rheumatoid patients treated with gold sodium thiomalate (GSTM) show decreased E-selectin expression (16).

Despite the extensive body of literature on the mechanism by which gold compounds alleviate rheumatoid arthritis (17, 18), no dominant paradigm has emerged. GSTM inhibits monocyte oxygen radical generation (19) and Fc and C3 receptor expression and chemotaxis (20). Moreover, GSTM has effects on endothelial cells including inhibition of cell proliferation (21) and HLA-DR expression (22).

Studies in mice and humans indicate that GSTM readily dissociates in vivo. The gold component rapidly binds to sulfhydryl groups on proteins while free thiomalate (TM) is either rapidly excreted or taken up by tissues. In mice, 6 h after injection, only 0.1% of the thiomalate remains circulating, while ~ 50% is incorporated into tissues (23). These observations, and the lack of correlation between blood gold concentrations and therapeutic effect, have led to the proposal that TM is responsible for some or all of the antiinflammatory effect of GSTM (24, 25). In this study, we have investigated the in vitro effect of GSTM and TM on TNF-stimulated expression of ICAM-1, VCAM-1, and E-selectin on endothelial cells.

Methods

Reagents. Bovine lung heparin, BSA, di-sodium EDTA, Tween 20, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid), and 3,4,5 dimethyl-

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1. Abbreviations used in this paper: BSO, DL-buthionine-[S,R]-sulfoximine; D-pen, D-penicillamine; GSTM, gold sodium thiomalate; GTG, gold thioglucose; HUVE, human umbilical vein endothelium; ICAM-1, intercellular adhesion molecule-1; MTT, 3,4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide; TM, thiomalate; VCAM-1, vascular cell adhesion molecule-1.

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thiazol-2,5 diphenyl tetrazolium bromide (MTT) were all supplied by Sigma Chemical Co. (St Louis, MO). Gelatine and phenol were purchased from BDH Chemicals Ltd. (Poole, United Kingdom). Chloroform and Triton X-100 were from BDH (Kilsyth, Victoria, Australia). Collagenase was obtained from Worthington Biochemical Corp. (Freehold, NJ). Medium 199 with Hanks' salts was supplied by ICN Biomedicals, Inc. (Costa Mesa, CA). Recombinant TNF was a generous gift from Dr. Debbie Rathjen (Peptide Technology, Sydney, Australia). Recombinant IL-1 α and IL-4 were supplied by Boehringer Mannheim GmbH (Mannheim, Germany). 75-cm² plastic cell culture flasks were purchased from Corning Glass Works (Corning, NY). 60-cm-diameter culture dishes (LUX brand) were obtained from Nunc, Inc. (Naperville, IL). 96-well plates were manufactured by Nunc (Kamstrup, Denmark) or by Biofusion (New South Wales, Australia). FCS was bought from Cytosystems (New South Wales, Australia). Glutaraldehyde and formaldehyde were supplied by Ajax Chemicals (New South Wales, Australia). Monoclonal antibodies to VCAM-1 and E-selectin were purchased from British Biotechnology (Abingdon, United Kingdom) and Becton Dickinson Immunocytometry Systems (San Jose, CA), respectively. Anti-ICAM-1 was a generous gift from Dr. Andrew Boyd (Walter and Eliza Hall Institute, Melbourne, Australia). The Vectastain kit was obtained from Vector Labs, Inc. (Burlingame, CA), and the peroxidase-conjugated goat anti-mouse was purchased from Tago, Inc. (Burlingame, CA). Sheep anti-mouse F(ab')₂ conjugated to FITC was from Silenus (Hawthorn, Victoria, Australia). ⁵¹Cr-labeled sodium chromate was supplied by Amersham International (Little Chalfont, Buckinghamshire, United Kingdom). Guanidine thiocyanate was manufactured by International Biotechnologies, Inc. (New Haven, CT). Agarose was bought from Promega Corp. (Madison, WI). GeneScreen nylon membrane was from New England Nuclear Research Products (Boston, MA). SDS was manufactured by ICN Biomedicals (Cleveland, OH). Dextran sulfate was purchased from Pharmacia AB (Uppsala, Sweden). Sheared salmon sperm DNA was supplied by Calbiochem-Novabiochem (La Jolla, CA). All DNA probes were obtained as gifts. ICAM-1 (1.5 kb excised from pGEM7 with XbaI and HindIII) was from Dr. Andrew Boyd. VCAM-1 (3 kb of clone VCAM1E11 excised from CDM8 with NotI) was from Biogen (Cambridge, MA). E-selectin (1,841 bp from XhoI digest of pCDM8) was from Dr. Eugene Butcher (Department of Pathology, Stanford University, Stanford, CA). Mouse actin (~600 bp excised from Blue Scribe with PstI) was from Dr. Bruce Spiegelman (Dana Farber Cancer Institute, Boston, MA). Probes were labeled with 3,000 Ci/mmol [α -³²P]dCTP supplied by Amersham International using a random primer kit (Boehringer Mannheim GmbH).

Preparation of drugs. GSTM (May and Baker, Melbourne, Australia) was dissolved in water at 30 mM (based on monomeric weight) and allowed to stand at room temperature until the yellow color disappeared (~1 h). DL-buthionine-[S,R]-sulfoximine (BSO; Sigma Chemical Co.) was dissolved in water at 6 mM. Thiocetate, thioglycolate, gold thioglucose (GTG), thioglucose (all from Sigma Chemical Co.), TM (mercaptosuccinic acid; Aldrich Chemical Co., Milwaukee, WI), D-penicillamine (D-pen) (Eli Lilly, Sydney, Australia), and L-cysteine hydrochloride (Ajax, Sydney, Australia) were dissolved in water at 30 mM and adjusted to pH 6–8 with NaOH if necessary. Buffering to physiological pH occurred upon mixing with basal medium and placement in the 5% CO₂ atmosphere of the cell culture incubator. These solutions were diluted at least 30-fold in basal medium before incubating with cells. L-cystine has a low solubility at pH 7 (26), so it could not be prepared as a concentrate in water. Instead, a solution was prepared by dissolving cystine (0.072 mg/ml; Hopkin and Williams, Essex, United Kingdom) in basal medium (10% FCS in Medium 199) and adding NaOH to 10 mM. Upon dissolution, the medium was returned to neutral pH by the addition of HCl. Basal medium contains 100 μ M cystine as an essential ingredient (ICN Biomedicals) so the concentration of this solution was 400 μ M. A final concentration of 300 μ M cystine was realized when 400 μ M cystine was diluted 2:1 with basal medium containing TNF. The effect of 300 μ M cystine was compared with that of basal medium which had undergone the same rise and fall in pH.

