Immunomodulatory Effects of Therapeutic Gold Compounds

Gold Sodium Thiomalate Inhibits the Activity of T Cell Protein Kinase C

Keisuke Hashimoto,* Charles E. Whitehurst,* Tsukasa Matsubara,‡ Kazushi Hirohata,‡ and Peter E. Lipsky*

**The Harold C. Simmons Arthritis Research Center, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75235; and ‡Department of Orthopedic Surgery, Kobe University School of Medicine, Kobe 650, Japan*

Abstract

Previous studies have shown that the gold compounds, gold sodium thiomalate (GST) and auranofin (AUR), which are effective in the treatment of rheumatoid arthritis, inhibit functional activities of a variety of cells, but the biochemical basis of their effect is unknown. In the current studies, human T cell proliferation and interleukin 2 production by Jurkat cells were inhibited by GST or AUR at pharmacologically relevant concentrations. Because it has been documented that protein kinase C (PKC) is involved in T cell activation, the capacity of gold compounds to inhibit PKC partially purified from Jurkat cells was assayed in vitro. GST was found to inhibit PKC in a dose-dependent manner, but AUR caused no significant inhibition of PKC at pharmacologically relevant concentrations. The inhibitory effect of GST on PKC was abolished by 2-mercaptoethanol. To investigate the effect of GST on the regulation of PKC in vivo, the levels of PKC activity in Jurkat cells were examined. Cytosolic PKC activity decreased slowly in a concentration- and time-dependent manner as a result of incubation of Jurkat cells with GST. To ascertain whether GST inhibited PKC translocation and down-regulation, PKC activities associated with the membrane and cytosolic fractions were evaluated after phorbol myristate acetate (PMA) stimulation of GST incubated Jurkat cells. Translocation of PKC was markedly inhibited by pretreatment of Jurkat cells with GST for 3 d, but the capacity of PMA to down-regulate PKC activity in Jurkat cells was not altered by GST preincubation. The functional impact of GST-mediated downregulation of PKC in Jurkat cells was examined by analyzing PMA-stimulated phosphorylation of CD3. Although GST preincubated Jurkat cells exhibited an increased density of CD3, PMA-stimulated phosphorylation of the γ chain of CD3 was markedly inhibited. Specificity for the inhibitory effect of GST on PKC was suggested by the finding that GST did not alter the mitogen-induced increases in inositol trisphosphate levels in Jurkat cells. Finally, the mechanism of the GST-induced inhibition of PKC was examined in detail, using purified PKC subspecies from rat brain. GST inhibited type II PKC more effectively than type III PKC, and also inhibited the enzymatic activity of the isolated catalytic fragment of PKC. The inhibitory effect of GST on PKC activity could not be explained by competition with phospholipid or nonspecific interference with the substrate. These data suggest that the immunomodulatory effects of GST may result from its capacity to inhibit PKC activity. (*J. Clin. Invest.* 1992 89:1839–1848.) Key words: anti-rheumatic drugs • gold compounds • protein kinase C • rheumatoid arthritis • T cells

Introduction

Gold compounds have been employed as therapeutic agents for rheumatoid arthritis (RA)[1] for many years. The most commonly used drugs are water-soluble, parenterally administered salts of gold thiol complexes, such as gold sodium thiomalate (GST) and gold sodium thioglucose (ATG), and the orally active agent, auranofin (AUR) [(2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-S) (triethylphosphine) gold (I)]. A characteristic feature of therapy with these drugs is a delayed onset of clinical effect, a decrease in acute phase reactants and, possibly, an ability to modify the course of the disease (1, 2).

Based on various effects of gold compounds in a number of different model systems, a number of mechanisms have been proposed to explain the mechanisms of action of gold compounds in RA, but none has been generally accepted as the mechanism by which gold compounds alter the course of RA. The possibility that gold compounds may suppress immune responsiveness was suggested by the clinical observations that immunoglobulin levels (3) and rheumatoid factor titers (4) often decrease in patients treated with gold compounds. In addition, gold therapy has been shown to cause a reduction in the number of circulating lymphocytes in patients with RA (5). The capacity of gold compounds to suppress immune responses has been confirmed with in vitro studies. These drugs were found to inhibit antigen- and mitogen-induced proliferation of human lymphocytes indirectly by inhibiting the accessory function of monocytes (6). A variety of other functions of monocytes are also inhibited by gold compounds, including their capacity to produce superoxide anions (7) and complement components after activation (8). In addition, gold compounds have been shown to inhibit the differentiation of monocytes into effector cells (9). The effects of gold compounds are not uniquely directed to monocytes, however, in that inhibition of endothelial cell and lymphocyte proliferation has also been observed (10, 11). Gold compounds have also been observed to inhibit a variety of enzymatic and cellular processes, but many of these effects require concentrations of gold that are much greater than those achieved by in vivo administra-

Address reprint requests to Dr. Lipsky, Department of Internal Medicine, Simmons Arthritis Research Center, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8884.

Received for publication 22 April 1991 and in revised form 9 January 1992.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/92/06/1839/10 $2.00
Volume 89, June 1992, 1839–1848

1. *Abbreviations used in this paper:* AUR, auranofin; GST, gold sodium thiomalate; IP_3, inositol trisphosphate; 2-ME, 2-mercaptoethanol; PKA, PKC, and PKM, protein kinase A, C, and M, respectively; RA, rheumatoid arthritis; TMA, thiomalic acid.

tion. Of importance, the biochemical basis for these various cellular effects has not been identified.

The following studies were undertaken to identify a biochemical basis for the various cellular effects of gold compounds. Because many of the cellular activities altered by gold compounds are associated with inositol phospholipid breakdown, protein kinase C (PKC) activation, and mobilization of internal Ca^{2+} stores, it seemed reasonable that gold compounds might inhibit the generation or activity of one of these second messengers. Inasmuch as induction of PKC activity is involved in many cellular activation event, the current studies explored the possibility that gold compounds might be active as a result of interference with the action of this critical enzyme. Previous reports have suggested that AUR might inhibit PKC activity isolated from platelets (12) or PKC-mediated cell responses of neutrophils (13). However, there are a number of concerns about these studies, including the concentrations of AUR employed and the failure to use a specific substrate for PKC for analysis of enzymatic activity, that question the relevance and specificity of the findings. Moreover, in one of the previous studies, GST was not found to have a significant effect on PKC activity in vivo (13), suggesting that the effects noted with AUR may not be characteristic of all therapeutic gold compounds. In the light of these issues, the current studies sought to determine whether gold compounds interfere with the activity of PKC in vitro and in vivo. The results clearly show that modulation of PKC is a mechanism by which GST and gold compounds other than AUR may exert their immunosuppression and anti-inflammatory effects in patients with RA.

Methods

Chemical compounds. The following compounds were utilized: GST (Merck Sharp & Dohme, Rahway, NJ), gold sodium thioglucose (Sigma Chemical Co., St. Louis, MO) AUR (Smith Kline & French Laboratories, Philadelphia, PA), thiomalic acid (TMA; Merck Sharp & Dohme), and 2-mercaptoethanol (2-ME, Eastman Kodak Co., Rochester, NY).

Miscellaneous reagents. Several MAbs were used in these studies, including OKT8 (American Type Culture Collection [ATCC], Rockville, MD), an IgG2 MAb directed at the CD8 molecule on the suppressor/cytotoxic T cell subset; L243 (ATCC), an IgG2a MAb directed at monomorphic HLA-DR determinants (14); OKT3 (ATCC), an IgG2a MAb directed to the CD3 complex on mature T cells (15); 64.1, an IgG2a MAb directed to the CD3 complex on mature T cells (16); and W6/32, an IgG2a MAb directed at class I encoded gene products (17). Recombinant IL-2 was obtained from Hoffmann-La Roche Inc., Newark, NJ. Homogeneous calpain 2 was prepared from rat kidney by the method of Yosimura et al. (18). Calf thymus H1 histone was prepared by the method of Oliver et al. (19). Phosphatidyl serine (bovine brain) and diolein were purchased from Serdary Research Laboratories, Port Huron, MI. Protamine sulfate (histone free) was purchased from Sigma Chemical Co. [γ-^{32}P]ATP was obtained from Amersham Corp., Arlington Heights, IL. [^{32}P]Orthophosphoric acid was purchased from ICN Radiochemicals, Irvine, CA. Phytohemagglutinin (PHA) was purchased from Wellcome Research Laboratories, Beckenham, England.

