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Research Article

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Inhibition of Antigen- and Mitogen-Induced Human Lymphocyte Proliferation by Gold Compounds

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ABSTRACT Gold sodium thiomalate (GST) inhibited *in vitro* antigen- and mitogen-triggered human lymphocyte DNA synthesis. Inhibition of responsiveness was observed with concentrations of GST equivalent to gold levels found in serum or tissues of patients receiving chrysotherapy. Inhibition was dependent upon the gold ion itself since GST and gold chloride were both inhibitory whereas thiomalic acid was not. Inhibition could not be explained by nonspecific killing of cells or by an alteration in the kinetics of the responses. GST inhibited mitogen-induced proliferation most effectively when present from the initiation of culture and could not inhibit the responsiveness of cells which previously had been activated by concanavalin A. These findings indicated that GST blocked a critical early step in lymphocyte activation. The degree of GST-induced inhibition of proliferation was increased in cultures of cells partially depleted of monocytes. Moreover, inhibition was reversed by supplementation of these cultures with purified monocytes. These observations suggested that GST blocked thymus-derived (T)-lymphocyte activation by interfering with a requisite function of the monocyte population in initiating such responses. Prolonged incubation of peripheral blood mononuclear cells with GST resulted in diminished mitogen responsiveness upon subsequent culture in the absence of gold. The addition of fresh monocytes restored responsiveness to these populations. Furthermore, preincubation of purified monocytes with GST rendered them deficient in their ability to support mitogen-induced T-lymphocyte proliferation

on subsequent culture. These observations indicate that the major effect of GST results from interference with the functional capability of the monocyte population.

INTRODUCTION

Rheumatoid arthritis is a disease characterized by chronic inflammation of synovial tissues. Although its etiology remains unknown, immunological processes have been implicated in the pathogenesis and maintenance of the chronic synovitis (1). Thus, cells of bone marrow origin, along with their secretory products, play a role in this process as indicated by the presence, within rheumatoid synovial tissue, of cells which synthesize and secrete both immunoglobulin and rheumatoid factor locally (2-4). More recently, a role for thymus-derived (T)¹ lymphocytes also has been suggested by the demonstration of large numbers of T lymphocytes as compared to bone-marrow derived lymphocytes (B lymphocytes), both in synovial fluid (5-7) and digests of synovial tissue (8, 9).

Chrysotherapy has been used in the treatment of rheumatoid arthritis for nearly 50 years. Recent clinical trials have established that treatment with gold compounds not only can ameliorate the symptoms of rheumatoid arthritis, but also can slow or even halt the progress of the disease (10-13). These observations imply that gold compounds exert a potent anti-inflammatory effect *in vivo*. Since immune mechanisms

¹Abbreviations used in this paper: B lymphocyte (or B cell), bone-marrow derived lymphocyte; Con A, concanavalin A; Δ cpm, difference in counts per minute between the means of triplicate-stimulated and control cultures; FBS, fetal bovine serum; GST, gold sodium thiomalate; 2-ME, 2-mercaptoethanol; MLR, mixed leukocyte reaction; M Φ , monocyte or macrophage; PBL, peripheral blood lymphocyte depleted of adherent cells; PBM, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SK/SD, streptokinase-streptodornase; T lymphocyte (or T cell), thymus-derived lymphocyte; TMA, thiomalic acid.

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are thought to mediate the chronic inflammatory synovitis of rheumatoid arthritis, it seems reasonable to suggest that the therapeutic efficacy of gold compounds may relate to their ability to modify immunological processes.

The studies reported here were undertaken, therefore, to delineate the effect of gold compounds on certain *in vitro* correlates of immune responsiveness. Specifically, the action of gold sodium thiomalate (GST) has been investigated, with respect to its ability to modify *in vitro* antigen- and mitogen-induced proliferative responses in populations of normal human peripheral blood mononuclear cells. These proliferative responses share two characteristics. First, the primary responding cells are T lymphocytes (14–16), and, second, the initiation of each of these responses is dependent upon the active participation of a nonresponding accessory cell (17–22). In populations of human peripheral blood mononuclear cells, the monocyte subserves this function (15, 18, 19, 21, 22).