Human umbilical vein endothelial (HUVE) cell culture. HUVE cells

were isolated as previously described (27). Briefly, human umbilical veins were filled with collagenase solution, incubated at 37°C for 10 min, flushed with Medium 199, and cells were collected by centrifugation. Cells were grown in gelatin (0.2%) coated 75-cm² flasks and maintained in growth medium consisting of Medium 199 supplemented with 20% FCS, 50 μ g/ml bovine lung heparin, and 1:100 dilution of bovine brain extract (28). HUVE cells were used up to passage five.

Cell ELISA. Endothelial cells were plated at confluent density into the wells of gelatin-coated 96-well plates. After overnight incubation, the growth medium was removed and replaced with basal medium (10% FCS in Medium 199) containing TNF (10 ng/ml) and test drugs or with basal medium alone. Cells were incubated for 24 h (unless otherwise stated), then washed in PBS with 0.05% Tween 20 (PBS/Tween), fixed in 0.025% glutaraldehyde for 10 min, washed and stored for 1–3 d at 4°C in PBS/Tween containing 0.02% gelatin.

Adhesion molecules were detected using ELISA by incubating cells with a primary antibody to either ICAM-1 (1:3,000 ascites), VCAM-1 (0.1 μ g/ml), or E-selectin (0.1 μ g/ml). In the case of the ICAM-1 assay, cells were incubated with a peroxidase-conjugated goat anti-mouse secondary antibody. VCAM-1 and E-selectin antibodies were detected with the Vectastain ABC kit by incubating the cells with a biotinylated secondary antibody followed by avidin-conjugated peroxidase. Finally, the expression of ICAM-1, VCAM-1, and E-selectin was quantitated by the addition of the peroxidase substrate 2,2'-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid). The absorbance of each well was measured at 414 nm, and the background at 492 nm was subtracted. Within each experiment, wells containing endothelial cells were treated in quadruplicate with each dose of drug. At each dose, mean \pm SD was calculated.

Flow cytometry. HUVE cells were plated at confluent density onto 60-cm-diameter cell culture dishes. After overnight incubation, the growth medium was removed and replaced with basal medium with and without TNF and GSTM. After 24 h of incubation, the plates were washed with PBS, and the cells were detached with 5 mM EDTA. 10⁵ cells were incubated on ice with mAbs to ICAM-1 (1:4,000 ascites) and VCAM-1 (1 μ g/ml) for 30 min. Cells were washed twice with PBS containing 0.25% BSA and then treated on ice for half an hour with anti-mouse-FITC (1:50 dilution) secondary antibody. After two more washes cells were analyzed by flow cytometry (Coulter Corp., Hialeah, FL).

⁵¹Cr release assay. HUVE cells were plated at confluent density into the wells of gelatin-coated 96-well plates. After overnight incubation, cells were labeled with ⁵¹Cr by incubating with basal medium containing sodium chromate (320 kBq/ml) for 2 h. Each well was washed three times and then incubated with TNF \pm GSTM. After 24 h of incubation, the supernatant was sampled and counted in a γ -counter (LKB-Wallac, Turku, Finland). Maximal release (max counts) of ⁵¹Cr was assessed by the addition of 1% Triton X-100 detergent, while incubating with basal medium alone recorded minimum release (min counts). All treatments were performed in triplicate, and the percentage of ⁵¹Cr released was calculated from the means of the triplicates as follows:

Percent release

$$= 100 \times (\text{test counts} - \text{min counts}) / (\text{max counts} - \text{min counts}).$$

MTT cell viability assay. HUVE cells were incubated for 24 h with TNF and the highest concentration of each test drug as per cell ELISA. Cells were then washed with PBS and incubated 2–3 h with MTT (0.4 mg/ml) in basal medium (29). Cells were washed again with PBS, and formazan crystals were dissolved with 100 μ l DMSO. The absorbance of each well was measured at 540 nm. The effect of each drug on cell viability was calculated from the means of quadruplicates as follows:

$$\text{Percent viability} = 100 \times (\text{Test absorbance} / \text{Drug-free absorbance}).$$

Northern blot analysis. Endothelial cells were grown to confluence in 75-cm² flasks and treated in triplicate with TNF and either GSTM or TM at 0, 30, or 1,000 μ M in basal medium. One flask was treated with basal medium alone (no TNF). After 24 h of incubation, cells

