GOLD COMPOUNDS AND RHEUMATOID ARTHRITIS:
MURINE STUDIES OF THE IMMUNE RESPONSE
TO GOLD SODIUM THIOMALATE

THESIS

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By

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Balb/c normal mice were used to study the effects of gold sodium thiomalate (GST) on intact, nonadherent, and adherent mononuclear spleen cells. The three populations were tested for the following aspects: in vitro effects of GST on the mitogen-triggered DNA synthesis; intracellular levels of cyclic AMP; and chemotaxis ability. These studies showed that GST inhibited the proliferative responses of all three populations as the concentration of GST increased. Cyclic AMP levels in the nonadherent population increased as the GST concentration increased. GST had a biphasic effect on the adherent population. At concentrations of 5 and 10 μg/ml, GST suppressed the cyclic AMP levels, and at concentration of 50 μg/ml it enhanced the cyclic AMP levels. GST had no effect on the cyclic AMP levels in the intact mononuclear spleen cells. GST appeared to have an inhibitory effect on the chemotaxis ability of all three populations of spleen cells.
ACKNOWLEDGMENT

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Rheumatoid arthritis (also atrophic arthritis, chronic proliferative arthritis, chronic infectious arthritis) is a systemic disorder of unknown cause in which symptoms and inflammatory change predominate in articular and related structures (20). The disease tends to be chronic and to produce characteristic, histologic changes in the joints consisting of edema, proliferation of capillaries, fibrosis, and infiltration first by polymorphonuclear leukocytes, then by lymphocytes and plasma cells (36).

According to present concepts (20), an unidentified antigenic stimulus elicits antibody (IgM) production by the plasma cells in the synovium. As antigen-antibody complexes are formed, the antibody is altered, becomes foreign, and stimulates the production of antibody (IgG anti IgM) by synovial plasma cells (20) and regional lymph nodes. This antibody is known as rheumatoid factor. The antigen-antibody complexes fix complement (87) inducing phagocytosis and lysosomal release, which gives rise to tissue damage and inflammation (87).

The role of rheumatoid factor is uncertain. It may be protective (rather than pathogenic) by having the capacity to
localize antigen-antibody complexes (36). When large amounts of rheumatoid factor are given by repeated transfusion to normal volunteers, no disease ensues (36). Patients with agammaglobulinemia, and little or no rheumatoid factor, have an unusually high incidence of rheumatoid arthritis (36).

The incidence (20) of rheumatoid arthritis among those fifteen years of age or older is approximately one percent for "definite" rheumatoid arthritis and three percent for "probable" rheumatoid arthritis. The disease may begin (20) at any time from infancy through the ninth decade. It is generally held that (20) the disease is three times as common in women as in men, but this does not apply at all ages. There is no evidence for familial aggregation of the disease (20). Symptoms appear to be more frequent and severe in an environment of high relative humidity and low barometric pressure (20).

Chrysotherapy

Chrysotherapy comes from the Greek word for gold, "chrysos," and is the use of gold compounds in a variety of diseases including rheumatoid arthritis. Although a number of German physicians had tried gold salts for the treatment of rheumatoid arthritis, the first successful use of chrysotherapy in this disease was reported by Forestier, a Frenchman, in 1929 (24). This study was based on the knowledge of the successful treatment of tuberculosis by gold
salts and the mistaken assumption that a relationship between rheumatoid arthritis and tuberculosis existed. This empirical use of gold initiated considerable interest in chrysotherapy. The initial studies were fraught with complications, and toxicity often developed with large doses. In 1960, a double-blind trial conducted by the European Rheumatism Council (22) confirmed the effectiveness of parenterally administered gold salts in the treatment of rheumatoid arthritis.

Recent reviews evaluating numerous other drugs used for the treatment of rheumatoid arthritis (i.e., nonsteroidal and anti-inflammatory drugs, steroidal anti-inflammatory drugs, immunomodulators) unanimously suggest that gold salts are the only class of drug able to bring about remission of this disease (10,12,16,41,88). Despite the significant benefits of parenterally administered gold, this form of therapy has been reserved as a treatment of last resort, in view of its undoubted toxicity (79).

The toxicological manifestations of gold therapy are trivial; chrysotherapy may be complicated (68) by allergic and idiosyncratic reactions that are mild (pruritus, rash, cheilitis, and eosinophilia) or severe (blood dyscrasias and nephrotic syndrome). It has been suggested (21) that the number of patients responding favorably will increase if the gold salts are administered earlier in the course of the
disease and that this may also reduce the proportion of patients that fail to respond to gold (21). Nevertheless, best results are obtained with patients with definite rheumatoid arthritis which is active, progressive, erosive and seropositive despite treatment with other anti-inflammatory drugs (30,44).

Gold occurs in the group Ib of the periodic table as one of the transition metals. It is classified as one of the b-class cations which are considered to be "soft" ions. This term indicates that the metal has a large size (atomic number 79, atomic weight 196.97) with a low charge and outer electrons that are easily excited (52). Soft metal ions bind strongly to soft ligands (i.e., those with low electronegativity and having some covalent character) (52).

Of the several oxidation states for gold compounds that have been characterized (Viz., -I,0,I,II,III and V), only gold (I) and gold (III), the principal states, have been examined for their biological activities (52). Gold (0), also referred to as colloidal or metallic gold, is exceptionally stable and has been tried in arthritis therapy (52). Gold (III) complexes are too toxic for clinical use because they bind nonspecifically to numerous proteins (52). Gold (I) has received the greatest attention since it has been used extensively in different complexes and shown to be of therapeutic value
Some of the many varieties of gold (I) compounds that have been used therapeutically are shown in Table I (52).