GST inhibited antigen- and mitogen-induced human lymphocyte proliferation. Inhibition, which was mediated by the gold moiety itself and not the sulfur-containing ligand, was concentration dependent and demonstrable within the range of serum or tissue levels found in patients treated with gold compounds. Two major effects of GST were observed. The first was a reversible interference with lymphocyte activation which required the presence of the gold compound in the culture medium. The second was an irreversible diminution in the magnitude of responsiveness which developed after prolonged exposure to GST, but no longer required the presence of the gold compound in the culture to be manifest. Data are presented to indicate that neither effect resulted from an action of GST on the potential responsiveness of the lymphocyte population *per se*. Rather, in both cases, GST interfered with the ability of monocytes to serve as effective accessory cells in the initiation of these proliferative responses.

METHODS

Cell preparation. Venous blood was obtained from healthy young adults who were taking no medications at the time of study. The mononuclear cell population was obtained from heparinized whole blood by centrifugation on sodium metrizoate/Ficoll cushions (Lymphoprep, Nyegaard & Co., Oslo, Norway) as previously described (23). Resultant preparations of peripheral blood mononuclear cells (PBM) consisted of 70–85% lymphocytes and 15–30% monocytes.

Mitogens and antigens. The following stimuli were used: concanavalin A (Con A, ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp. Cleveland, Ohio), purified phytohemagglutinin (PHA, The Wellcome Research Laboratories, Beckenham, England), pokeweed mitogen (PWM, Grand Island Biological Co., Grand Island,

N. Y.), streptokinase-streptodornase (SK/SD, Lederle Laboratories, Pearl River, N. Y.).

Gold compounds. The following compounds were utilized: gold sodium thiomalate (GST, Merck Chemical Div., Merck, Sharp & Dohme & Co., Inc., Rahway, N. J.), Gold trichloride ($\text{AuCl}_3 \cdot \text{HCl} \cdot 3\text{H}_2\text{O}$, J. T. Baker Chemical Co., Phillipsburg, N. J.), and *d, l*-thiomalic acid (TMA, Merck, Sharp & Dohme & Co., Inc.).

Miscellaneous reagents. The following reagents were used where indicated: 2-mercaptoethanol (2-ME, Eastman-Kodak Co., Rochester, N. Y.) and α -methyl-D-mannoside and mitomycin-C (Sigma Chemical Co., St. Louis, Mo.).

Culture medium. Cultures were carried out in medium RPMI-1640 (Microbiological Associates, Bethesda, Md.) supplemented with 0.3 mg/ml fresh *l*-glutamine, 10 $\mu\text{g/ml}$ gentamicin, and 200 U/ml penicillin-G. The medium was further supplemented with either 10% fetal bovine serum (FBS, Microbiological Associates) or 10% heat-inactivated, pooled human AB serum.

Separation of PBM into adherent and nonadherent populations. In certain experiments, PBM were separated into adherent and nonadherent populations. This was accomplished by suspending PBM ($5 \times 10^6/\text{ml}$) in medium RPMI supplemented with 10% FBS and incubating them in $100 \times 20\text{-mm}$ glass Petri dishes for 60 min at 37°C . After this incubation, the nonadherent cells were removed by washing with warm medium and then pooled. The cells adherent to the glass Petri dish were then reincubated in medium containing 10% FBS for 30 min at 37°C after which they were vigorously washed with four changes of warm medium to remove loosely adherent cells. The adherent cells remaining after these washings were then bathed in phosphate-buffered saline (pH 7.2), chilled to 4°C , and dislodged from the Petri dish with a rubber policeman. After washing, the cells were suspended in medium, exposed to 40 $\mu\text{g/ml}$ mitomycin-C for 60 min at 37°C , and washed four times. More than 90% of the resulting cell population was monocyte/macrophages as judged either morphologically with Euchrysin staining (24) or by determination of phagocytic cells using polystyrene latex particles. Viability was greater than 95% as estimated by trypan blue exclusion.