Culture medium. All cultures were carried out in medium RPMI 1640 (Hazelton Biologics Inc., Lenexa, KS) supplemented with 0.3 mg/ml fresh glutamine, 10 μg/ml gentamicin, and 200 U/ml penicillin G. The medium was further supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY).

Cell preparation. The human T cell leukemia line Jurkat was maintained in RPMI medium supplemented with 10% FBS. Venous blood was obtained from healthy adult volunteers and peripheral blood mononuclear cells (PBMC) were obtained by centrifugation over sodium diatrizoate/ficoll gradients (Sigma Chemical Co.) (20). PBMC were deleted of monocytes and natural killer (NK) cells by incubation with 5 mM L-leucine methyl ester HCl (Sigma Chemical Co.) in serum-free RPMI 1640 as described (21, 22). After washing twice, T cells were purified (23) by passing the cells that formed rosettes with neuraminidase-treated sheep red blood cells over a nylon wool column. HLA-DR–depleted $CD4^+$ T cells were purified by a panning technique (24) after reacting the T cell population with the MAb OKT8 and L243. Viability always exceeded 96%.

Technique of mitogen-induced $CD4^+$ T cell DNA synthesis. $CD4^+$ T cell cultures were carried out in 96-well round-bottomed microtiter plates, with each well containing 1×10^5 responding cells in 0.2 ml of culture medium. The cells were incubated for 72 h at 37°C with OKT3 (1 μg/ml) and PMA (10 ng/ml) as a mitogenic stimulus. 1 μCi [^{3}H]thymidine (6.7 Ci/mM; New England Nuclear, Boston, MA) was added for the last 6 h. The cells were harvested onto glass fiber filter paper, and [^{3}H]thymidine incorporation was determined by liquid scintillation spectroscopy.

IL-2 activity. Jurkat cells were cultured at a density of 2×10^5 in 0.2 ml of culture medium in 96-well flat-bottomed microtiter plates for various time intervals at 37°C with OKT3 (100 ng/ml) and PMA (10 ng/ml) as a mitogenic stimulus. The supernatants were harvested, and the IL-2 activity was assayed by using the IL-2–dependent mouse cell line CTLL-2 as described (25).

Partial purification of PKC from Jurkat cells. Jurkat cells ($\sim 1 \times 10^7$ cells) were suspended in 1 ml of 20 mM Tris–HCl (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA, 1 mM EDTA, 1 mM PMSF, and 100 μg/ml leupeptin. The cells were lyzed by sonication using three 15-s bursts, and centrifuged at 100,000 *g* for 30 min. The supernatant from this centrifugation was designated "cytoplasmic fraction." The pellet was resonicated in 1 ml of the same buffer containing 1% (vol/vol) Triton X-100 and recentrifuged as above. The supernatant from this centrifugation was designated "particulate fraction." These crude fractions were separately applied to a 1-ml DE-52 column equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.5 mM EDTA, 0.5 mM EGTA, and 10 mM 2-ME. After washing with two column volumes of the same buffer, PKC was eluted batchwise with three column volumes of the buffer containing 300 mM NaCl.

Immunofluorescence and flow cytometry. Jurkat cells ($2–5 \times 10^5$ per sample) were stained with saturating concentrations of anti–CD3 (64.1), anti–class I MHC (W6/32), or control MAb (P117) and incubated at 0°C for 45 min. The cells were then washed in cold PBS containing 1% normal human serum and incubated with FITC-goat anti–mouse immunoglobulin for an additional 30–45 min. Cell-associated fluorescence was then analyzed by flow microfluorimetry.

Immunoprecipitation and detection of protein phosphorylation. Control and GST preincubated Jurkat cells were biosynthetically labeled with [^{32}P]orthophosphoric acid. To accomplish this, cell samples were washed three times in 20 mM Tris–HCl, pH 7.5, containing 0.15 M NaCl and resuspended (40×10^6/ml) in phosphate-free RPMI medium. After 60 min, 60×10^6 cells were incubated with 1.0 mCi/ml of [^{32}P]orthophosphoric acid in the same medium at 37°C for 60 min. The cells were then stimulated with PMA (3 ng/ml) at 37°C for 10 min. After PMA stimulation, the cells were washed and solubilized with a lysis solution containing 1% nonidet P40 in 20 mM Tris-HCl buffer, pH 7.5, 50 mM NaF, 1 mM EGTA, 1 mM Na vanadate, 20 mM *p*-nitrophenylphosphate, 100 μg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 mM iodoacetamide and were allowed to stand on ice for 10 min. After centrifuging to remove insoluble material, lysates were precleared with pansorbin (Calbiochem-Behring Corp., San Diego, CA) for 1 h at 4°C. Precleared lysates were precipitated with monoclonal antibody to CD3 or class I MHC, covalently coupled to protein A-agarose (Bethesda Research Laboratories, Gaithersburg, MD). The antibody-agarose complexes were incubated at 4°C with the detergent lysate for 60 min. The resins were then washed four times with the buffer as above but containing 0.2% nonidet P40 and 150 mM NaCl. The associated ^{32}P-labeled proteins were eluted from the beads

by boiling for 5 min in Laemmli reducing buffer, and then analyzed by SDS-PAGE on a 15% gel run under reducing conditions.

Purification of PKC subspecies. PKC subspecies were purified from the soluble fraction of rat brain, and separated into three distinct fractions, type I, type II, and type III, by hydroxyapatite column chromatography as described (26). The PKC preparations were pure on SDS-PAGE.

Assay of PKC. Using a PKC enzyme assay system (Amersham Corp.), PKC was assayed with synthetic peptide as a phosphate acceptor in the presence of 0.67 mol% phosphatidylserine, 2 μg/ml PMA, and 0.1 mM Ca^{2+} as described (26). Dithiothreitol was not used. Control incubations were performed by replacing Ca^{2+} with 0.5 mM EGTA and without addition of lipids.

To measure the activity of purified PKC subspecies, myelin basic protein residues 4 through 14 (MBP_{4-14}, kindly provided by Dr. Kishimoto, Kobe University School of Medicine, Kobe, Japan) was used (27). The reaction mixture (0.05 ml) contained Tris-HCl at pH 7.5, 25 μM MBP_{4-14}, 2.5 nmol [γ-^{32}P]ATP, 1.25 μmol magnesium acetate, 25 nmol $CaCl_2$, 8 μg of phosphatidyl serine, 0.8 μg/ml diolein, and PKC to be assayed (26).

Purification of protein kinase M and cyclic AMP-dependent protein kinase. When PKC was subjected to calpain, a catalytic fragment (protein kinase M, PKM) was produced that was not affected by Ca^{2+}, phospholipid, or diacylglycerol (28). After this fragment was produced, it was purified using a TSK gel DEAE-5PW column, and assayed with MBP_{4-14} as substrate in the presence of 0.5 mM EGTA instead of Ca^{2+}, phospholipid, and diacylglycerol. Cyclic AMP-dependent protein kinase A (PKA) was partially purified from rabbit skeletal muscle and was assayed as described (29).

Determination of inositol trisphosphate (IP_3) production in Jurkat cells. Jurkat cells were incubated for 72 h in the presence or absence of GST at 20 μg/ml. For the last 12 h, the cells were incubated in serum-free medium but still in the presence or absence of GST. This was done to decrease the baseline level of IP_3. The cells were then incubated for various lengths of time at 37°C with PHA at 4 μg/ml and 10 mM LiCl. PHA was used as a stimulus in these studies because it induced larger increases in IP_3 content than other stimuli. The incubation was terminated by the addition of ice-cold 15% TCA. Samples were then centrifuged at 12,000 *g* for 15 min at 4°C and the supernatants were extracted with water-saturated diethyl ether and pH was neutralized by the addition of sodium bicarbonate, pH 8.5. IP_3 levels were then measured by competitive binding assay using a kit obtained from a commercial source (Amersham Corp.).