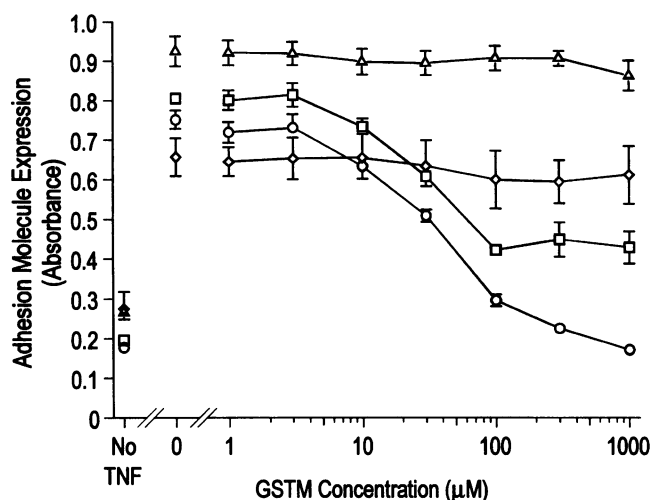


Figure 1. Adhesion molecule expression on HUVE cells incubated with TNF and GSTM. VCAM-1 expression after 24 h (○), E-selectin expression after 24 h (□), E-selectin after 6 h (△), and ICAM-1 expression after 24 h (◇) were measured using a cell ELISA. Mean ± SD was calculated from four replicate wells. Where SD is not shown, it is less than or equal to the size of the markers. The slope of the linear regression line, based on three independent experiments, was statistically significant for VCAM-1 and E-selectin after 24 h ($P < 0.0001$) but not for ICAM-1 or E-selectin after 6 h.

were removed using trypsin/EDTA, and total RNA was isolated using a guanidine thiocyanate-phenol-chloroform method based on that of Chomczynski and Sacchi (30).

15 µg of denatured total RNA was electrophoresed through a 1.4% agarose gel containing 1.25 M formaldehyde and then was transferred to GeneScreen nylon membrane and ultraviolet cross-linked (Stratalinker; Stratagene, La Jolla, CA). The replicate treatments were processed independently throughout the RNA isolation and electrophoresis.

DNA probes of VCAM-1, mouse β-actin, E-selectin, and ICAM-1 were labeled with [α - 32 P]dCTP by the random primer method and incorporated at least 10^9 cpm/µg. The membrane was prehybridized overnight in 5× sodium chloride sodium phosphate EDTA buffer (SSPE) (31), 2% SDS, 10% dextran sulfate, and 1 µg/ml sheared salmon sperm DNA at 65°C. The membrane was hybridized overnight with VCAM-1 ($> 10^6$ cpm/ml) in the prehybridization solution and was then washed at high stringency in $0.1 \times$ SSPE, 0.1% SDS at 65°C. Autoradiography and quantification of band intensities was performed using a Phosphor-Imager (Molecular Dynamics, Inc., Sunnyvale, CA). The same procedure was used to probe with actin, E-selectin, and then ICAM-1. To avoid overlapping bands, the membrane was stripped by boiling in 10 mM Tris, 1 mM EDTA, 1% SDS for a half an hour before probing with E-selectin and ICAM-1. The relative transcript accumulation for a given band was calculated from the ratio of counts in the band to counts in the actin band of that same lane.

Statistical analysis. All experiments examining the effect of a drug on adhesion molecule expression were performed at least twice. Results were obtained using HUVE cells derived from different individuals and gave very similar results. Therefore, one representative experiment is shown in the figures. However, data from replicate experiments were pooled before undertaking statistical analysis. Adhesion molecule expression was standardized using the formula:

Standardized expression

$$= (\text{Test Abs} - \text{Min Abs}) / (\text{Max Abs} - \text{Min Abs});$$

where Test Abs is mean absorbance (of quadruplicate wells) at test concentration of drug, Min Abs is mean absorbance (of quadruplicate

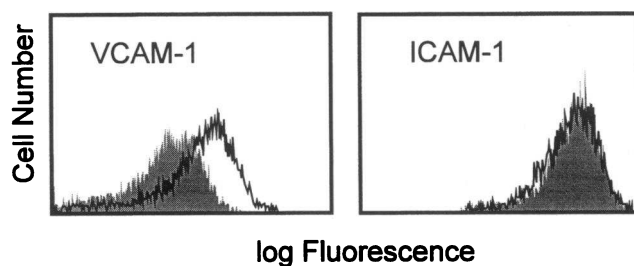


Figure 2. Flow cytometry profile of HUVE cells incubated for 24 h with TNF (black trace) or TNF + 1,000 µM GSTM (gray area). Indirect immunofluorescence was used to identify cells labeled with monoclonal antibodies to either VCAM-1 or ICAM-1.

wells) without TNF, and Max Abs is mean absorbance (quadruplicate wells) when drug concentration is zero.

Standardized measures of adhesion molecule expression were plotted against the \log_{10} of the drug concentration, and a linear regression line was fitted using least squares (32). A t test was used to determine if the slope of this regression line was significantly (i.e., $P < 0.05$) different from zero (32). A significant slope is evidence that the drug exerts a dose-dependent effect.

Results

The effect of GSTM on the cytokine-stimulated expression of endothelial adhesion molecules was investigated using a cell ELISA (Fig. 1). Incubation of endothelial cells with TNF for 24 h increased the expression of ICAM-1, VCAM-1, and E-selectin. However, when GSTM was also added, VCAM-1 and E-selectin expression was suppressed in a concentration-dependent manner (Fig. 1). By contrast, the expression of ICAM-1 remained unaffected. TNF stimulation for 6 h also elevated E-selectin expression but no dose-dependent effect of GSTM was observed over this period (Fig. 1).

The cell ELISA method used to detect E-selectin and VCAM-1 differed slightly from that used for ICAM-1. To exclude this as the explanation for the difference between the effect of GSTM on VCAM-1 compared with ICAM-1, another assay system was evaluated. Flow cytometry was used to compare the effect of GSTM on VCAM-1 and ICAM-1 expression. Fig. 2 illustrates that 1,000 µM GSTM decreases VCAM-1 expression but not ICAM-1 expression, a finding consistent with the cell ELISA result.

To ascertain whether the effect of GSTM was specific for TNF-activated cells, we investigated the GSTM response of HUVE cells stimulated with IL-1 or IL-4. When IL-1 was used (in place of TNF) to stimulate adhesion molecule expression, GSTM again exhibited a selective dose-dependent inhibition of VCAM-1 and E-selectin, but not ICAM-1, expression after 24 h (Fig. 3). Similarly, GSTM inhibited the IL-4-stimulated expression of VCAM-1. IL-4 did not elevate ICAM-1 or E-selectin expression.