The nonadherent cells removed from the Petri dish on the first wash were pooled and further depleted of adherent cells by passage over nylon-wool (Fenwal Laboratories, Inc., Morton Grove, Ill.) columns, as previously described (20). The resultant population contained about 85–90% T lymphocytes as judged by rosette formation with sheep erythrocytes (25), and 0.1–0.8% monocytes as detected either morphologically with Euchrysin staining or by determination of phagocytically active cells.

Technique of cell culture and assay of lymphocyte DNA synthesis. Cultures were carried out in sterile microtiter plates with round-bottomed wells (Cooke Engineering Co., Alexandria, Va.). In some experiments, as noted in the text, cells were cultured in sterile microtiter plates with flat-bottomed wells (Falcon Plastics, Division of BioQuest, Oxford, Calif.). Routine cultures were done in triplicate, with each well containing 1×10^5 responding cells in 0.2 ml of culture medium. In some experiments, the number of cells cultured per well was varied; in others, the responding cells were supplemented with mitomycin-C-treated monocyte/macrophages. The stimulating agent or an equal volume of medium as control was added directly to the cultures at the indicated final concentrations. Two-way mixed leukocyte reactions (MLR) were set up by mixing 5×10^4 PBM from one individual with 5×10^4 PBM from another unrelated individual in triplicate 0.2-ml cultures. Mitogen-stimulated

TABLE I
GST Inhibition of Human PBM Proliferation

GST† concn. μg/ml	Lymphocyte DNA synthesis*			
	PHA (n = 15)	Con A (n = 19)	SK/SD (n = 4)	MLR (n = 4)
Δcpm §				
0	85,105±6,013	56,648±3,857	26,844±2,953	29,685±9,298
% Inhibition				
1	4.3±2.8	5.6±2.7	13.7±5.3	0.8±2.9
10	15.9±1.5	20.0±2.2	27.0±4.3	21.1±4.8
25	21.5±1.9	23.8±1.9	42.5±6.0	47.6±11.7
50	26.1±1.7	34.4±2.6	75.2±3.8	89.7±3.8
100	35.6±3.9	61.3±2.9	96.9±2.9	99.4±0.4

* [³H]Thymidine incorporation observed in cultures containing 1×10^5 responding cells per round-bottomed well using previously determined optimal concentrations of stimuli (PHA = 0.5 μg/ml, Con A = 5.0 μg/ml, SK/SD = 50 μg/ml). Mitogen-stimulated cultures were incubated for 3 days, antigen-stimulated cultures for 5 days, and MLR cultures for 7 days. Data are expressed as mean±SEM from *n* separate experiments.

† Refers to concentration of GST of which gold constituted 50.5%.

§ Background [³H]thymidine incorporation of unstimulated cultures from 19 separate experiments, Δcpm = 1,252±180. Background Δcpm in cultures containing GST: 1 μg/ml, 1,343±249; 10 μg/ml, 1,320±224; 25 μg/ml, 1,248±259; 50 μg/ml, 1,192±254; 100 μg/ml, 809±169.

^{||} Inhibition by 1 μg/ml GST not significant. Each response is significantly inhibited by all other concentrations of GST (10–100 μg/ml). Inhibition is significant at $P < 0.001$ (paired sample *t* test) for each situation except for inhibition of SK/SD response by 10 μg/ml GST ($P < 0.05$) and 25 μg/ml ($P < 0.01$), and inhibition of MLR by 10 and 25 μg/ml GST ($P < 0.02$).

cultures were carried out in medium supplemented with 10% FBS whereas antigen-stimulated and MLR cultures were supplemented with 10% human serum. Cultures were incubated (72 h for mitogen stimulation, 120 h for antigen stimulation, and 168 h for MLR) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. 18 h before harvesting, 1 μCi of tritiated thymidine (6.7 Ci/mM, New England Nuclear, Boston, Mass.) was added to each well. Cells were harvested onto glass fiber filter paper using a semi-automated microharvesting device. Tritiated thymidine incorporation was then determined by liquid scintillation spectroscopy. All data are expressed as the difference in counts per minute between the means of triplicate stimulated and control cultures (Δcpm). In some experiments, data are expressed as percent inhibition compared to control stimulation calculated according to the following formula: % inhibition = $(1 - [\Delta\text{cpm}_i / \Delta\text{cpm}_c]) \times 100$, where Δcpm_i = proliferative response in the presence of a putative inhibitor and Δcpm_c = proliferative response in control cultures.