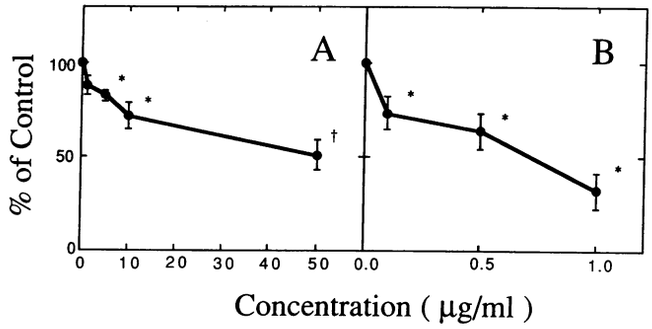

Figure 1. Gold compounds inhibit the proliferative response of CD4+ T cells to OKT3 and PMA. CD4+ T cells (1×10^5 per well) were incubated with 10 ng/ml PMA and 1 μg/ml OKT3, or an equal volume of medium as control for 72 h at 37°C in the presence of (*A*) GST or (*B*) AUR at the indicated concentrations, and assayed for [^{3}H]-thymidine incorporation. Each point represents the mean±SEM of four separate experiments. Student's *t* test of each point was performed by comparison with the control. $^*P < 0.05$; $^\dagger P < 0.01$ compared to control.

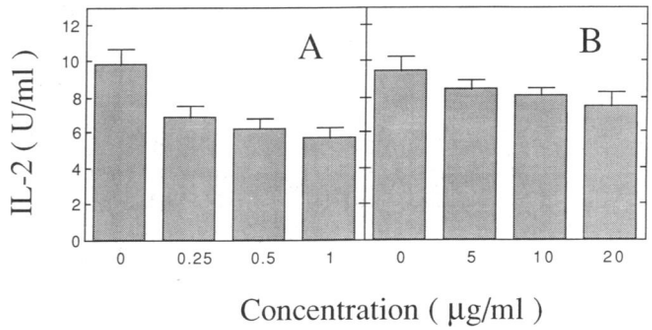

Figure 2. Gold compounds inhibit IL-2 production by Jurkat cells. Jurkat cells (2×10^5 per well) were incubated with 10 ng/ml PMA and 100 ng/ml OKT3 in the presence of (*A*) AUR or (*B*) GST at the indicated concentration, or an equal volume of medium as control for 24 h at 37°C. Culture supernatants were harvested and assayed for IL-2 activity with CTLL-2 cells. Each point represents the mean±SEM of four separate experiments.

Results

GST and AUR inhibit CD4$^+$ T cell proliferation. GST or AUR inhibited the proliferative responses of CD4$^+$ T cells induced by PMA and OKT3 in a dose-dependent manner as shown in Fig. 1. The degree of suppression observed was dependent on the concentration of GST or AUR, with significant inhibition seen with 5 and 0.1 μg/ml, respectively. Incubation of CD4$^+$ T cells with these concentrations of drugs did not significantly affect their viability. To determine whether other gold compounds also inhibited CD4$^+$ T cells, we also examined the effect of ATG on CD4$^+$ T cell proliferation. Significant inhibition was observed at a concentration of 5 μg/ml (data not shown). By contrast, TMA, the ligand of GST, did not exert a significant inhibitory effect on CD4$^+$ T cell proliferation at concentrations between 1 and 50 μg/ml (data not shown).

GST and AUR inhibit IL-2 production by Jurkat cells. Jurkat cells were activated with PMA and OKT3 in the presence or absence of gold compounds and the amount of IL-2 produced was measured. As the results demonstrate (Fig. 2 *A*), AUR inhibited IL-2 production by Jurkat cells. Compared with AUR, GST was much less potent in inhibiting IL-2 production (Fig. 2 *B*). In that previous reports have shown that prolonged incubation of cells with GST may be necessary for an inhibitory effect to become apparent (6), Jurkat cells were incubated with GST for 72 h to determine whether this would inhibit their subsequent capacity to produce IL-2. As can be seen in Fig. 3, a 72-h preincubation with GST markedly inhibited the capacity of Jurkat cells to produce IL-2 in response to PMA and OKT3. IL-2 production was inhibited in a concentration-dependent manner with significant inhibition observed with concentrations of GST as small as 10 μg/ml. These findings could not be explained by nonspecific toxic effects of GST because each population manifested similar viability as assessed by trypan blue exclusion. Moreover, GST had no effect on the growth of Jurkat cells during the 72-h preincubation (data not shown).

Effect of gold compounds on partially purified PKC. Direct evidence that gold compounds interfered with signal transduction at the level of PKC was obtained by analyzing the effect of GST on the activity of PKC partially purified from Jurkat cells. Results illustrated in Fig. 4 *A* demonstrate that GST directly

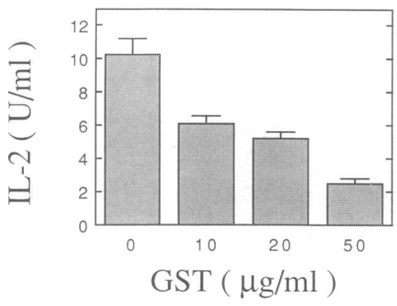

Figure 3. GST inhibits IL-2 production by Jurkat cells. Jurkat cells (2×10^5 per well) were incubated with GST at the indicated concentration. After a 72-h incubation at 37°C, the cells were washed three times, exposed to 10 ng/ml PMA and 100 ng/ml OKT3. After an additional 24-h incubation at 37°C without GST, culture supernatants were harvested and assayed for IL-2 activity using CTLL-2 cells. Each point represents the mean±SEM of four separate experiments.

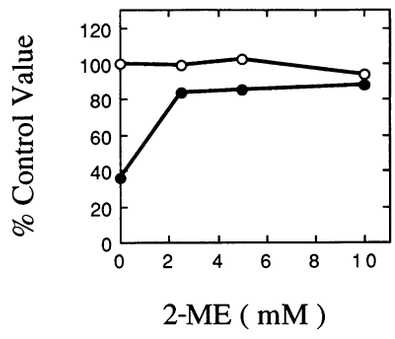

Figure 5. Influence of 2-ME on PKC activity in the presence or absence of GST. PKC partially purified from Jurkat cells was assayed as described in Methods with 2-ME at the indicated concentration in the presence (●) or absence (○) of GST at 20 μg/ml. PKC activity without GST and 2-ME was taken as control (100% value). Points are means of duplicate assays.

inhibited PKC in a dose-dependent manner, whereas TMA had no significant effect on PKC activity. By contrast to the action of GST, AUR exhibited only minimal effects on PKC activity (Fig. 4 *B*). The inhibitory effect of GST on PKC activity was abolished by 2-ME at concentrations as low as 2.5 mM (Fig. 5). Therefore, the inhibitory effect of GST on PKC activity may be related to an interaction with a sulfhydryl group on the PKC molecule.

Effect of GST on the enzymatic activity of PKC in intact Jurkat cells. The next experiments were carried out to determine whether CST altered PKC activity in intact Jurkat cells. Initially the effect of GST preincubation on cytosolic PKC activity was examined and the results are shown in Fig. 6. Cytosolic PKC activity in Jurkat cells decreased in a concentration- and time-dependent manner during the incubation with GST. To ascertain whether GST also affected PMA-induced PKC translocation, PKC activity in the particulate and cytosolic fractions isolated from cells preincubated with GST was measured. After a 72-h preincubation with 20 μg/ml GST, the cells were washed extensively and treated with PMA. Fig. 7 shows the time course of translocation of PKC in Jurkat cells in response to 10 ng/ml PMA. At time 0 in control cells (Fig. 7 *A*), ~ 90% of the PKC activity was found in the cytosolic fraction. There was little or no change in the enzyme levels in the cytosolic and membrane fraction for the entire 2-h incubation time in the absence of PMA (data not shown). In the presence of PMA, however, PKC activity in the cytosolic fraction decreased by ~ 55% after 30 min. The rapid decrease in activity of PKC in the cytosol was associated with a significant increase in activity in the membrane fraction, which reached a peak within 30 min. During the 2-h incubation, total PKC activity in the cells did not change. PMA also caused translocation of the residual PKC activity in GST-preincubated Jurkat cells (Fig. 7 *B*). However, the initial velocity of translocation was decreased and the percentage of PKC translocated was less.

In contrast, PMA-induced down-regulation of PKC was not significantly affected by preincubation of Jurkat cells with GST. After a 72-h preincubation with GST, the cells were washed extensively and treated with PMA at 100 ng/ml. As can be seen in Fig. 8, activation of PKC with high concentrations of PMA altered the levels of total PKC activity in control Jurkat cells in a time-dependent manner. The decrease in the enzyme activity in GST-pretreated cells was comparable.