Mode of Action of Gold Compounds

Despite the many significant contributions over the years concerning the therapeutic use of gold salts, we are still faced with a problem, "...namely the mode of action of aurotherapy in chronic rheumatism cannot yet be defined with certainty" (87). There have been a number of hypotheses regarding the mechanism of action of gold in inflammatory processes, particularly those involving protein and enzyme interaction (20). However, a view rapidly gaining support is that gold possesses immunomodulatory activity (52). Figure 1 shows the major postulated sites of biological action for gold compounds (52).

The importance of protein sulphydryl (SH) or mercapto groups in biology is well established. There are several cellular constituents containing SH substitutes (84) namely coenzyme A, glutathione, cysteine, homocysteine, ergothioneine, cytoplasmic proteins, plasma membranes, structural proteins and finally many types of enzyme. Consequently, modification of, or reaction with, these SH groups may directly or indirectly alter cell metabolism and function (84). Gold is one of the heavy metals known to interact with SH groups to form mercaptides (9). The gold-SH interaction and the role of the ligand in these interactions
<table>
<thead>
<tr>
<th>Generic name</th>
<th>Trade names</th>
<th>%Au</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gold sodium thiomalate (GST), disodium aurothiomalate</td>
<td>Myochrysin, Myocrisin, Tauredon</td>
<td>50.5</td>
<td><img src="image1" alt="Formula 1" /></td>
</tr>
<tr>
<td>Gold thioglucone, aurothioglucose</td>
<td>Solganal</td>
<td>50.3</td>
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<tr>
<td>Gold thioglycoanilid, aurothioglycolanilid</td>
<td>Lauron</td>
<td>54.2</td>
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<tr>
<td>Gold sodium thiosulphate, aurothiosulphate sodium</td>
<td>Sanochrysin, Sanocrisin, Aurothion, Crisalbine, Solfocrisol, Thiochrysin</td>
<td>40.2</td>
<td>Na₃ Au(S₂ O₃)₂</td>
</tr>
<tr>
<td>Sodium 2-aurothiobenzimidazole-4-carboxylate, aurothiol</td>
<td>Triphal</td>
<td>47.8</td>
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<td>Sodium auroallythiourea-m-benzoate</td>
<td>Lopion</td>
<td>43.4</td>
<td><img src="image5" alt="Formula 5" /></td>
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Fig. 1--Major postulated sites of biological action for gold compounds.
has been the subject of several investigations. The \textit{in vitro} binding of gold to protein with concomitant release of free thiomalate has been reported by Jellum, Munthe, Guldal, and Aaseth (42,43). In these studies performed in mice using double-isotope labelled $^{195}\text{Au, }^{14}\text{C}$ gold sodium thiomalate(GST), fifty percent of the thiomalate was bound to tissue membranes and cells and fifty percent was excreted in the urine. It was concluded that at least a part of the effects of GST is due to thiomalate, since this carrier has demonstrable immunologic activity similar to D-penicillamine in animals (2).

Free thiol levels are known to be reduced in sera from patients with a number of connective tissue diseases including rheumatoid arthritis (59). The reason for this is not established although it is possibly due to a disturbance of the SH-disulphide (S-S) exchange reaction (52):

\[ 2RS^- \rightleftharpoons RSSR + 2e \]

This might result in aggregation of serum proteins, including IgG, and formation of new antigenic sites that may eventually lead to the production of autoantibodies. Gerber (27) postulated that inhibition of IgG-aggregation by gold occurs \textit{in vivo}, thus preventing the formation of immune complexes between IgG and rheumatoid factor in the rheumatoid joint.

In view of the ubiquitous presence of SH groups on most enzymes, it is not surprising that gold (I) has been shown to inhibit a large number of enzymes. Both PMN leukocytes
and macrophages play extremely important roles in immunologically-induced tissue injury (52) and this is largely a result of the release of the hydrolytic enzymes they have sequestered in their cytoplasmic granules or lysosomes.

Finkelstein, Roisman, Ladizesky, and Walz (23) have reported that auranofin (AF) completely blocked beta-glucuronidase secretion and markedly reduced acid phosphatase (88 percent) and lysosome (72 percent) release in human peripheral PMN leukocytes. In contrast, gold sodium thiosulfate (GSTS) had no inhibitory activity on lysosome released at equivalent gold concentration. These same people in their investigation (50) on the effect of AF and gold sodium thiomalate (GST) on superoxide radical production by human leukocytes reported that AF (0.5-1.0 μg/Au/ml) showed a potent inhibitory activity on superoxide generation, while GST showed only moderate inhibition at higher concentrations, (100 μM). The thiol protecting agent, dithiothreitol, completely blocks the inhibitory effect of AF, suggesting that AF may interact with sulphydryl groups at the cellular membrane level (50).

Complement is a self-assembling cascade (52) of proteins that constitutes the major humoral mediator of antigen-antibody interactions. Complement activation may result both in the irreversible structural and functional alteration of biological membranes leading to cell death and consequently
to extensive tissue injury (52), and in the release of products that are capable of releasing histamine from mast cells, releasing lysosomal enzymes, and acting as chemoattractants for PMN leukocytes and contracting smooth muscle (66).

Analysis of rheumatoid joints has shown that complement activation and depletion do occur, and the presence of complement in synovial membrane and cartilage further confirms its importance in arthritis (13,15). Schultz et al found that gold inactivated native C1 and bound to both C\textsubscript{1q} and C\textsubscript{1s} (81). They postulated that gold, interfered with immune complex-mediated activation of the classical complement pathway in rheumatoid synovitis.