RESULTS

GST inhibition of human lymphocyte proliferation. GST inhibited the proliferative responses of normal human lymphocytes induced by either of the mitogens PHA or Con A, the antigen SK/SD or allogenic lymphocytes in a MLR as depicted in Table I. The

degree of suppression observed was dependent on the concentration of GST, with significant inhibition seen with 10 μg/ml. The data shown in Table I were obtained using concentrations of mitogens or antigens which gave maximum responses in control cultures containing no gold. When suboptimal concentrations of mitogen were employed, the degree of GST inhibition of lymphocyte proliferation was markedly greater than that observed in cultures stimulated with concentrations yielding maximal [³H]thymidine incorporation (Table II). Moreover, the concentration or nature of the serum used to support the cultures appeared to have little effect on GST inhibition. Thus, varying the concentration of FBS from 5 to 20% had no significant effect on GST inhibition of the PHA response. Furthermore, GST suppression of PHA responsiveness was similar in cultures supported by either human serum or FBS (data not shown).

Experiments were carried out to determine whether GST caused an actual depression in the degree of mitogen-induced lymphocyte proliferation or merely altered the kinetics of these responses (Fig. 1). In these studies, PBM were cultured for 2–6 days with

TABLE II
GST Inhibition of Optimal and Suboptimal
Mitogenic Stimulation

GST concn. $\mu\text{g/ml}$	Lymphocyte DNA synthesis*			
	Con A		PHA	
	1 $\mu\text{g/ml}$	5 $\mu\text{g/ml}$	0.1 $\mu\text{g/ml}$	0.5 $\mu\text{g/ml}$
Δcpm				
0	15,315	49,800	51,198	63,220
% Inhibition				
10	48.9	19.5	28.4	13.9
25	68.8	42.1	50.5	34.8

* $[^3\text{H}]$ Thymidine incorporation observed in cultures containing 1×10^5 responding cells per round-bottomed well. Each term represents the mean of triplicate determinations.

PHA, Con A, or medium control in the presence or absence of $10 \mu\text{g/ml}$ GST, and $[^3\text{H}]$ thymidine incorporation was assayed daily. Lymphocyte DNA synthesis induced by PHA and Con A in the absence of GST is shown in Fig. 1 (insert) as a function of the length of culture. Peak response to both mitogens is seen after a 3-day incubation. GST-mediated suppression of each of these responses was observed throughout the period of observation. There was no tendency for the degree of lymphocyte proliferation in GST-inhibited cultures to approach that manifested by control cultures. On the contrary, as incubation was prolonged beyond 3 days, the degree of GST-mediated inhibition became more marked, suggesting the possibility that prolonged incubation in GST may have an adverse effect on lymphocyte responsiveness. This point will be addressed in more detail in a subsequent section. For the remaining experiments, a standard 3-day incubation was employed to analyze the effect of GST on mitogen stimulation.

To determine whether suppression of lymphocyte proliferation required the intact gold thio molecule or merely the sulfur-containing ligand, the inhibitory effect of GST was compared to that of thiomalic acid (TMA). Fig. 2 depicts the percent inhibition of the Con A response as a function of the molar concentration of each of these reagents. TMA caused no significant inhibition of the Con A response and GST produced significant suppression at concentrations as low as $26 \mu\text{M}$ ($10 \mu\text{g/ml}$).