Inhibitory effect of GST on phosphorylation of the γ and δ chains of CD3. It has been reported that PMA can induce phosphorylation of the γ and δ subunit of the CD3 complex (30, 31). The next experiments were therefore carried out to determine whether GST-mediated downregulation of PKC inhibited the phosphorylation of the CD3 complex. Initial experiments examined whether GST affected the cell surface expression of CD3. To accomplish this, Jurkat cells were treated with GST at 20 μg/ml for 3 d and CD3 expression was monitored by indirect immunofluorescence using anti-CD3 mAb. The expression of CD3 by Jurkat cells was enhanced as a result of the incubation with GST (Fig. 9). The enhancing effect of GST on CD3 expression was dependent on both the concentration of

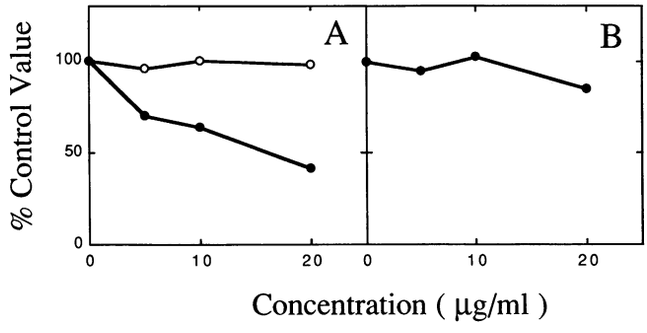

Figure 4. Effect of GST and AUR on the activity of PKC isolated from Jurkat cells. PKC was partially purified and assayed as described in Methods (*A*) in the presence of GST (●) or in the presence of TMA (○); (*B*) in the presence of AUR (●). PKC without GST, TMA, or AUR was taken as control (100% value). Points are means of duplicate assays.

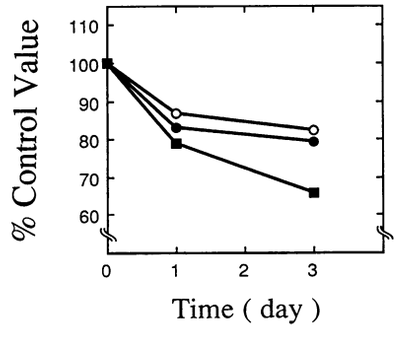

Figure 6. Effect of GST on cytosolic PKC activity of Jurkat cells. Jurkat cells were preincubated for 24 and 72 h with GST at 10 μg/ml (○), 20 μg/ml (●), and 50 μg/ml (■) before cellular disruption and ultracentrifugation. The 100,000-*g* supernatant was partially purified and assayed as described in Methods. Each point represents the mean±SEM of three experiments and is expressed as percentage of control activity found in cells that had not been preincubated with GST.

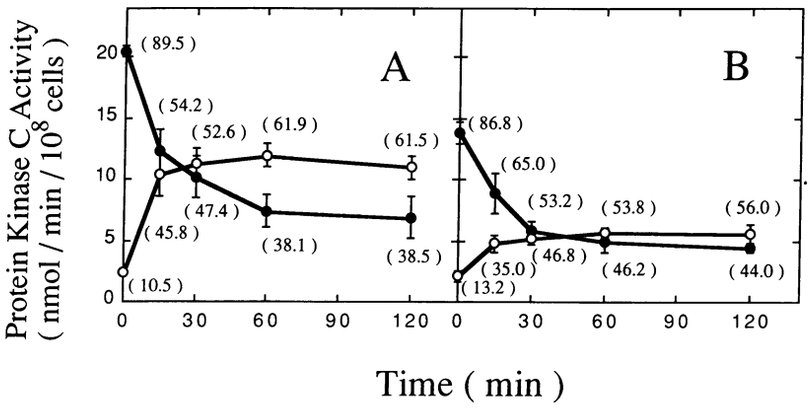

Figure 7. Time course of PKC translocation in Jurkat cells. Jurkat cells were preincubated in the absence (*A*) or presence (*B*) of 20 μg/ml GST for 3 d. At the end of the incubation, the cells were washed with PBS, suspended in fresh medium containing 10% FBS, and the cells were treated with 10 ng/ml PMA at 37°C for the times indicated, and cellular fractions were prepared and assayed as described in Methods. The PKC activity in the soluble (●) or particulate (○) fractions is shown. Each point represents the mean±SEM of four experiments. Numbers in parentheses indicate percentage of total cellular PKC activity in each fraction at each time point.

GST and the length of incubation, with a significant effect observed with concentrations of GST as small as 10 μg/ml after a 24-h incubation (data not shown). By contrast, GST did not affect the expression of the class I MHC molecule (Fig. 9).

Despite the increased expression of CD3, constitutive phosphorylation of both CD3γ and CD3δ were markedly diminished. Moreover, the increase in CD3γ phosphorylation noted with PMA stimulation was prevented by preincubation with GST (Fig. 10). By contrast, GST preincubation had no effect on the constitutive phosphorylation of class I MHC molecules. These results are consistent with the conclusion that GST preincubation inhibited the functional consequences of PKC activation in that PMA-induced phosphorylation of the γ chain of CD3 was prevented.

Inhibitory effect of GST on PKC subspecies. To investigate the effect of GST on PKC in greater detail, purified PKC from rat brain was utilized. Results illustrated in Fig. 11 demonstrate that GST inhibited both type II and type III PKC subspecies in a dose-dependent manner. The concentrations of GST that produced 50% inhibition of types II and III PKC were estimated to be 3 and 10 μg/ml, respectively. TMA did not have a significant effect on PKC activity in the range of concentrations in which GST inhibited significantly.

Consistent with previous observations, limited proteolysis of types II and III PKC by calpain yielded two major fragments, a regulatory and a protein kinase fragment (PKM). The latter was fully active without added Ca^{2+}, phospholipid, and diacylglycerol (26). As can be seen in Fig. 12, PKMs purified from both types II and III PKC were directly inhibited by GST. These results suggest that GST can directly interact with the protein kinase domain of PKC and inhibit its activity. In addition, these data indicate that GST did not interfere with phospholipid stimulation since PKM activity was assayed in the absence of phospholipid. To exclude the possibility that GST interacted with the substrate and not the enzyme, a variety of other substrates were utilized. Using H1 histone or protamine as a substrate, GST inhibited PKC-mediated phosphorylation (Fig. 13). Finally, specificity of the effect of GST was examined by determining its action on other protein kinases. Modest inhibition of cAMP-dependent protein kinase (PKA) was also observed with 20 μg/ml GST (Fig. 12), suggesting that the inhibition was not completely specific for PKC.

Effect of GST on mitogen-induced IP_3 production. To examine whether GST has selective effects on PKC activity or whether it also inhibits other enzymes involved in the phosphatidyl inositol signaling pathway, the effect of GST preincubation on the levels of IP_3 produced in Jurkat cells stimulated with PHA was measured. As can be seen in Table I, PHA induced comparable levels of IP_3 in GST-preincubated and control Jurkat cells. More detailed kinetic analysis indicated that GST preincubation had no effect on the production of IP_3 after mitogen stimulation (data not shown). These results are consistent with the conclusion that GST predominantly inhibits PKC activity and not the production of IP_3.

Discussion

Several pharmacological agents have been used in an attempt to treat RA. Because the cause of the disease is unknown, therapy has largely been directed at suppressing the inflammatory process, with the aim of diminishing symptoms and preventing damage to articular structures. One group of agents that has been shown to be effective in the treatment of RA is the gold compounds. Despite documentation of clinical efficacy, there is no adequate explanation for the mechanism of action of gold compounds in RA.

The studies reported in this communication were carried out to determine a biochemical basis for the action of gold compounds in RA. Because $CD4^+$ T cells are felt to play a critical role in the pathogenesis of this disease (32–37), initial experiments were designed to examine the effect of gold compounds on the function of these cells. The data demonstrate that GST inhibits mitogen-induced DNA synthesis of $CD4^+$ T

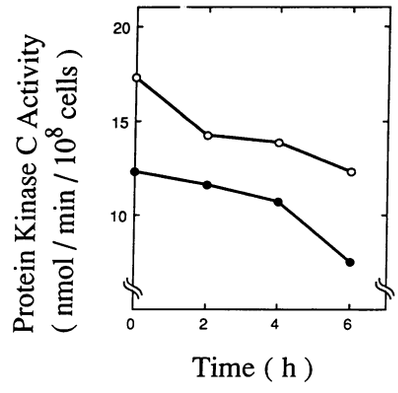

Figure 8. Time course of PKC down-regulation in Jurkat cells. Jurkat cells were preincubated in the presence (●) or absence (○) of 20 μg/ml GST for 3 d. At the end of their incubation, the cells were washed with PBS, suspended in fresh medium containing 10% FBS, and then were treated with 100 ng/ml PMA at 37°C for the lengths of time indicated. The total cellular PKC activity was determined as described in Methods. Results shown are representative of three experiments.