To determine which component(s) of GSTM was responsible for the inhibition of VCAM-1 and E-selectin expression, we examined the effect of GTG and TM. Like GSTM, GTG is an antirheumatic gold(I) compound, but the thiol ligand binding the gold is based on glucose rather than malic acid. D-pen was also examined because it is used clinically and like TM has a reduced thiol group. Fig. 4 illustrates that thiomalate exhibited

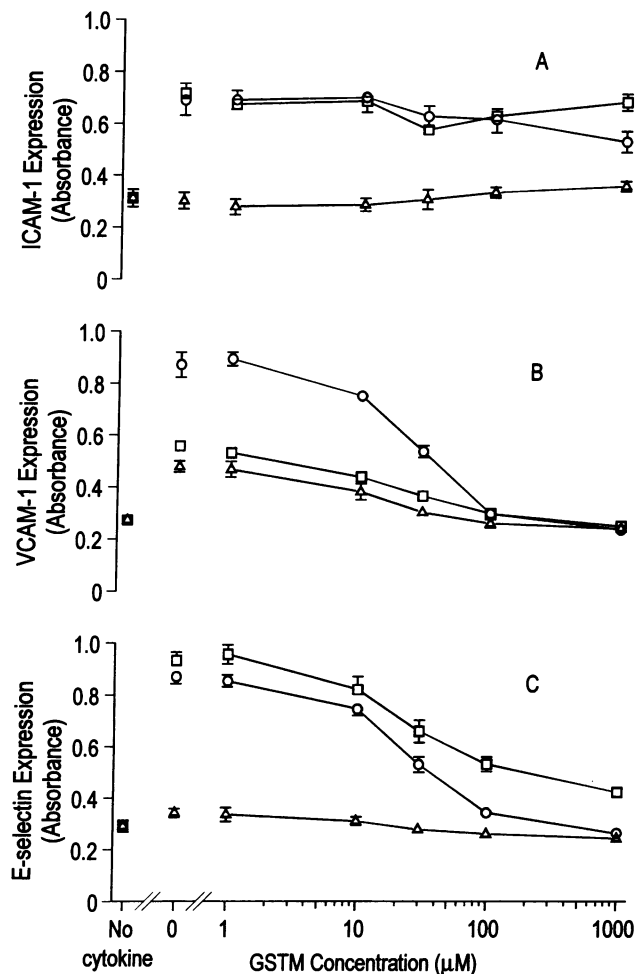


Figure 3. Adhesion molecule expression on HUVE cells incubated with GSTM and either TNF (○), IL-1 (□), or IL-4 (Δ) for 24 h. (A) ICAM-1 expression, (B) VCAM-1 expression, (C) E-selectin expression. Mean±SD was calculated from four replicate wells. Where SD is not shown, it is less than or equal to the size of the markers. The slope of the linear regression line, based on two independent experiments, was statistically significant when VCAM-1 expression was stimulated by TNF ($P < 0.005$), IL-1 ($P < 0.05$), or IL-4 ($P < 0.005$). GSTM also had a significant effect on E-selectin, but not ICAM-1, expression during TNF ($P < 0.0005$) or IL-1 ($P < 0.0005$) stimulation. IL-4 did not stimulate either ICAM-1 or E-selectin expression.

a similar effect to GSTM while D-pen and GTG showed no effect over the range of 1–1,000 μ M.

A range of other thiol compounds was studied in an attempt to elucidate the specificity and mechanism of GSTM and TM inhibition of VCAM-1 and E-selectin expression. Thioglycolate and thioglycolate were chosen as they are structurally related to TM. The possible importance of a reduced thiol group was further investigated using cysteine and cystine. Cysteine has a free SH group, while cystine is composed of two cysteine molecules joined by a disulfide bond. Thioglucose, which is related to GTG, was also evaluated.

Thioglycolate and cysteine had a modest inhibitory effect at high concentrations, while thioglucose had no significant effect on VCAM-1 or E-selectin expression (Fig. 5). Thioglycolate had a slight effect on VCAM-1 but not E-selectin expression.

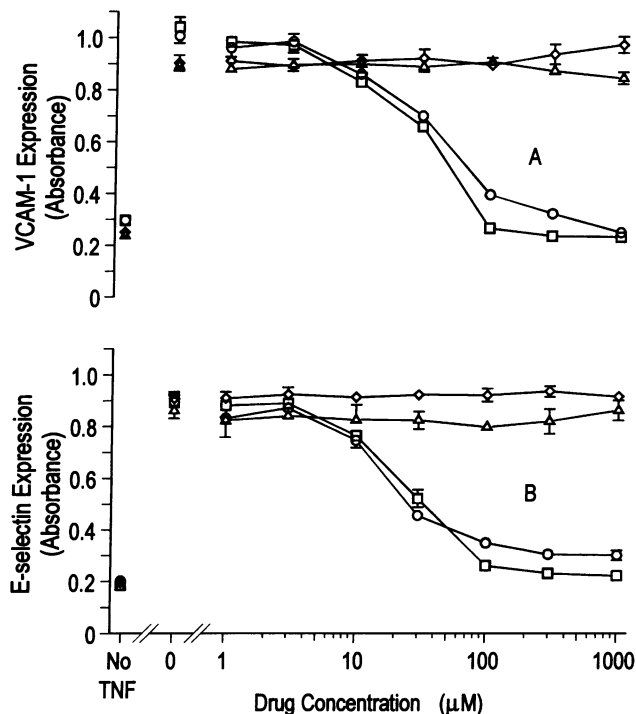


Figure 4. Adhesion molecule expression on HUVE cells incubated with TNF and either GSTM (○), TM (□), GTG (Δ), or D-pen (◇) for 24 h. (A) VCAM-1 expression, (B) E-selectin expression. Mean±SD was calculated from four replicate wells. Where SD is not shown, it is less than or equal to the size of the markers. The slope of the linear regression line, based on at least two independent experiments, was statistically significant for VCAM-1 and E-selectin with either GSTM or TM ($P \leq 0.0005$) but not significant for GTG or D-pen.