In a study of gold sodium thiomalate (GST), Littman (57) found that GST irreversibly inhibited lymphokine-mediated C2 production by human peripheral monocytes only when GST treatment coexisted with or proceeded exposure of monocytes to lymphokine. Monocytes already stimulated by lymphokine were resistant to the effects of GST. Littman, thus, makes the conclusion that the important factor determining how quickly rheumatoid inflammation could be controlled by gold would be the life span of the macrophages that are already involved in the inflammatory response.

Both polymorphonuclear leukocytes and mononuclear phagocytes (peripheral blood monocytes and tissue macrophages) play important roles in the mediation of
inflammatory degradation of connective tissue (52). This results from the ability of the phagocytic mononuclear system to ingest and degrade foreign materials and to secrete a number of biologically active substances which enhance the inflammatory process (79).

Scheinberg, Santos, and Finkelstein (74) compared the effect of GST and AF on some function of human peripheral blood monocytes. Both agents were capable of inhibiting monocyte chemotaxis and the expression of FC and C3 receptors. This inhibition occurred in association with an increase of the intracellular levels of cyclic AMP. The increase in cyclic AMP levels point to a possible effect of the membrane level interfering with the activation of the enzyme adenyl cyclase. Sallin and coworkers (26) have shown that agents that increase cyclic AMP inhibit accumulation of cyclic GMP and depress human monocyte locomotion.

One of the features of rheumatoid arthritis is the presence of activated lymphoid cells within the synovial tissue of involved joints (1). Lipsky (56) has demonstrated that parenteral gold compounds (i.e., gold sodium thiomalate, aurothioglucose, and aurothiosulphate) can inhibit in vitro antigen and mitogen triggered human lymphocytes DNA synthesis (56). Gold compounds have also been shown to inhibit pokeweed mitogen (PWM) induced immunoglobulin production from human peripheral blood mononuclear (PBM) cells in vitro (55). The observed inhibition in both studies appeared to be
mediated by the gold moiety itself and was not dependent on the ligand (55,56).

The in vitro study of human peripheral blood lymphocytes by Lorber, Jackson and Simon (58) demonstrated that AF inhibited $^3$H-thymidine uptake and nearly abolished DNA synthesis by Epstein Barr virus transformed lymphocytes. They concluded that AF may exert antiproliferative cellular action as well as suppression of lymphocyte, macrophage, and effector function.

Rheumatoid arthritis is characterized by excessive production of antibodies to a variety of antigens (namely, altered IgG, collagen, nuclear protein) and subsequent local immune complex deposition (87).

Walz and his coworkers (83) using spleenic lymphocytes from adjuvant arthritic rats demonstrated that AF is capable of reducing levels of antibody involved in antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent complement lysis (ADCL), and hemagglutination reactions. In contrast, gold sodium thioglucose (GSTG) is inactive or stimulatory in these assay systems.

The complexity of the immune response suggests that a simple explanation for the effect of an immune active drug (in this case gold compounds) is very unlikely. From the literature review just presented, it is evident that gold compounds have immunomodulatory or immunoregulatory activity and that this activity may contribute greatly to their
beneficial effects in treatment of rheumatoid arthritis. Most researchers have looked at the effects of gold compounds using cells from human peripheral blood. It seems reasonable though, to look at the effects of gold compounds using cells from the reticuloendothelial system, namely the spleen, where gold has a tendency to concentrate (7,51).

The present project employed spleen cells of Balb/c mice and three different cell populations of intact, adherent, and nonadherent were tested for the following:

1. *in vitro* effects of gold sodium thiomalate on the mitogen-induced blastogenesis
2. *in vitro* effects of gold sodium thiomalate on the levels of cyclic-AMP
3. *in vitro* effects of gold sodium thiomalate on the chemotaxis ability

It has been shown that gold binds to the membrane of lymphocytes (60) and specifically interferes with an early step in the inductive phase of lymphocyte activation even before DNA synthesis begins (56). The question was asked: "Do gold compounds affect different cell functions by interfering with the activation of the second messenger system, cyclic AMP dependent-adenylate cyclase?". To answer this question, the cyclic AMP levels were determined under the influence of gold. To confirm the obtained levels of cyclic AMP, one of the end results of such levels, namely chemotaxis ability of the cells, was tested.
MATERIALS AND METHODS

Cell Source

Male or female balb/c mice four to six weeks of age (Harlan, Sprague, Dawley, Indianapolis, Indiana) were killed by cervical dislocation and the spleens were aseptically harvested. Single-cell suspension was made by grinding the spleens in Hanks Balance Salt Solution (HBSS) (Gibco Labs, Chagrin Falls, Ohio), pH 7.2, using a tissue grinder. The mononuclear cells were separated by pipeting 3 ml of Lymphocyte Separation Media (LSM) (Bionetics, Kensington, Maryland) into a 15 ml conical tube (Corning) and gently layering 10 ml of single-cell suspension on top of the LSM. The tubes then were centrifuged at 1400 RPM for 30 min (brake off). The "buffy coats" at the LSM and HBSS interface were pooled from all the tubes and washed twice using HBSS (1800 RPM for 10 min). At the end of the second wash, the tube was decanted and blotted on a sterile gauze. The cell button was resuspended in 2 ml of RPMI-1640 (Flow Labs, McLean, Virginia) containing added L-glutamine (2 mM), penicillin G (Sigma, St. Louis, Missouri) (100 µg/ml), streptomycin (100 µg/ml), 10% fetal-ovine serum (heat inactivated at 56°C for 30 min) pH 7.2. The concentration of
cells was determined using a Coulter counter (model ZBI) and adjusted to $5 \times 10^7$ cells/ml using RPMI-1640.