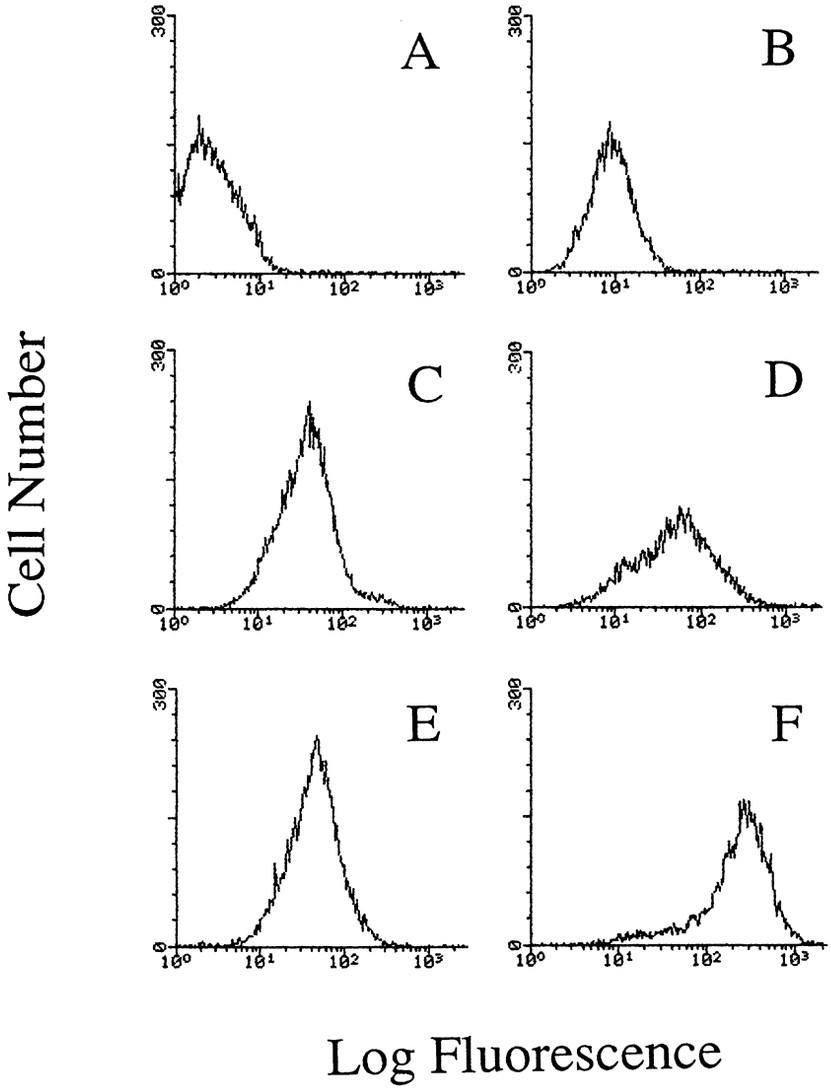

Figure 9. Surface expression of CD3 and class I MHC molecules after GST preincubation. Jurkat cells were incubated in the absence (*A, B, C,* and *D*) or presence (E and F) of GST at 20 μg/ml for 3 d and stained with anti-CD3 (OKT3) (*D* and *F*), anti-class I MHC (W6/32) (*C* and *E*) or a control antibody (P117) (*A* and *B*), followed by goat anti-mouse IgG conjugated to FITC as described in Methods.

cells in a dose-dependent manner. TMA, in contrast, did not significantly affect $CD4^+$ T cell proliferation, indicating that the inhibitory effects depend on the gold moiety itself and not on the TMA residue. This conclusion is also supported by the observation that similar inhibition of $CD4^+$ T cell DNA synthesis was exerted by AUR and ATG. Significant inhibitory effects of GST and AUR on $CD4^+$ T cell proliferation were observed at concentrations of 5 and 0.1 μg/ml, respectively. A number of studies have quantitated the levels of gold present in various body fluids and tissues after therapy with different gold com-

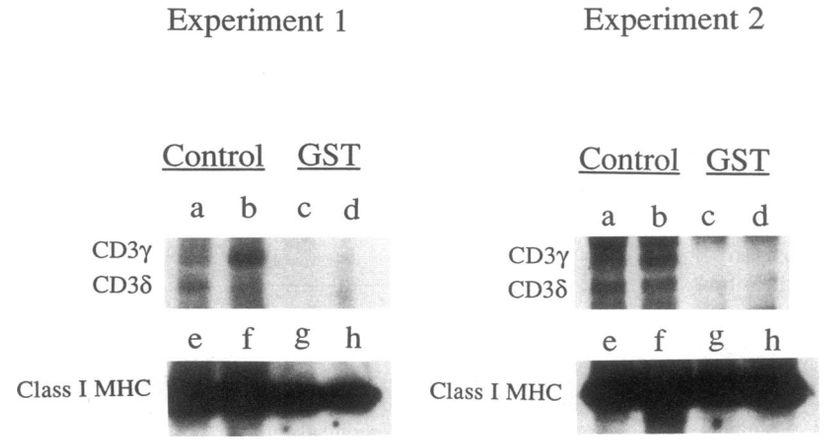

Figure 10. Effect of GST on phosphorylation of the γ and δ chains of CD3 and class I MHC molecules. Jurkat cells were cultured in the absence (*a, b, e,* and *f*) or presence (*c, d, g,* and *h*) of GST at 20 μg/ml for 3 d. The cells were washed and prelabeled for 1 h with 1.5 mCi of $[^{32}P]PO_4$ in phosphate-free medium and treated with (*b, d, f,* and *h*) or without (*a, c, e,* and *g*) 3 ng/ml PMA for 10 min. After incubation with PMA, cells were washed and lyzed as described in Methods. Each cell lysate was then immunoprecipitated with anti-CD3 (64.1) (*a, b, c,* and *d*) or anti-class I MHC (W6/32) (*e, f, g,* and *h*), coupled to protein A-agarose. The immunoprecipitates were eluted and analyzed by SDS-PAGE using a 15% gel. Results shown are two representatives of five experiments with similar results.

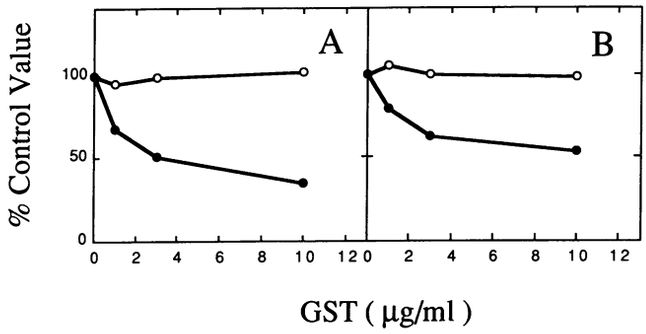

Figure 11. Effect of GST and TMA on activity of type II or type III PKC. PKC was purified from rat brain and assayed as described in Methods. PKC activity in the presence of GST (●) or in the presence of TMA (○) is shown. (*A*) Type II PKC; (*B*) type III PKC. PKC activity without GST or TMA was taken as control (100% value).

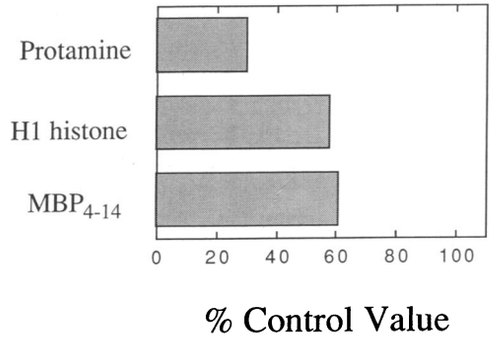

Figure 13. Phosphorylation of protamine, H1 histone, and MBP_{4-14} catalyzed by PKC. Type III PKC activity was measured in the presence of 10 μg/ml GST using several substrates. PKC activity without GST was taken as control (100% value).

pounds. It is generally accepted that serum gold levels attained in GST-treated patients tend to be in the range of 2–5 μg/ml (38–43), which is equivalent to the gold in 4–10 μg/ml GST. It has also been reported that the blood concentration range of AUR gold is 0.3–1.0 μg/ml (44). The levels of gold in the synovial tissue of patients treated with parenteral gold are much higher, i.e., 21–25 μg/g of tissue, wet wt (45, 46), equivalent to 42–50 μg/ml GST. Therefore, concentrations of GST and AUR, observed to suppress the proliferation of $CD4^+$ T cells in the studies reported here, are easily attainable in the serum and synovium of treated patients.