None of these thiol compounds approached the potency of TM in the inhibition of VCAM-1 and E-selectin expression.

The concentration range over which cystine could be studied was limited. Cystine (100 μ M) is an ingredient of the basal medium (ICN Biomedicals), and the solubility of cystine (26) limited the maximum useable concentration to 300 μ M. HUVE cells incubated for 24 h with TNF and 300 μ M cystine displayed significant ($P < 0.0001$, t test, $n = 4$) reductions in VCAM-1 (less 14%) and E-selectin (less 16%) expression relative to the basal concentration of 100 μ M cystine.

The effect of BSO (an inhibitor of glutathione synthesis) was studied to test the hypothesis that glutathione was involved in the inhibition of VCAM-1 and E-selectin expression by GSTM and TM. Over the concentration range of 0.2–200 μ M, the slope of the linear regression line, based on three independent experiments, was not significantly different from zero. In a typical experiment, the absorbance due to VCAM-1 expression ranged between 1.11 and 1.12, while the absorbance due to E-selectin ranged from 0.82 to 0.84.

Kinetic studies were undertaken to further evaluate the similarities in the effects of GSTM and TM on endothelial cell adhesion molecule expression. Fig. 6 illustrates the time course for the inhibition of VCAM-1 and E-selectin expression by 30 μ M GSTM or TM. In the absence of TNF and GSTM or TM, the background expression of the adhesion molecules remained low. In the presence of TNF alone, VCAM-1 expression reached a plateau at 12 h and remained elevated at 24 h. By contrast,

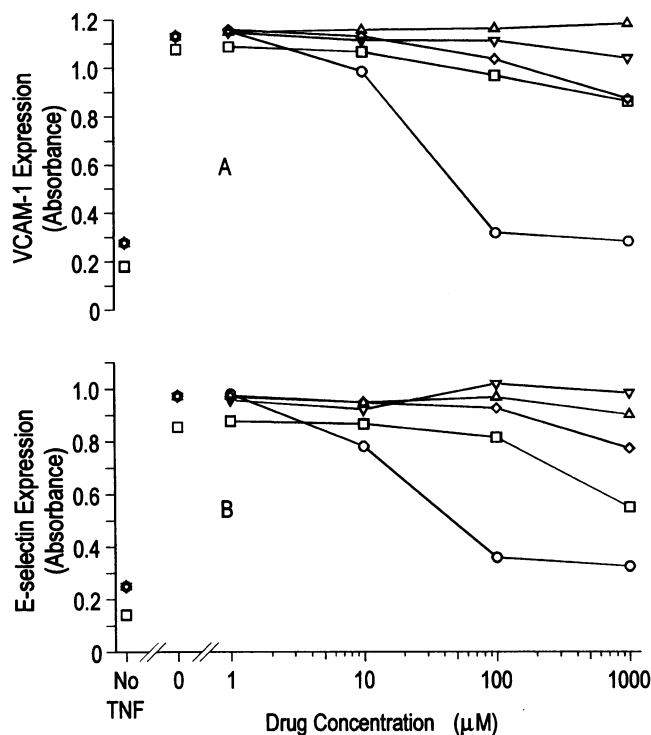


Figure 5. VCAM-1 (A) and E-selectin (B) expression on HUVE cells incubated for 24 h with TNF and either thiolactate (▽), thioglycolate (◇), TM (○), cysteine (□), or thioglycolate (Δ). Mean was calculated from four replicate wells. SD is not shown, to maintain clarity. The slope of the regression line, based on at least three independent experiments, was significant for TM ($P \leq 0.0005$) and thioglycolate ($P < 0.005$) but not for thioglycolate in both the VCAM-1 and E-selectin assays. Thiolactate had a statistically significant effect on VCAM-1 ($P < 0.005$) but not on E-selectin expression. Cysteine had a more significant effect on E-selectin ($P < 0.005$) than VCAM-1 ($P < 0.05$) expression.

E-selectin expression peaked between 6 and 12 h and then declined. When cells were incubated with TNF and either GSTM or TM, the expression of both VCAM-1 and E-selectin decreased compared with cells treated with TNF alone. This effect was noted at 6 h and became more marked by 12 h.

A ^{51}Cr release assay was used to investigate whether GSTM or TM had cytotoxic effects on endothelial cells that could explain the reduction in adhesion molecule expression. There was no significant difference ($P = 0.105$, ANOVA) in ^{51}Cr release between cells incubated with or without GSTM or TM (Fig. 7). An MTT cytotoxicity assay, which relies on the activity of mitochondrial dehydrogenases (29), recorded a 20% drop in cell viability at 1,000 μM GSTM or TM. 1,000 μM cysteine and 300 μM cystine both decreased viability by 10% relative to 0 μM concentrations. None of the other drugs investigated displayed any toxicity in the MTT assay.