Cell Viability

The percentage of viable white cells can be determined by staining cells with trypan blue. Viable cells exclude the dye, while nonviable cells take up the dye. After being stained with trypan blue, the cells must be counted within three minutes using a hemacytometer. The staining procedure (65) is as follows: One part trypan blue [0.2% (w/v) in distilled water; Flow Laboratories] was mixed with one part cell suspension and the hemacytometer was loaded. The number of viable and nonviable cells were counted per 200 cells. Then the percent viability was calculated. Cell suspensions with 80% or greater viability were used for all the experiments.

Cell Populations

The adherent property of monocytes was used to remove these cells from lymphoid cell populations. A modification of the method described by Mishell and Herscomitz (39,65) was used to separate the mononuclear spleen cells into adherent and nonadherent populations. The nonadherent population obtained by this method, according to Mishell (65) contained 98% T cells. The adherent population contained 8-10% macrophages as determined by nonspecific esterases stain described by Lamand Yam (49).
Briefly, a 35 ml syringe was packed to 18 ml mark using 1.8 gm of nylon wool, an 18 gauge needle was attached to a 3-way stopcock which in turn was attached to the syringe. The column was then autoclaved at 110°C for 15 min. After autoclaving, the column was washed with 100 ml of RPMI-1640 and incubated at 37°C at least 1 hr prior to the addition of the cells. The stopcock was opened and the column was allowed to run dry. After the stopcock was closed, 5 ml of single-cell suspension at 5 x 10^7 cells/ml was added to the column. The cells were allowed to penetrate, then an additional 1 ml RPMI-1640 was added and the column was incubated for 1.5 hrs at 37°C. At the end of the incubation period, the nonadherent cells were eluted by dropwise addition of 60 ml prewarmed RPMI-1640. Care was taken not to generate a fluid head by adjusting the elution speed to one drop per second. The adherent cells were recovered by addition of 10 ml cold (4°C) HBSS without Ca^{++} and Mg^{++} to the column and compression of the nylon wool with a sterile syringe barrel. This was repeated four times and the cells were pooled. Both populations were centrifuged and the cell concentrations were adjusted to 5 x 10^6 cells/ml for further use.

Blastogenesis Assay

Blastogenesis assays were performed aseptically using 96-well flat-bottomed culture plates (Corning). Appendixes A
and B show the details of how each well was set-up. Briefly, each test well received 0.1 ml of proper cell population (intact, nonadherent, or adherent lymphocytes) at concentration of $5 \times 10^6$ cells/ml, 0.1 ml of mitogen (Concanavalin A, Sigma, Lot #77C-7390), and 0.010 ml of GST (Myochrysine, Merck, Sharpe and Dohme, West Point, Pennsylvania). Con A control wells received only mitogen and cells, gold sodium thiomalate (GST) control wells received only GST and cells, and cell control wells received only cells and media. The plates were agitated using a micro-shaker for 18 sec and then incubated at $37^\circ C$ with 5% CO$_2$ for 72 hrs. The cultures were pulsed with 0.5 μCi $^3$H thymidine (ICN, Irvine, California, Lot #2093112, 6.7 μCi/mM) after 66 hrs of incubation. The cells then were harvested onto glass fiber filter paper using a Tetrak harvester and the filter papers were allowed to air dry before they were placed in scintillation vials. Four ml of toluene-based scintillation fluid (3.8 liters of toluene, 19 gms 2,5-diphenyloxazol, 1.14 gms dimethyl POPOP) were added to each vial and counted for one minute on a Packard Liquid Scintillation Spectrometer.

**Intracellular Cyclic AMP Assay**

The assay of cyclic AMP is based upon the work of Gilman (29). The cyclic AMP kit was developed by Amersham Corporation (2636 South Clearbrook Drive, Arlington Heights,
Illinois, 60005). This assay was performed using the optimum mitogen concentration determined by blastogenesis assay (7.5 µg/ml). Briefly, each Eppendorf micro test tube received 0.2 ml of proper single-cell suspension (5 x 10⁶ cells/ml) 0.2 ml of mitogen and 0.020 ml of GST. The GST control tubes received only GST and cells. Con A control tubes received Con A and cells, and the cell control tubes received cells and media only. Appendix C shows the contents of each test and control tubes. The tubes were incubated for 10 min at 37°C with 5 percent CO₂. At the end of the incubation period, the tubes were placed in 4°C ice bath to halt the enzymatic reaction and then the reaction was stopped by membrane disruption using ultrasound (74). The tubes were sonified for 1 min. After sonification, 0.6 ml of 20 percent trichloroacetic acid was added to each tube and the tubes were incubated at 4°C for 30 min. The tubes then were centrifuged for 10 min using an Eppendorf microfuge. The supernatants were placed in 13 x 100 mm test tubes and washed five times with 2 ml of water saturated ether (add 2 ml of ether, vortex, discard top layer) (14,17,86). To remove traces of ether, the tubes were placed under a stream of nitrogen. At this point, the procedure given in the instruction pamphlet on pages 10-11, steps 1-12 provided with the kit was followed:

"1. Label a number of small tubes sufficient for the number of unknowns to be run in triplicate and an
additional 14 tubes which will be used for standards and blanks. Place the tubes in racks in a 0°C water bath.