The effect of gold chloride ($\text{AuCl}_3 \cdot \text{HCl} \cdot 3\text{H}_2\text{O}$) on Con A responsiveness was next examined to ascertain whether the inhibitory capacity of gold compounds was limited to those in which gold was complexed to a sulfur-containing ligand (Table III). The ability of gold chloride to inhibit Con A-induced lymphocyte proliferation was similar to that manifested

by GST at a low concentration ($10 \mu\text{g/ml}$) but exceeded the inhibitory capacity of GST at higher concentrations ($25, 50 \mu\text{g/ml}$). These data suggest that inhibition of lymphocyte proliferation induced by gold compounds is mediated by the gold moiety itself and is not dependent on the ligand.

Effect of immediate and delayed addition of GST on lymphocyte proliferation induced by Con A. Lymphocyte DNA synthesis assayed by $[^3\text{H}]$ thymidine incorporation is a relatively late occurrence in a complex series of biological events triggered by mitogens (26, 27). To determine whether there was a particular step in this sequence of events which was sensitive to inhibition by gold, GST was added to control and Con A-stimulated cultures either at the initiation of the incubation or at varying times thereafter (Fig. 3). The total length of incubation was 72 h for each experimental point. The stippled area indicates the Con A response of these cells (mean $\Delta\text{cpm} \pm \text{SEM}$) when incubated for 72 h with no added gold. When GST was added to the cultures at their inception, concen-

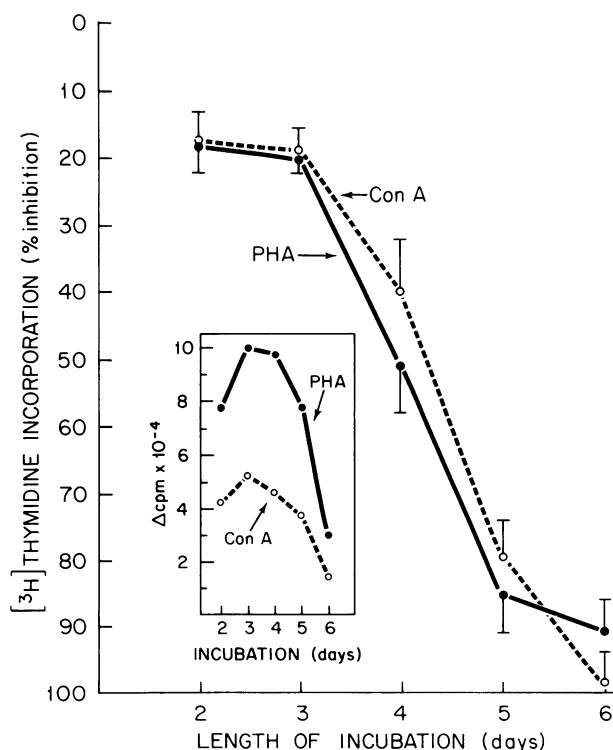


FIGURE 1 GST-mediated inhibition of the response of PBM to Con A and PHA as a function of the length of incubation. PBM (1×10^5 /well) were incubated with $5 \mu\text{g/ml}$ Con A, $0.5 \mu\text{g/ml}$ PHA, or an equal volume of medium as control for 2-6 days at 37°C in the presence or absence of $10 \mu\text{g/ml}$ GST and assayed for $[^3\text{H}]$ thymidine incorporation. Insert depicts proliferative response (Δcpm) of control cells cultured without gold. Each point represents the mean \pm SEM of three separate experiments.

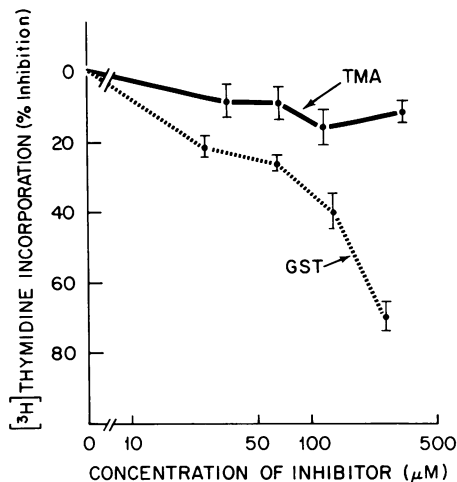


FIGURE 2 Comparison of the effect of GST and TMA on Con A-induced proliferative responses of PBM. Each point represents the mean \pm SEM of four separate experiments.