Having established that GSTM and TM inhibited the expression of VCAM-1 and E-selectin protein, we investigated their effect on adhesion molecule mRNA levels. Northern blot analysis showed that endothelial cells stimulated with TNF had higher levels of ICAM-1, VCAM-1, and E-selectin mRNA than cells incubated without TNF (Fig. 8). The presence of 1,000 μM GSTM or TM reduced the (TNF-stimulated) levels of VCAM-1 and E-selectin mRNA to near basal levels but had no effect on ICAM-1 transcript. Band intensities were used to quantitate

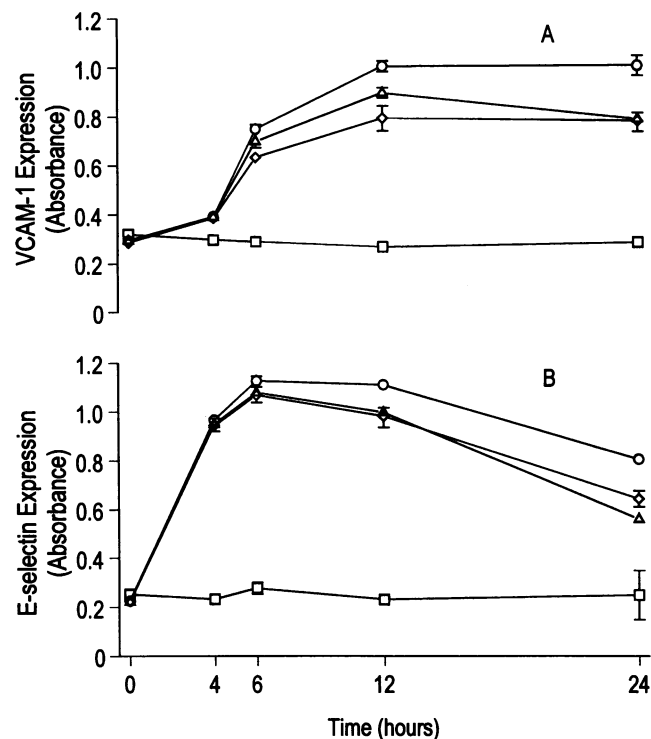


Figure 6. Kinetics of expression of VCAM-1 (A) and E-selectin (B) on HUVE cells incubated with TNF (○), TNF + 30 μM GSTM (Δ), TNF + 30 μM TM (◇), or basal medium alone (□). Mean \pm SD was calculated from three replicate wells. Where SD is not shown, it is less than or equal to the size of the markers.

mRNA species relative to the actin band (Fig. 9). Fisher's significant difference test (33) established that only at 1,000 μM GSTM or TM was VCAM-1 mRNA significantly ($P < 0.005$) reduced relative to the "TNF only" control. 30 μM GSTM or TM had no significant effect ($P > 0.05$) on VCAM-1 mRNA. In contrast, E-selectin transcript was significantly ($P < 0.005$) reduced by 30 μM concentrations. Incubation with 1,000 μM GSTM or TM resulted in a further significant ($P < 0.005$) reduction in E-selectin mRNA levels. ICAM-1 transcript accu-

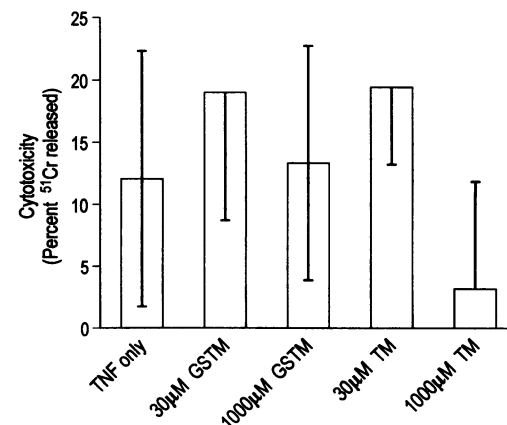


Figure 7. ^{51}Cr release assay of HUVE cells incubated for 24 h with TNF and 0, 30, and 1,000 μM concentrations of either GSTM or TM. Mean \pm SD was calculated from four replicate wells.

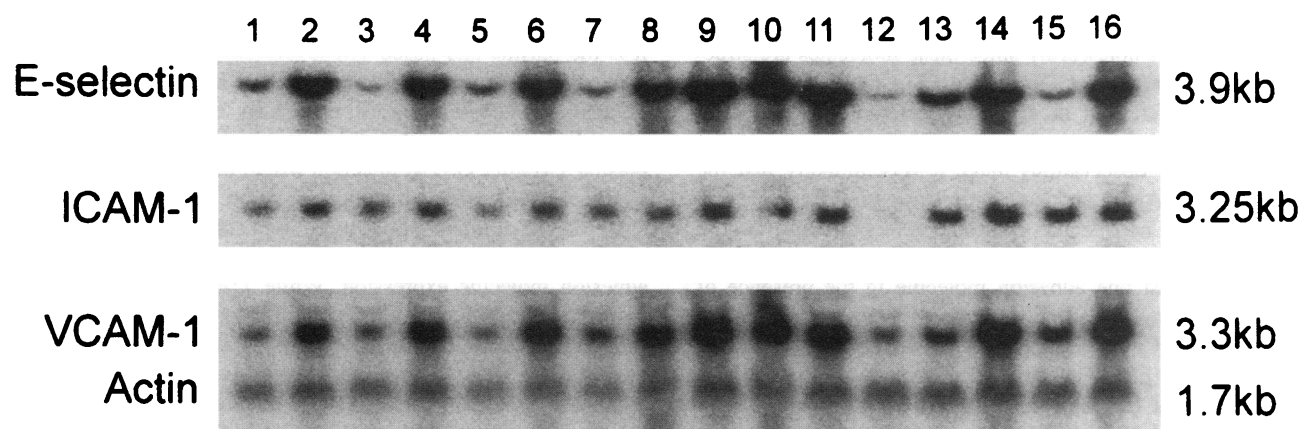


Figure 8. Northern blot analysis of HUVE cells incubated for 24 h with TNF and either GSTM or TM. The membrane was sequentially probed with VCAM-1, actin, E-selectin, and ICAM-1. Lanes 1, 5, and 13, TNF + 1,000 μ M TM; lanes 2, 6, and 14, TNF + 30 μ M TM; lanes 3, 7, and 15, TNF + 1,000 μ M GSTM; lanes 4, 8, and 16, TNF + 30 μ M GSTM; lanes 9–11, TNF only; and lane 12, basal medium alone.

mulation was not significantly affected by any of the concentrations of GSTM and TM tested. The effects of GSTM and TM on mRNA levels can be summarized: for VCAM-1 the effect of 1,000 μ M > 30 μ M \approx TNF only; for E-selectin 1,000 μ M > 30 μ M > TNF only; for ICAM-1 1,000 μ M \approx 30 μ M \approx TNF only.