Earlier reports from our laboratory showed that GST depressed the accessory function of monocytes, but appeared to have minimal inhibitory effects on the potential responsiveness of the T lymphocytes (6). The present study shows clearly that GST also can directly inhibit T lymphocyte function. The previous studies utilized mitogenic stimuli that required participation of accessory cells for T cell activation and proliferation and, therefore, could not directly analyze the effect of GST on T cell activation. The current studies employed the mitogenic combination of an anti-CD3 MAb and PMA that can activate T cells in the complete absence of accessory cells, so that direct effects on T cell function could be analyzed. The data clearly show that gold compounds including GST, ATG, and AUR inhibit T cell proliferation and IL-2 production. Although previous studies have suggested that gold compounds might inhibit the function of T cells (11), this was not analyzed in a system in which an action of accessory cells might not have contributed to the effect noted. The current data clearly indicate that the immunosuppressive effects of GST are, therefore, broader than previously appreciated with inhibitory effects on the function of both T cells and mononuclear phagocytes.

In order to explore the mechanism of action of gold compounds in greater detail, Jurkat cells were employed. Jurkat cells resemble resting T cells in their activation requirements (47, 48) and, therefore, are a useful model to probe the putative mechanisms of action of gold compounds. As was observed for T cell proliferation, AUR inhibited IL-2 production by Jurkat cells at very low concentrations and without preincubation, presumably because of the lipophilicity conveyed by the triethylphosphine group (49). By contrast, preincubation of Jurkat cells with GST was required before inhibition of IL-2 production became apparent. This may relate to the slow entry of GST into cells presumably because of its hydrophilic nature and tendency to bind free sulfhydryl groups (49). After a 3-d incubation with pharmacologically attainable concentrations of GST, however, Jurkat cells were significantly inhibited in their ability to secrete IL-2. The results obtained with Jurkat cells confirm that gold compounds exert a direct suppressive effect on T cell function.

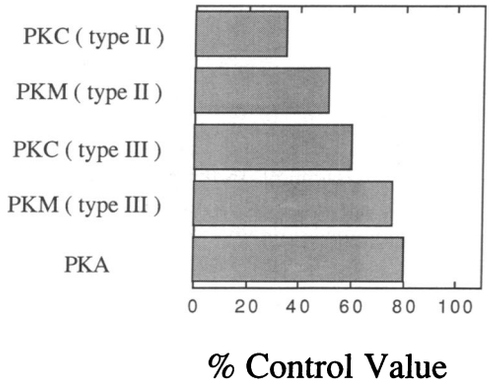

Figure 12. Effect of GST on PKC, PKM, and PKA activity. PKM and PKA were obtained by the procedure described in Methods. Type II PKC.PKM, type III PKC.PKM, and PKA were assayed in the presence of 10 μg/ml GST as described in Methods. Enzyme activity without GST was taken as control (100% value).

Table I. Lack of Effect of GST Preincubation on PHA-induced IP_3 Production

Preincubation	IP_3 0 min	IP_3 40 min
	pmol/10^7 cells	
Medium	0.6±0.6	18.5±0.3
GST (20 μg/ml)	6.4±0.9	25.9±2.5

Results indicate total IP_3 content of Jurkat cells before stimulation and 40 minutes after stimulation with PHA (4 μg/ml), and are expressed as the mean±SEM of three different experiments. Jurkat cells had been incubated in medium alone or with GST (20 μg/ml) for 72 h before stimulation.

Activation of T cells involves receptor-mediated hydrolysis of inositol phospholipids generating diacylglycerol and inositol 1,4,5-trisphosphate that activate PKC and elevate intracellular Ca^{2+}, respectively (50, 51). Therefore, it seemed reasonable to suggest that gold compounds might interfere with second messenger generation and thereby inhibit T cell responsiveness. Because gold compounds inhibited T cell responses that were co-stimulated with PMA to activate PKC directly, it seems more likely that gold compounds might interfere directly with the activity of PKC rather than with the production of second messengers. The experiments reported herein examined that possibility. The results indicated that GST inhibits PKC directly. GST not only directly inhibited the function of PKC in vitro, but also diminished PKC activity in vivo and inhibited PKC-mediated phosphorylation of the γ chain of CD3. The latter finding is of particular importance in that it demonstrates that GST can inhibit a functionally relevant PKC-dependent event in T cell activation and is consistent with the conclusion that the principal effect of GST on T cell activation results from inhibition of PKC. It was also of interest that GST increased expression of CD3 by Jurkat cells. Activators of PKC are known to decrease CD3 expression (30). It is therefore possible that the increased expression of CD3 resulting from GST preincubation may have also resulted from inhibition of PKC. The constitutive phosphorylation of CD3γ was also inhibited by GST preincubation, suggesting that this effect might also be mediated by PKC. Finally, the data establish that GST has selective effects on PKC-mediated signal generation in vivo. The incubation of Jurkat cells with GST did not affect the phosphorylation of class I MHC molecules and had no significant effect on mitogen-induced IP_3 production.

Somewhat unexpectedly, AUR did not inhibit PKC although it did suppress T cell proliferation and IL-2 production. These results appear to conflict with those of Parente et al. (13) and Froscio et al. (12), who previously reported that AUR inhibited PKC activity in vitro and in vivo in polymorphonuclear leukocytes (PMN). This discrepancy is difficult to understand, but may relate to various features of the systems examined. Both of the aforementioned studies analyzed the in vitro effect of AUR on PKC activity partially purified from platelets. The characteristics of PKC subspecies in platelets are quite different from PKC subspecies in other cells (52) and may explain the disparate results. The studies also utilized type III-S histone as substrate to measure PKC activity. This substrate does not appear to provide a specific measure of PKC activity in crude fractions, because other kinases, such as cyclic AMP–dependent protein kinase or cyclic GMP–dependent protein kinase, can phosphorylate this histone (53). Moreover, basic proteins such as this substrate are known to enhance casein kinase–catalyzed phosphorylation of other proteins (53). Thus, the use of this substrate may have affected the results. It is also possible that there are differences in the sensitivities of PKC from different cells to inhibition by gold compounds. Although type III-S histone was used as a substrate, it remains possible that the PKC expressed by PMN or platelets is more sensitive to the action of AUR than the PKC found in T cells. Regardless of the explanation for the discrepancy, the current results clearly indicate that AUR at concentrations, as large as 20 μg/ml (29.5 μM) had minimal effect on T cell PKC activity.

One question not answered by the present studies was the mechanism by which AUR might inhibit T cell function but not the action of PKC. Because of the triethylphosphine group, AUR has a number of properties not shared by other gold compounds (54–57). It is a highly lipophilic compound that exerts inhibitory effects on a number of cell types not usually affected by other gold compounds. One of these appears to be the capacity to inhibit T cell function but not T cell PKC activity.

The results of the current studies clearly indicate that GST inhibits T cell PKC activity directly. The explanation for the inability of previous investigators to detect this inhibition (13) is unclear, but may relate to some of the same considerations discussed above. Additional analysis was carried out to determine the mechanism by which GST inhibited PKC. A characteristic of gold compounds is their ability to bind to sulfhydryls on various cellular proteins. Because both the regulatory, and the catalytic domains of PKC contain thiols (58), it seemed likely that the action of GST might relate to gold binding to these sulfhydryl groups. The finding that 2-ME reversed the inhibitory action of GST on PKC supports this contention. The observation that AUR is not a potent thiol reagent in aqueous medium (55) may explain its inability to inhibit PKC in the current studies.

To understand the action of GST on PKC in greater detail, purified PKC subspecies were prepared and analyzed. PKC is known to be a family of multiple subspecies with closely related structures. PKC from rat brain can be resolved into three subfractions, types I, II, and III, upon hydroxyapatite column chromatography. Type III PKC is present ubiquitously in tissues and cell types so far examined, whereas type II PKC is found in varying amounts in most tissues and cell types with distinct regional expression and intracellular localization (50). In contrast, type I PKC is expressed only in the central nervous system. Such tissue- and cell-specific expression of PKC subspecies suggests that each member of the enzyme family has a different function in cellular responses to external stimuli. Human T cells and Jurkat cells express type II and type III PKC (59, 60). We, therefore, examined the inhibitory effect of GST on types II and III PKC subspecies, by using a synthetic peptide, $MBP_{4\text{-}14}$, which is a specific substrate for these enzymes (27). Using $MBP_{4\text{-}14}$ as substrate, both types II and III PKC subspecies were inhibited by GST in a concentration-dependent manner, with type II PKC somewhat more sensitive to the inhibitory effects of GST. This suggests the possibility that GST may have a greater inhibitory effect on functional activities mediated by this specific PKC pathway.