tration-dependent inhibition of the Con A response was observed with 25 μ g/ml resulting in a 30.8% inhibition, and 100 μ g/ml suppressing the response by 68.5%. When the addition of GST was delayed by progressively longer intervals from the beginning of the culture, as indicated on the abscissa, the degree of resultant inhibition became progressively less marked. There was no effect on proliferation when GST was present only for the terminal 24 h of culture. These findings indicate that GST inhibition does not result merely from a nonspecific blocking of either thymidine uptake or DNA synthesis by lymphocytes. Moreover, the observation that inhibition by gold was most effective when GST was present from the initiation of culture suggests that gold acts by blocking an early step in the sequence of events resulting in lymphocyte DNA synthesis. Similar results were observed when PHA was used as the stimulus or when gold chloride was used as the inhibitor (data not shown).

Inability of GST to inhibit DNA synthesis by Con A-activated lymphocytes. The previous experiments suggested that the major effect of gold was to inhibit an early step in the sequence of mitogen-triggered events resulting in lymphocyte DNA synthesis. To investigate this possibility further, the effect of GST on the degree of lymphocyte proliferation, manifested by a population of PBM previously activated by Con A in the absence of gold, was investigated (Table IV). PBM were incubated with 5 μ g/ml Con A or an equal volume of medium as control for 24 h at 37°C. At the end of this period of activation, the cells were incubated twice with 40 mM α -methyl-D-mannoside for 10 min at room temperature and washed to remove excess Con A (28). The cells

TABLE III
Gold Inhibition of Con A-Induced Lymphocyte Proliferation:
Comparison of GST and Gold Chloride

Concentration of inhibitor μ g/ml	$[^3\text{H}]$ Thymidine incorporation	
	GST*	Gold chloride†
	% Inhibition §	
10	17.0 \pm 3.2	14.5 \pm 4.5
25	24.8 \pm 4.7	52.0 \pm 8.3
50	42.3 \pm 6.6	91.4 \pm 3.3

* GST; gold content: 50.5%.

† Gold Trichloride ($\text{AuCl}_3 \cdot \text{HCl} \cdot 3\text{H}_2\text{O}$); gold content: 49.2%.

§ Each term represents the mean \pm SEM of five separate experiments.

then were suspended in fresh medium, supplemented with 10% FBS, and aliquoted into the wells of microtiter plates (1×10^5 /well). If the cells were assayed for $[^3\text{H}]$ thymidine incorporation at this point, no significant difference was noted between Con A-activated and control cells (data not shown). However, when the cells were incubated at 37°C for 48 h after the period of mitogen exposure, significant $[^3\text{H}]$ thymidine incorporation developed in the Con A-activated population ($\Delta\text{CPM} = 22,179 \pm 1,545$). As

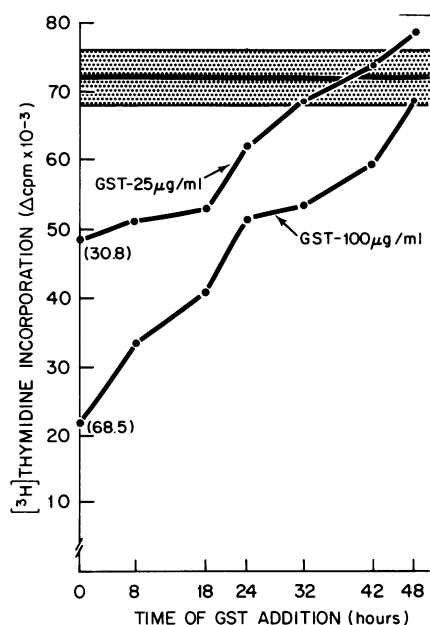


FIGURE 3 Effect of delayed addition of GST on Con A-induced lymphocyte DNA synthesis. PBM were cultured for 72 h with or without 5 μ g/ml Con A. The proliferative response of these cells cultured without GST is indicated by the stippled area ($\Delta\text{cpm} = 71,924 \pm 4,036$). 25 or 100 μ g/ml GST was added either at the initiation of culture or at varying times thereafter. Each point represents the mean of triplicate determinations.