Discussion

Many mechanisms have been proposed for the antirheumatic action of GSTM. Recent *in vivo* studies have suggested that GSTM may act by reducing endothelial adhesion molecule expression. Corkill et al. (16) found decreased E-selectin (ELAM-

1) expression on the synovial endothelium of rheumatoid arthritis patients treated with GSTM. However, it is unclear whether this is a direct effect of GSTM on synovial endothelium or an indirect effect by reducing inflammation and cytokine levels. We used an *in vitro* model to investigate the effect of GSTM on endothelial adhesion molecule expression and found that GSTM exhibited a selective and dose-dependent inhibition of VCAM-1 and E-selectin expression (Fig. 1). From this we conclude that GSTM can act directly on endothelial cells to inhibit VCAM-1 and E-selectin expression.

Cronstein et al. (15) observed that the inhibition of ICAM-1 and E-selectin by dexamethasone occurred with IL-1- and endotoxin-, but not TNF-, stimulated endothelial cells. We found that GSTM inhibited both the TNF- and IL-1-stimulated expression of VCAM-1 and E-selectin (Fig. 3). GSTM was also effective when IL-4 was used to selectively induce VCAM-1. This suggests that GSTM acts at a step common to the pathways by which these cytokines induce VCAM-1 and E-selectin expression. The effectiveness of GSTM, regardless of cytokine, is consistent with the hypothesis that the inhibition of adhesion molecule expression is a mechanism by which GSTM exerts some of its antiinflammatory properties *in vivo*.

GSTM dissociates into protein-bound gold and free thiomalate *in vivo* (23, 24). It is likely that a similar reaction occurs *in vitro*. The effect of GSTM on VCAM-1 and E-selectin expression could be due to the gold component, the thiomalate, or both. We demonstrated that TM, without gold, is sufficient to inhibit VCAM-1 and E-selectin expression (Fig. 4). As GTG showed no effect, we conclude that gold(I) is not required for the inhibition of adhesion molecule expression.

The thiol group is the chemical group that distinguishes GSTM and TM from most other compounds in the cell. To examine the importance of this group in the inhibitory activity of GSTM and TM on adhesion molecule expression, a number of other thiol compounds were tested. Neither D-pen, cysteine, cystine, thioglucose, thiolactate, nor thioglycolate at equimolar concentrations approached the degree of inhibition of VCAM-1 and E-selectin expression that is produced by TM (Fig. 5). Thus, the mere presence of a thiol moiety per se is unlikely to explain the effect of GSTM and TM.

The observation that thiolactate ($\text{CH}_3\text{CH}(\text{SH})\text{COO}^-$) and

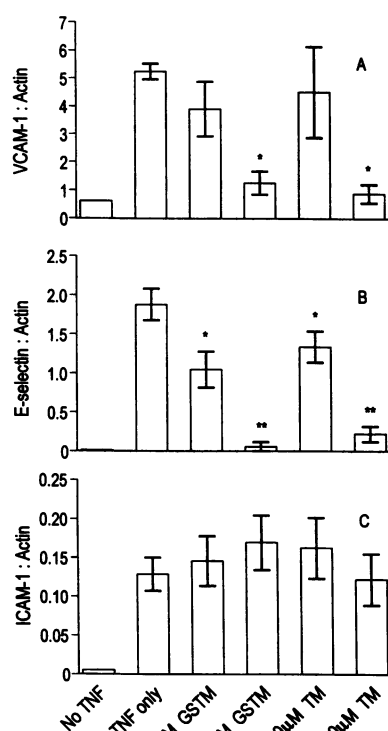


Figure 9. Analysis of band intensities on the Northern blot (Fig. 8). Relative transcript accumulation is expressed as the ratio of counts in the adhesion molecule band to counts in the actin band. Means \pm SD were calculated from triplicates, except in the case of No TNF where the single sample was omitted from any statistical analysis. Graphs show relative transcript accumulation for VCAM-1 (A), E-selectin (B), ICAM-1 (C). Fisher's least significant difference test was used to compare means within each graph. *Means are significantly different ($P < 0.005$) from the TNF only control. **Means are significantly below ($P < 0.005$) 30 μ M GSTM- or TM-treated cells.

thioglycolate ($\text{HSCH}_2\text{COO}^-$) had little effect on adhesion molecule expression is noteworthy since these molecules are structurally related to TM ($^- \text{OOCCH}(\text{SH})\text{CH}_2\text{COO}^-$). Thus, relatively small differences in chemical structure may result in marked biological differences.

A number of observations make it very unlikely that the inhibition of VCAM-1 and E-selectin expression by GSTM or TM is due to a nonspecific cytotoxic effect. Firstly, GSTM does not inhibit ICAM-1 expression. Thus, the cells are able to synthesize at least one adhesion molecule in the presence of GSTM. Secondly, TNF-stimulated endothelial cell viability, assessed using a ^{51}Cr release assay, was unaltered by GSTM or TM up to $1,000 \mu\text{M}$ (Fig. 7). An MTT assay was used as a third independent indicator of cell viability. A small drop in cell viability at $1,000 \mu\text{M}$ GSTM and TM was noted but is unlikely to account for the marked effect on VCAM-1 and E-selectin expression.