To analyze the effect of GST on PKC more completely, the catalytic domain of the molecule, PKM, was purified from both types II and III PKC. Both of these catalytic domains were directly inhibited by GST. It is likely, therefore, that GST directly inhibits the catalytic activity of PKC by interacting with thiol groups of the catalytic fragment. In that GST also inhibited PMA-induced translocation of PKC in intact cells, an activity that involves the regulatory domain (58), it is likely that GST can also inhibit the function of this fragment of PKC, presumably by binding its sulfhydryl group.

It should be noted that the action of GST was not completely specific for PKC. Thus, GST also caused some modest inhibition of cyclic AMP–dependent protein kinase (PKA). It has previously been shown that modification of one sulfhydryl group in the catalytic subunit of PKA brings about inactivation of this enzyme (61). GST may therefore interact with this thiol group and inhibit PKA. Although the magnitude of the inhibitory effect on PKA was not as great as on PKC, these data show

that GST is not a specific inhibitor of PKC, but rather may alter a number of cellular kinases that may play a role in regulating immune responses.

Different substrates, including protamine and H1 histone, which can be used for the assay of PKC, were also used to test whether GST might act on the substrate rather than the kinase. Although quantitative differences were observed, GST inhibited phosphorylation of protamine and H1 histone substrate by PKC. Many known inhibitors of PKC such as chloropromazine (62) appear to compete with phospholipids and not to be direct inhibitors of the enzyme. However, the inhibition of PKC caused by GST was not reversed by increasing concentrations of phosphatidylserine (data not shown). Moreover, GST also inhibited PKM activity in the absence of phospholipid. These results indicate that GST directly inhibited the catalytic center of PKC and did not compete for the substrate or cofactors.

In summary, GST inhibited PKC in vitro and in vivo in T cells probably by interacting with thiol groups in the catalytic domain and possibly the regulatory domain of PKC. In contrast, AUR did not inhibit PKC. The interaction of GST and PKC and the inhibition of the catalytic activity of PKC in T cells may be responsible or co-responsible for the therapeutic antirheumatic action of this drug. Whether GST also inhibits PKC activity in other inflammatory cells that may contribute to its anti-inflammatory effects remains to be documented, but seems likely.

Acknowledgments

We are grateful to Dr. T. D. Geppert for this expert advise on the phosphorylation experiments.

This work was supported by National Institutes of Health grants AR-39169 and AR-09989.

References

1. Huskisson, E. C. 1976. Specific therapy for rheumatoid arthritis. *Rheumatol. Rehabil.* 15:133–135.

2. Wright, V., and R. Amos. 1980. Do drugs change the course of rheumatoid arthritis? *Br. Med. J.* 280:964–966.

3. Lorber, A., T. Simon, J. Leeb, A. Peter, and S. Wilcox. 1978. Chrysotherapy: suppression of immunoglobulin synthesis. *Arthritis Rheum.* 21:785–791.

4. Research Subcommittee of the Empire Rheumatism Council. 1960. Gold therapy in rheumatoid arthritis: report of a multi-center controlled trial. *Ann. Rheum. Dis.* 19:95–119.

5. Hanly, J. G., and B. Bresnihan. 1985. Reduction of peripheral blood lymphocytes in patients receiving gold therapy for rheumatoid arthritis. *Ann. Rheum. Dis.* 44:299–301.

6. Lipsky, P. E., and M. Ziff. 1977. Inhibition of antigen- and mitogen-induced human lymphocyte proliferation by gold compounds. *J. Clin. Invest.* 59:455–466.

7. Mirabelli, C.-K., C. P. Sung, D. H. Picker, C. Barnard, P. Hydes, and A. M. Badger. 1988. Effect of metal containing compounds on superoxide release from phorbol myristate acetate stimulated murine peritoneal macrophage: inhibition by auranofin and spirogermanium. *J. Rheumatol.* 15:1064–1069.

8. Littman, B. H., and P. Schwartz. 1982. Gold inhibition of the production of the second complement component by lymphokine-stimulated human monocytes. *Arthritis Rheum.* 25:288–296.

9. Littman, B. H., P. L. Carlson, L. D. Loose, and K. M. Sanders. 1990. Effects of gold sodium thiomalate and tenidap sodium (CP-66, 248-2) on a model of macrophage differentiation using HL-60 cells. *Arthritis Rheum.* 33:29–36.

10. Matsubara, T., and M. Ziff. 1987. Inhibition of human endothelial cell proliferation by gold compounds. *J. Clin. Invest.* 79:1440–1446.

11. Wolf, R. E., and V. C. Hall. 1988. Inhibition of in vitro proliferative response of cultured T lymphocytes to interleukin-2 by gold sodium thiomalate. *Arthritis Rheum.* 31:176–181.

12. Froscio, M., A. W. Murray, and N. P. Hurst. 1989. Inhibition of protein kinase C activity by the antirheumatic drug auranofin. *Biochem. Pharmacol.* 38:2087–2089.

13. Parente, J. E., M. P. Walsh, P. R. Girard, J. F. Kuo, S. N. David, and K. Wong. 1989. Effects of gold coordination complexes on neutrophil function are mediated via inhibition of protein kinase C. *Mol. Pharmacol.* 35:26–33.

14. Lampson, L. A., and R. Levy. 1980. Two population of Ia-like molecules on a human B cell line. *J. Immunol.* 125:293–299.

15. Nel, A. E., M. W. Wooten, and R. M. Galbraith. 1987. Molecular signaling mechanisms in T-lymphocyte activation pathways: a review and future prospects. *Clin. Immunol. Immunopathol.* 44:167–186.

16. Kung, P., G. Goldstein, E. Reinherz, and S. F. Schlossman. 1979. Monoclonal antibodies defining distinctive human T cell surface antigens. *Science (Wash. DC).* 206:347–349.

17. Brodsky, F. M., and P. Parman. 1982. Monomorphic anti-HLA-A, B, C monoclonal antibodies detecting molecular subunits and combinatorial determinants. *J. Immunol.* 128:129–135.

18. Yoshimura, N., T. Kikuchi, T. Sasaki, A. Kitahara, M. Hatanaka, and T. Murachi. 1983. Two distinct Ca^{2+} proteases (calpain I and calpain II) purified concurrently by the same method from rat kidney. *J. Biol. Chem.* 258:8883–8889.

19. Oliver, D., K. R. Sommer, S. Panyim, S. Spiker, and R. Chalkley. 1972. A modified procedure for fractionating histones. *Biochem. J.* 129:349–353.

20. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation and sedimentation of 1 *g*. *Scand. J. Clin. Lab. Invest.* 21(Suppl. 97):77–89.

21. Thiele, D. L., M. Kurosaka, and P. E. Lipsky. 1983. Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: delineation by its sensitivity to the lysosomotrophic agent, L-leucine methyl ester. *J. Immunol.* 131:2282–2290.

22. Thiele, D. L., and P. E. Lipsky. 1985. Regulation of cellular function by products of lysosomal enzyme activity: elimination of human natural killer cells by a dipeptide methyl ester generated from L-leucine methyl ester by monocytes or polymorphonuclear leukocytes. *Proc. Natl. Acad. Sci. USA.* 82:2468–2472.

23. Galili, U., and M. Schlesinger. 1974. The formation of stable E rosettes after neuraminidase treatment of either human peripheral blood lymphocytes or of sheep red blood cells. *J. Immunol.* 112:1628–1634.

24. Wysocki, L. J. and V. L. Sato. 1978. "Panning" for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci. USA.* 75:2844–2848.

25. Stohl, W., and M. Linker-Israeli. 1989. Inhibitory effects of anti-CD2 monoclonal antibodies on interleukin 2 production and interleukin 2 receptor expression in anti-CD3-induced T cell activation. *Cell. Immunol.* 120:351–365.

26. Sekiguchi, K., M. Tsukuda, K. Ase, U. Kikkawa, and Y. Nishizuka. 1988. Mode of activation and kinetic properties of three distinct forms of protein kinase C from rat brain. *J. Biochem. (Tokyo).* 103:759–765.