2. Pipette 150 µl of the buffer, reagent 1, into the assay tubes 1 and 2. These tubes are for the determination of the blank counts for the assay.

3. Pipette 50 µl of the buffer, reagent 1, into the assay tubes 3 and 4 for determination of binding in the absence of unlabeled cyclic AMP.

4. Starting with the lowest level of standard cyclic AMP, add 50 µl of each dilution into each successive pair of assay tubes (tubes 5-14).

5. Add 50 µl of each unknown, in triplicate, into the additional assay tubes as appropriate.

6. Add 50 µl of the labeled cyclic AMP, reagent 3, to every assay tube.

7. Add 100 µl of the binding protein, reagent 2, to assay tubes 3-14 and to every assay tube containing an unknown.

8. Vortex mix all tubes for about 5 seconds.

9. Place the ice bath containing the tubes into a cold room or refrigerator at 2 to 4°C and leave for 2 hours.

10. At least 15 minutes before the end of the incubation time, add 20 ml of ice cold distilled water to the
charcoal reagent, stand the container in an ice bath and stir the reagent continuously.

11. Remove ice bath containing the assay tubes from the cold room and replenish the ice if necessary.

12. Add 100 µl of the charcoal suspension to all tubes, vortex mix for 10-12 seconds and replace tubes in ice bath or refrigerated centrifuge. Do not add charcoal to more tubes than can be centrifuged in one batch."

The tubes were then centrifuged for 2 min using an Eppendorf microfuge. Without disturbing the sediment, 0.2 ml of supernatant was removed from each tube and placed in 5 ml of water base scintillation fluid and counted for 4 min using a Packard Liquid Scintillation Spectrometer. The calculation procedure given on page 12 of the pamphlet provided with the kit was used to determine the concentration of cAMP in each sample tube as outlined below:

"1. To determine the blank counts per minute (CPM) for the assay, average the CPM for tubes 1 and 2.

2. Average the CPM for tubes 3 and 4 and subtract the blank CPM. This result is the CPM bound in the absence of unlabeled cyclic AMP (Co).

3. Average the CPM for each pair of duplicates in tubes 5-14 and the additional pairs of tubes which contain unknowns. Subtract the blank CPM from each result
to give the CPM bound in the presence of standard or unknown unlabeled cyclic AMP (Cx).

4. Calculate \( \frac{C^O}{C^x} \) for each level of cyclic AMP and the unknowns.

5. Plot \( \frac{C^O}{C^x} \) against picomoles of inactive cyclic AMP per tube on linear graph paper. A straight line should be obtained with an intercept of 1.0 on the ordinate.

6. From the \( \frac{C^O}{C^x} \) value for the unknown read the number of picomoles of cyclic AMP from the standard curve."

Chemotaxis Assay

Chemotaxis assays were performed using a modification of Boyden's original technique, which studies the unidirectional locomotion of a cell population (11). The chemoattractant (Zymosan-activated allogeneic serum) was prepared following Herscomitz's procedure (38). In short, 5 mg/ml serum incubated 1 hr at 37°C, 30 min at 56°C, centrifuged at 300 x g and pellet discarded, used at 20 percent V/V. The assay employed blind-well chemotaxis chambers (Neroprobe Corp.), 0.1 ml volume size and polyvinylpyrrolidone micropore filters (Neroprobe Corp.), 5 µm pore size; 13 mm diameter (#NMF-5 µm-PVRF). Appendix D shows how each chamber was set up. Briefly, the lower compartments of the plastic chambers were filled with chemoattractant or media alone, the
micropore filters were placed between the lower and the upper compartments. Then the upper test compartments were filled with 0.1 ml proper cell suspension at concentration of $5 \times 10^6$ cells/ml, 0.1 ml Con A (7.5 µg/ml), and 0.010 ml GST. The upper compartment of the control chambers received only 0.1 ml of Con A and 0.1 ml of cell suspension. The upper compartment of random chamber received cells and Con A only. The chambers then were incubated for 4 hrs at 37°C and 5% CO$_2$ in humidified air. At the end of the incubation period, the filters were removed and stained using the following procedure (11): methanol, 5 sec.; rinse in distilled water; Erhlich's hemotoxylin, 6 min; rinse in distilled water twice; 70% ethanol, 1 min; 95% ethanol, 1 min; absolute ethanol 1 min; clear in xylene, 40 sec; air dry. The filters were than mounted on glass slides. The chemotactic cell activity was expressed as the total number of cells that migrated completely to the lower surface of the micropore filter in 20 oil fields (75).

Statistics

Data are given as mean ± 1 S.D. Results were analyzed by mean of student's two-tailed t test (available in any statistics textbook). P values < 0.05 were regarded as significant.
RESULTS

Blastogenesis Assay

The blastogenesis assays using normal murine intact mononuclear spleen cells were conducted to determine the concentration of Con A under which the maximum percent inhibition by GST is obtained. Table II shows the counts per minute (CPM) after seventy two hours incubation with GST.

### TABLE II

**THE EFFECTS OF GOLD SODIUM THIOMALATE ON $[^3H]$ THYMIDINE UPTAKE BY MITOGEN STIMULATED MONONUCLEAR MURINE SPLEEN CELLS***

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Concentration of ion Au pg/ml</th>
<th>Con A** pg/ml</th>
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<tr>
<td></td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>IM*** Spleen Cells</td>
<td>3112±223</td>
<td>560±35</td>
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<td></td>
<td>186±15</td>
<td>180±16</td>
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<td>885±53</td>
<td>458±32</td>
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<td>Adherent Spleen Cells</td>
<td>5904±201</td>
<td>1755±36</td>
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<td>388±21</td>
<td>309±18</td>
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</tbody>
</table>

*Each value represents mean cpm ± SD of six flat bottomed wells containing $5 \times 10^5$ responding cells.