The inhibitory effect of GSTM and TM was further characterized by kinetic studies (Fig. 6). The time course of TNF-stimulated VCAM-1 expression is consistent with previous reports (2, 4). The effect of GSTM and TM is apparent after 12 h incubation, although there is a small difference in VCAM-1 expression after just 6 h between cells treated with TNF only and those with GSTM. Continuous TNF stimulation induced E-selectin expression to increase rapidly and peak between 6 and 12 h. E-selectin expression decreased to 60% of the peak level by 24 h. The prolonged E-selectin expression observed in our experimental system, compared with others (2), remains unexplained. After 6 h of incubation there is negligible difference between cells treated with and without GSTM despite E-selectin expression being maximal (Fig. 6). It is only after 12 and 24 h that the difference is evident. This suggests that there is a delay between the addition of GSTM or TM to the cells and the time when these drugs exert their effect. The entry of TM into cells is not rapid. Aguilar et al. (34) found that the intracellular concentration of thiomalate was only 15% of the 30 mM concentration in the medium after 1 h of incubation. When we pretreated endothelial cells by incubating for 24 h with GSTM or TM alone, and then washed the cells and incubated them with TNF for 6 h, a dose-dependent inhibition of E-selectin by GSTM and TM was observed (data not shown). Thus, by allowing TM and GSTM time to penetrate the cell and interact with its target molecule(s), we were able to demonstrate that these drugs inhibit E-selectin after 6 h of TNF stimulation.

Quantitative analysis of RNA isolated from HUVE cells showed that GSTM and TM inhibited VCAM-1 and E-selectin mRNA accumulation relative to actin mRNA (Fig. 9). ICAM-1 mRNA was unaffected. Thus GSTM and TM selectively reduce both the protein and mRNA accumulation of E-selectin and VCAM-1. We propose that the reduction in protein expression of these molecules is due, at least in part, to the decrease in their mRNA levels. Whether GSTM and TM act by inhibiting transcription or by promoting transcript degradation remains to be determined.

Conventional GSTM therapy results in serum gold concentrations in the range of $15\text{--}35 \mu\text{M}$ and is associated with a decrease in E-selectin expression on synovial endothelium. In our *in vitro* assay, $15 \mu\text{M}$ GSTM decreased E-selectin and VCAM-1 expression by 20 and 30%, respectively. Thus, it is plausible that GSTM exerts some of its antiinflammatory properties by decreasing VCAM-1 and E-selectin expression. However, as the decreased expression of adhesion molecules may

be attributable to TM and not the gold component of GSTM, it is the plasma and tissue TM levels that are relevant for this discussion. Plasma TM peaks at $0.5 \mu\text{M}$, a half hour after GSTM injection, and declines almost to zero by 2 h (24). From our *in vitro* data, it appears that the concentration and duration of plasma TM after GSTM injection would be insufficient to have an effect on adhesion molecule expression. However, intramuscular GSTM administration results in a substantial store of tissue-bound TM (25) that may be available to inhibit endothelial adhesion molecule expression. While TM is rapidly excreted during the first 6 h after GSTM administration, it then plateaus such that after 24 h only 60% of the injected dose has been voided (25). Thus, in rheumatoid arthritis, TM bound to cells within the synovium may be slowly released and may be available to reduce the expression of E-selectin and VCAM-1 on endothelial cells.

The observation that both GSTM and TM reduce endothelial adhesion molecule expression suggests that TM may be an effective antirheumatic compound. Previous workers have cited the structural similarities of TM to D-pen and the fact that GSTM dissociates into TM *in vivo* as supporting evidence (24). A preliminary trial of oral TM for the treatment of rheumatoid arthritis was very encouraging (35). However, these results have not been published as a complete paper. Furthermore, when the trial was independently repeated, oral TM was found to be ineffective and to cause adverse side effects (36). No trials involving parenteral administration of TM (analogous to GSTM therapy) have been reported. It may be that the route of delivery, and/or the association with gold, is critical for TM to decrease adhesion molecule expression *in vivo*.

TM is known to be a potent inhibitor of the enzyme glutathione peroxidase, as is D-pen, in a cell-free system (37). However, we observed that D-pen did not inhibit the expression of any of the adhesion molecules tested. Thus, assuming D-pen can penetrate the cell membrane to a similar degree as TM, it is unlikely that TM inhibits VCAM-1 expression by blocking glutathione peroxidase. In a further experiment, we evaluated the effect of BSO (an inhibitor of glutathione synthesis) on endothelial adhesion molecule expression. $200 \mu\text{M}$ BSO eliminates glutathione, the substrate for glutathione peroxidase, after 24 h of incubation (38). Thus, by eliminating its substrate, the activity of glutathione peroxidase within the cells can be indirectly inhibited. However, we observed that $0.2\text{--}200 \mu\text{M}$ BSO had no effect on VCAM-1 or E-selectin expression on TNF-stimulated endothelial cells. Thus, it is unlikely that the inhibition of VCAM-1 and E-selectin by TM is due to the inhibition of glutathione peroxidase.

A possible mechanism for the inhibition of adhesion molecule expression involves the second messenger cAMP. Elevated cAMP, induced by forskolin or isobutyl methylxanthine, inhibits VCAM-1 and E-selectin but not ICAM-1 expression on TNF-stimulated endothelial cells (39). The similarity of this effect, to that which we observed with GSTM, suggests that GSTM may inhibit adhesion molecule expression by increasing cAMP in endothelial cells. In fact, Scheinberg et al. (20) observed increased cAMP in monocytes incubated *in vitro* with GSTM and suggested that this may be responsible for inhibited chemotaxis. However, in contrast, Lazarevic et al. (40) observed that GSTM inhibited the activity of adenylate cyclase from lymphocyte membranes. This would result in decreased intracellular cAMP. Thus, to resolve whether or not cAMP is involved in the inhibition of VCAM-1 and E-selectin by GSTM, it would

be necessary to determine the effect of GSTM on endothelial cAMP and to correlate this with adhesion molecule expression.

In summary, we have demonstrated that GSTM inhibits the in vitro endothelial expression of VCAM-1 and E-selectin, but not ICAM-1. We propose that this inhibition is due to the thiomalate and not the gold component of GSTM and involves the reduction of mRNA for VCAM-1 and E-selectin. The decrease in endothelial adhesion molecule expression by GSTM may reduce the recruitment of leukocytes to rheumatoid synovium and thus may contribute to the antiinflammatory effects of this compound.

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