27. Yasuda, I., A. Kishimoto, S. Tanaka, M. Tominaga, A. Sakurai, and Y. Nishizuka. 1990. A synthetic peptide substrate for selective assay of protein kinase C. *Biochem. Biophys. Res. Commun.* 166:1220–1227.

28. Kishimoto, A., N. Kajikawa, M. Siota, and Y. Nishizuka. 1983. Proteolytic activation of calcium-activated, phospholipid-dependent protein kinase by calcium-dependent neutral protease. *J. Biol. Chem.* 258:1156–1164.

29. Kumon, A., K. Nishiyama, H. Yamaura, and Y. Nishizuka. 1972. Multiplicity of adenosine 3',5'-monophosphate-dependent protein kinases from rat liver and mode of action of nucleotide 3',5'-monophosphate. *J. Biol. Chem.* 247:3726–3735.

30. Cantrell, D. A., A. A. Davies, and M. J. Crumpton. 1985. Activators of protein kinase C down-regulate the T3/T-cell antigen receptor complex of human T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 82:8158–8162.

31. Alexander, D. R., J. M. Hexham, S. C. Lucas, J. D. Graves, D. A. Cantrell, and M. J. Crumpton. 1989. A protein kinase C pseudosubstrate peptide inhibits phosphorylation of the CD3 antigen in streptolysin-*O*-permeabilized human T lymphocytes. *Biochem. J.* 260:893–901.

32. Duke, O., G. S. Panayi, G. Janossy, and L. W. Poulter. 1982. An immunohistological analysis of lymphocyte subpopulations and their microenvironment in the synovial membranes of patients with rheumatoid arthritis using monoclonal antibodies. *Clin. Exp. Immunol.* 49:22–30.

33. Klareskog, L., U. Forsum, A. Wigren, and H. Wigzell. 1982. Relationship between HLA-DR expression and T lymphocytes of different subsets in rheumatoid synovial tissue. Scand. *J. Immunol.* 15:501–507.

34. Degre, M., O. J. Mellbye, and O. Clarke-Jenssen. 1983. Immune interferon in serum and synovial fluid in rheumatoid arthritis and related disorders. *Ann. Rheum. Dis.* 42:672–676.

35. Husby, G. and R. C. Williams, Jr. 1985. Immunohistochemical studies of interleukin-2 and γ-interferon in rheumatoid arthritis. *Arthritis Rheum.* 28:174–181.

36. Paulus, H. E., H. I. Machleder, S. Levine, D. Yu, and N. S. MacDonald. 1977. Lymphocyte involvement in rheumatoid arthritis. Studies during thoracic duct drainage. *Arthritis Rheum.* 20:1249–1262.

37. Kotzin, B. L., S. Strober, E. G. Engleman, A. Calin, R. T. Hoppe, G. S. Kansas, C. P. Terrell, and H. S. Kaplan. 1981. Treatment of intractable rheumatoid arthritis with total lymphoid irradiation. *N. Engl. J. Med.* 305:969–976.

38. Lorber, A., R. L. Cohen, C. C. Chang, and H. E. Anderson. 1968. Gold determination in biological fluids by atomic absorption spectrophotometry: ap-

plication to chrysotherapy in rheumatoid arthritis patients. *Arthritis Rheum.* 11:170–177.

39. Mascarenhas, B. R., J. L. Granda, and R. H. Freyberg. 1972. Gold metabolism in patients with rheumatoid arthritis treated with gold compounds: reinvestigated. *Arthritis Rheum.* 15:391–402.

40. Jessop, J. D., and R. G. S. Johns. 1973. Serum gold determinations in patients with rheumatoid arthritis receiving sodium aurothiomalate. *Ann. Rheum. Dis.* 32:228–232.

41. Rubinstein, H. M., and A. A. Dietz. 1973. Serum gold. II. Levels in rheumatoid arthritis. *Ann. Rheum. Dis.* 32:128–132.

42. Billings, R., R. Grahame, V. Marks, P. J. Wood, and A. Taylor. 1975. Blood and urine gold levels during chrysotherapy for rheumatoid arthritis. *Rheumatol. Rehabil.* 14:13–18.

43. Gerber, R. C., H. E. Paulus, R. Bluestone, and C. M. Pearson. 1972. Clinical response to serum gold levels in chrysotherapy. Lack of correlation. *Ann. Rheum. Dis.* 31:308–310.

44. Gottlieb, N. L. 1982. Comparative pharmacokinetics of parenteral and oral compounds. *J. Rheumatol.* 8(Suppl.):99–109.

45. Grahame, R., R. Billings, M. Laurence, V. Marks, and P. J. Wood. 1974. Tissue gold levels after chrysotherapy. *Ann. Rheum. Dis.* 33:536–539.

46. Gottlieb, N. L., P. M. Smith, and E. M. Smith. 1972. Tissue gold concentration in a rheumatoid arthritic receiving chrysotherapy. *Arthritis Rheum.* 15:16–22.

47. Berry, N., and Y. Nishizuka. 1990. Protein kinase C and T cell activation. *Eur. J. Biochem.* 189:205–214.

48. Manger, B., A. Weiss, C. Weyand, J. Goronzy, and J. D. Stobo. 1985. T cell activation: differences in the signals required for IL 2 production by nonactivated and activated T cells. *J. Immunol.* 135:3669–3673.

49. Lewis, A. J., and Walz, D. T. 1982. Immunopharmacology of gold. *Prog. Med. Chem.* 19:1–58.

50. Nishizuka, Y. 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature (Lond.).* 334:661–665.

51. Berridge, M. J., and R. F. Irvine. 1989. Inositol phosphates and cell signalling. *Nature (Lond.).* 341:197–205.

52. Tsukuda, M., Y. Asaoka, K. Sekiguchi, U. Kikkawa, and Y. Nishizuka. 1988. Protein kinase C subspecies in human platelets. *Biochem. Biophys. Res. Commun.* 155:1387–1395.

53. Yamamoto, M., W. E. Criss, Y. Takai, H. Yamamura, and Y. Nishizuka. 1979. A hepatic soluble cyclic nucleotide-independent protein kinase. *J. Biol. Chem.* 254:5049–5052.

54. Sutto, B. M., E. McGusty, D. T. Waltz, and M. J. DiMartino. 1972. Oral gold. Antiarthritic properties of alkylphosphinegold coordination complexes. *J. Med. Chem.* 15:1095–1098.

55. Walz, D. T., M. J. DiMartino, L. W. Chakrin, B. M. Sutton, and A. Misher. 1976. Antiarthritic properties and unique pharmacologic profile of a potential chrysotherapeutic agent: SK & FD-39162. *J. Pharmacol. Exp. Ther.* 197:142–152.

56. Crooke, S. T., R. M. Snyder, T. R. Bott, D. J. Ecker, H. S. Allaudeen, B. Monia, and C. K. Mirabelli. 1986. Cellular and molecular pharmacology of auranofin and related gold complexes. *Biochem. Pharmacol.* 35:3423–3431.

57. Walz, D. T., M. J. DiMartino, and D. E. Griswold. 1982. Comparative pharmacology and biological effects of different gold compounds. *J. Rheumatol.* 9(Suppl. 8):54–60.

58. Parker, P. J., L. Coussens, N. Totty, L. Rhee, S. Young, E. Chen, S. Stabel, M. D. Waterfield, and A. Ullrich. 1986. The complete primary structure of protein kinase C: the major phorbol ester receptor. *Science (Wash. DC).* 233:853–859.

59. Shearman, M. S., N. Berry, T. Oda, K. Ase, U. Kikkawa, and Y. Nishizuka. 1988. Isolation of protein kinase C subspecies from a preparation of human T lymphocytes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 234:387–391.

60. Lucas, S., R. Marais, J. D. Graves, D. Alexander, P. Parker, and D. A. Cantrell. 1990. Heterogeneity of protein kinase C expression and regulation in T lymphocytes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 260:53–56.

61. Jimenez, J. S., A. Kupfer, V. Gani, and S. Shaltiel. 1982. Salt-induced conformational changes in the catalytic subunit of adenosine cyclic 3',5'-phosphate dependent protein kinase. Use for establishing a connection between one sulfhydryl group and the γ-P subsite in the ATP site of this subunit. *Biochemistry.* 21:1623–1630.

62. Mori, T., Y. Takai, R. Minakuchi, B. Yu, and Y. Nishizuka. 1980. Inhibitory action of chlorpromazine, dibucaine, and other phospholipid-interacting drugs on calcium-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* 255:8378–8380.