**Concanavalin A

***Intact Mononuclear
These CPM values were used to calculate the percent inhibition given in Tables III and IV. Table III shows that at Con A concentration of 3.8 µg/ml the maximum percent inhibition was obtained.

**TABLE III**

GOLD SODIUM THIOMALATE INHIBITION OF OPTIMAL AND SUBOPTIMAL MITOGENIC STIMULATION OF NORMAL MURINE INTACT MONONUCLEAR SPLEEN CELLS

<table>
<thead>
<tr>
<th>Au ion Concentration µg/ml</th>
<th>Lymphocyte DNA Synthesis* (percent inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concanavalin A Concentration µg/ml</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>61</td>
</tr>
<tr>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>50</td>
<td>82</td>
</tr>
</tbody>
</table>

*[^3]H Thymidine incorporation observed in cultures containing 5 x 10^5 responding cells per flat-bottomed well. Each value represents the mean of six determinations.

When suboptimal concentrations of mitogen were employed, the degree of GST inhibition was decreased. Table IV shows that GST also, inhibited the proliferative responses of normal murine adherent and nonadherent spleen cells induced by the mitogen Con A (3.8 µg/ml).
TABLE IV
GOLD SODIUM THIOMALATE INHIBITION OF MITOGEN-STIMULATED MURINE SPLEEN CELLS PROLIFERATION, USING CONCANAVALIN A AT 3.8 µg/ml

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Percent Inhibition*</th>
<th>Au ion concentration µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact Mono-nuclear Spleen</td>
<td>87</td>
<td>5</td>
</tr>
<tr>
<td>Cells</td>
<td>94</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>50</td>
</tr>
<tr>
<td>Nonadherent Spleen Cells</td>
<td>160</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>163</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>188</td>
<td>50</td>
</tr>
<tr>
<td>Adherent Spleen Cells</td>
<td>75</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>102</td>
<td>50</td>
</tr>
</tbody>
</table>

*[^3]H] Thymidine incorporation observed in cultures containing 5 x 10⁵ responding cells. Mitogen-stimulated cultures were incubated for 3 days and were pulsed at 66 hours of incubation.

The percent inhibition increased as a function of gold concentration (Figure 2) and it was calculated using the following formula:

\[
\% \text{ Inhibition} = (1 - \frac{\text{CPM}_t}{\text{CPM}_c}) \times 100
\]

Where:

- \(\text{CPM}_t\) = mean CPM from six wells containing gold and Con A (test wells)
- \(\text{CPM}_c\) = mean CPM from six wells containing Con A (gold control wells)
- \(\text{CPM}_c\) = mean CPM from six wells containing cells only (cell control wells)
- \(\text{CPM}_c\) = mean CPM from six wells containing cells only (cell control wells)
Fig. 2--Percent inhibition of $[^3$H]$\text{thymidine}$ incorporation increases as the concentration of Au increases. The intact, adherent and nonadherent populations have the same pattern of inhibition. The Con A concentration for all three populations is 3.8 $\mu$g/ml. The data presented is the mean of six flat-bottomed wells containing $5 \times 10^5$ cells per well.
Cyclic AMP Assay

All three populations were tested for the effects of GST on the intracellular levels of cyclic AMP at the optimum mitogen (Con A) concentration (3.8 μg/ml). The calculation scheme provided in the methodology section was used to calculate \( \frac{C_0}{C_x} \) ratio where \( C_0 \) = the CPM found in the absence of unlabelled cyclic AMP and \( C_x \) = the CPM bound in the presence of standard or test (unknown) unlabelled cyclic AMP. The \( \frac{C_0}{C_x} \) ratio was determined for each level of cyclic AMP standards and unknowns; and plotted against picomoles of inactive cyclic AMP per tube on linear graph paper. A best fitted straight line was obtained with an intercept of 1.0 on the ordinate. From the \( \frac{C_0}{C_x} \) value for the test and control tubes the number of picomoles of cyclic AMP was of the standard curve. The cyclic AMP levels are presented in Table V.
<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Concentration of Au pg/ml</th>
<th>Con A** µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>IM***</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Spleen Cells</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Nonadherent</td>
<td>4.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Spleen cells</td>
<td>6.1</td>
<td>2.9</td>
</tr>
<tr>
<td>Adherent</td>
<td>3.4</td>
<td>12.8</td>
</tr>
<tr>
<td>Spleen Cells</td>
<td>4.8</td>
<td>5.1</td>
</tr>
</tbody>
</table>

*Each term represents the mean of triplicate determination.

**Concanavalin A

***Intact Mononuclear

The Con A control tubes indicate a decrease in the level of cyclic AMP caused by the Con A present in the tubes. This effect is the same for all populations. However, when the GST is added the three populations behave differently. The non-adherent cell population shows an increase in the levels of intracellular cyclic AMP as the concentration of GST increases. This increase is large enough to mask the decreasing effect of Con A on the cyclic AMP levels. The GST have a biphasic effect on the adherent population. At concentrations of 5 and 10 µg/ml GST suppresses the cyclic AMP levels, and at concentration of 50 µg/ml it enhances the
cyclic AMP production. The cyclic AMP levels of the intact cell population was not affected by GST (Figure 3).

Chemotaxis Assay

The data presented in Table VI is derived from six individual chemotaxis chambers. The spontaneous migration of the cell preparation was assessed by determining the number of cells that had migrated toward the lower chamber filled with medium alone. This value was subtracted from the response of the cell suspension to the chemotactic factor activity. GST appears to have an inhibitory effect on the chemotaxis ability of all three populations of spleen cells. However, the inhibition is more pronounced in the intact mononuclear spleen cells (Figure 4).
Fig. 3--The effects of GST on the levels of intracellular cyclic AMP. Each point represents mean of triplicate determination.
TABLE VI

THE EFFECT OF GOLD SODIUM THIOMALATE ON CHEMOTAXIS MIGRATION OF MURINE SPLEEN CELLS*

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Concentration of Au ion (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>0</strong></td>
</tr>
<tr>
<td>IM*** Spleen Cells</td>
<td>46.7±6.5</td>
</tr>
<tr>
<td>Nonadherent Spleen Cells</td>
<td>72.0±7.8</td>
</tr>
<tr>
<td>Adherent Spleen Cells</td>
<td>105.7±30</td>
</tr>
</tbody>
</table>

*Mean of six individual chemotaxis chambers.

**Random migration for intact mononuclear spleen cells is 8.5 ± 0.5; for nonadherent cells is 0.5 ± 0.5; for adherent cells is 18.5 ± 2.5. These values were subtracted from the test and control chambers.

***Intact Mononuclear

a: Compare to control  p < 0.001

b: Compare to control  p < 0.005
Fig. 4--The GST has an inhibitory effect on the migration of the intact, adherent and nonadherent spleen cells.
DISCUSSION

A number of clinical trials have demonstrated that chrysotherapy leads not only to amelioration of the symptoms of rheumatoid arthritis but also to actual remissions of disease activity (70-72,76). Since the pathogenesis of chronic rheumatoid arthritis involves immunologic mechanisms (36), it seems logical to suggest that the therapeutic efficacy of gold compounds lies in their ability to modulate immune responsiveness. A number of studies have shown that gold compounds inhibit nonspecific inflammatory responses in a variety of experimental models (46,78,80). However, the evidence as to whether these agents exert a direct effect on lymphocyte responsiveness remains conflicting (82).

To evaluate the effects of gold compounds observed in vitro such effects should be tested with concentrations attainable in treated patients. A number of studies have quantitated the levels of gold present in various body fluids and tissues after therapy with different gold compounds. The results indicated that the concentration of gold in the blood does not correlate with either the therapeutic or toxic effects of gold compounds in man (8,28,45,63,73), suggesting that the anti-inflammatory action of gold compounds is not related to serum gold levels. Gold compounds following
administration, rapidly distribute throughout the body with a tendency to concentrate in areas of inflammation as well as in organs rich in reticuloendothelial elements (7,51). Thus, tissue gold levels provide more meaningful estimation of both pharmacologically attainable and therapeutically effective gold concentrations. Mean synovial tissue gold concentrations of 21.1 µg/g of tissue (wet weight) have been reported in a group of 14 patients recently treated with a mean total dose of 1.84 g of gold (32). These findings are consistent with those reported in a patient who had received a total gold dose of 2,530 mg over a five year period and at postmortem examination had a mean synovial tissue gold concentration of 25 µg/g wet weight (32). These data suggest that the maximal concentration of gold attainable at the relevant tissue site is equivalent to 42-50 µg/ml of GST (56).

GST was found to inhibit mitogen-induced murine spleen lymphocyte proliferation. This finding is similar to results reported by others in human lymphocytes, using Con A (56), PHA (55), and allogeneic lymphocytes (37) as stimuli. Inhibition of responsiveness was dependent on the concentration of GST in the culture with significant suppression observed well within the range of serum and tissue levels found in patients treated with gold compounds. This is also similar to observation made by Lipsky (56).
GST was found to inhibit the mitogen-induced proliferation of murine spleen adherent and nonadherent cell populations. Inhibition of responsiveness was directly related to the concentration of GST present in the culture. These findings in the nonadherent population disagree with the observation of Petersen (69). He showed that highly purified human T cells pulsed with aurothiomalate were resistant to the gold salt. However, the findings concerning the adherent population were in agreement with Petersen (69).

It has been shown that the fluidity of the membrane increases within minutes after the addition of mitogen to lymphocytes (33). Several mechanisms have been proposed to explain the transmission of these membrane changes to an intracellular process that initiates cell activation. One of these has focused on the role of cyclic nucleotides as "second messengers" through their activation of specific cyclic nucleotide-dependent protein kinases. While the expression of some genes is dependent upon elevated levels of cyclic AMP, the expression of other genes can be antagonized by similar levels of this cyclic nucleotide (33). It has been shown, for example, that cyclic AMP can either inhibit (53,64) or stimulate (35,54) the formation of prostaglandins and thromboxanes in various cells. Moreover, following mitogen stimulation of lymphocytes, a decrease in intracellular levels of cyclic AMP occurs, but intracellular levels of cyclic GMP may rise fifty-fold (3). It is known
that agents that increase levels of intracellular cyclic AMP also suppress mitogen-induced lymphocyte proliferation (3).

Although cAMP has an essential role to play in certain cells, there is increasing evidence that many effects of cAMP are mediated indirectly through effects on Ca$^{2+}$ (5). The ability of a variety of mitogens to stimulate cell division in lymphocytes appears to depend on an early rise in the intracellular level of Ca$^{2+}$ (6). During the action of mitogens, such as phytohemagglutinin and concanavalin A, there is an increase in the uptake of Ca$^{2+}$ (25). When a wide range of compounds was tested, a close correlation was shown between the ability of agents to stimulate both the uptake of Ca$^{2+}$ and cell division (67). The increase in Ca$^{2+}$ concentration which is thought to occur during the action of mitogens, may account for the observed increases in cGMP levels (34). Ca$^{2+}$ concentration and cell division may be linked through cGMP which may initiate early events in the cell cycle by phosphorylating nuclear acidic proteins (47). There also appears to be an important interaction operating between cAMP and Ca$^{2+}$. Elevation of the intracellular level of cAMP by direct application of the nucleotide or by treatment with prostaglandin E, cholera toxin or theophyline can prevent lectin-induced transformation of lymphocytes (19,85). This ability to inhibit cell division seems to depend on the ability of cAMP to inhibit the influx of Ca$^{2+}$ as described earlier (25). It was, therefore, of interest to
study the effects of gold sodium thiomalate on the intracellular levels of cyclic AMP. The results obtained from these studies indicated a strong agreement with Bellanti (3) showing an increase in the levels of cyclic AMP in the nonadherent cell population which in turn explains the inhibition of mitogen-induced lymphocyte proliferation. This explanation also holds true for the adherent population at gold concentration of 50 µg/ml. The unchanged levels of cyclic AMP in the intact spleen lymphocytes, however, remains unexplainable.

The increase observed in the cyclic AMP levels in nonadherent and adherent cell populations, as was mentioned before, points to a possible effect at the membrane level interfering with the activation of the enzyme adenyl cyclase. In this direction, Gallin and coworkers have shown that agents that increase cyclic AMP inhibit accumulation of cGMP and depress human monocyte locomotion (26). This finding is similar to the findings in the present project using murine spleen cells. All three populations; intact, adherent and nonadherent showed an inhibitory effect caused by GST on the chemotaxis ability of cells.

From the findings in the present project and what already exists in the literature, it seems reasonable to suggest that the therapeutic efficacy of gold compounds may relate to their ability to bind to the mononuclear cell membranes (60) disturbing the delicate balance between intracellular cyclic
AMP and cyclic GMP levels. Depending on the system being considered, Ca\(^{2+}\) increases or decreases cAMP and cGMP levels by activating or inhibiting adenylate cyclase and guanylate cyclase respectively. Therefore, monitoring the levels of cGMP and Ca\(^{2+}\) in the lymphocytes under the influence of gold compounds should be considered as a step forward for future research. Up to the present time, the studies have tended to concentrate on individual second messengers, but the above discussion should underline the importance of integrated studies which consider not a single component of the control loop in isolation but rather the dynamic structure of the overall system.
<table>
<thead>
<tr>
<th></th>
<th>GST 1 mg/ml</th>
<th>GST 0.2 mg/ml</th>
<th>GST 0.04 mg/ml</th>
<th>No GST</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Con A</strong></td>
<td>Test</td>
<td>Test</td>
<td>Test</td>
<td>Con A control</td>
</tr>
<tr>
<td>5 µg/ml</td>
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<td>Test</td>
<td>Test</td>
<td>Con A control</td>
</tr>
<tr>
<td>7.5 µg/ml</td>
<td>Test</td>
<td>Test</td>
<td>Test</td>
<td>Con A control</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>Test</td>
<td>Test</td>
<td>Test</td>
<td>Con A control</td>
</tr>
<tr>
<td>No Con A</td>
<td>GST control</td>
<td>GST control</td>
<td>GST control</td>
<td>Cell control</td>
</tr>
</tbody>
</table>

Appendix A--Culture plan for intact and nonadherent spleen cells. Each square represents a well in a 96 well microtiter plate.
Appendix B—Culture plan for adherent spleen cells. Each square represents a well in a 96 well microtiter plate.
<table>
<thead>
<tr>
<th>GST 1 mg/ml</th>
<th>GST 0.2 mg/ml</th>
<th>GST 0.04 mg/ml</th>
<th>No GST</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5 µg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con A</td>
<td>Test</td>
<td>Test</td>
<td>Test</td>
</tr>
<tr>
<td>No Con A</td>
<td>GST control</td>
<td>GST control</td>
<td>GST control</td>
</tr>
<tr>
<td>7.5 µg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con A</td>
<td>Test</td>
<td>Test</td>
<td>Test</td>
</tr>
<tr>
<td>No Con A</td>
<td>GST control</td>
<td>GST control</td>
<td>GST control</td>
</tr>
<tr>
<td>7.5 µg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con A</td>
<td>Test</td>
<td>Test</td>
<td>Test</td>
</tr>
<tr>
<td>No Con A</td>
<td>GST control</td>
<td>GST control</td>
<td>GST control</td>
</tr>
</tbody>
</table>

Intact Mononuclear Spleen Cells

Nonadherent Spleen Cells

Adherent Spleen Cells

Appendix C--Cyclic AMP assay set up plan. Each square represents an Eppendorf micro test tube.
### Appendix D -- Chemotaxis assay set up plan for the chemotaxis chambers.

Each square represents an upper compartment of a chemotaxis chamber. All the lower compartments contain chemoattractant except for the random migration chamber that contains media alone.

<table>
<thead>
<tr>
<th>GST 1 mg/ml</th>
<th>GST 0.2 mg/ml</th>
<th>GST 0.04 mg/ml</th>
<th>No GST</th>
</tr>
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<tbody>
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<td>Test</td>
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</tbody>
</table>

- **Intact mononuclear Spleen Cells**
- **Adherent Spleen Cells**
- **Nonadherent Spleen Cells**
BIBLIOGRAPHY