TABLE IV
Failure of GST to Inhibit DNA Synthesis of Lymphocytes
Previously Activated by Con A

GST concn. during last 48 h of incubation	DNA synthesis by Con A-activated PBM* cultured with:	
	Medium	Con A
$\mu\text{g/ml}$	$[^3\text{H}]$ Thymidine incorporation- Δcpm	
0	22,179 \pm 1,545	95,995 \pm 2,589
10	20,952 \pm 1,421	82,626 \pm 3,058
25	22,162 \pm 2,643	76,637 \pm 2,988
50	21,155 \pm 2,523	65,525 \pm 1,352

* PBM were incubated in test tubes ($1 \times 10^6/\text{ml}$ in medium containing 10% FBS) with 5 $\mu\text{g/ml}$ Con A or an equal volume of medium as control for 24 h at 37°C. At the end of this incubation, the cells were washed with 40 mM α -methyl-D-mannoside, suspended in fresh medium containing 10% FBS, and aliquoted into the wells of microtiter plates ($1 \times 10^5/\text{well}$). After an additional 48-h incubation at 37°C with GST at the indicated final concentration, [^3H]thymidine incorporation was assayed. During the final 48 h, cultures were supplemented with either 5 $\mu\text{g/ml}$ Con A or an equal volume of medium. Δcpm indicates the difference in [^3H]thymidine incorporation between Con A-activated and control PBM. Mean [^3H]thymidine incorporation of control PBM was 1,121 cpm. This was not significantly affected by presence of GST during the final 48 h of incubation.

† Each term represents the mean \pm SEM of four separate experiments.

shown in Table IV, when varying concentrations of GST were present in cultures of Con A-activated or control lymphocytes during this latter 48-h incubation, no significant inhibition of DNA synthesis was observed. If an additional 5- $\mu\text{g/ml}$ Con A was added to the Con A-activated cells during the last 48 h of incubation, a marked increase in the degree of [^3H]thymidine incorporation was observed ($\Delta\text{cpm} = 95,995 \pm 2,589$). This may have resulted from the induction of a response in cells which had not been activated initially or perhaps by inducing daughter cells of the original Con A-activated population to divide again. In either event, GST caused concentration-dependent inhibition of [^3H]thymidine incorporation induced by the addition of Con A to these cultures without affecting DNA synthesis resulting from the initial Con A activation. These data indicate that GST inhibition of lymphocyte proliferation does not result from nonspecific toxicity or interference with the assay system employed. Moreover, the observation that the addition of GST after 24 h of mitogen activation does not alter subsequent lymphocyte DNA synthesis supports the concept that gold exerts its major influence during the initial induction phase of lymphocyte activation before DNA synthesis actually begins.

Reversibility of GST inhibition of lymphocyte proliferation. PBM were incubated with 5 $\mu\text{g/ml}$ Con A or an equal volume of medium as control for 24 h at 37°C in the presence or absence of various concentrations of GST. At the end of this period, the cells were washed with α -methyl-D-mannoside as previously described and then washed four times with medium to remove both excess Con A and GST. The cells then were suspended in fresh medium containing 10% FBS, aliquoted into the wells of microtiter plates ($1 \times 10^5/\text{well}$), incubated for an additional 48 h at 37°C, and assayed for incorporation of [^3H]thymidine. As seen in Table V, the presence of GST during the period of activation resulted in no diminution in subsequent [^3H]thymidine incorporation if the inhibitor was removed after the first 24 h. These observations suggest that the inhibitory effect of GST did not result merely from an alteration in the uptake or handling of functionally relevant mitogen by PBM.

The inverse relationship between monocyte number and GST inhibition of lymphocyte proliferation. Two sets of observations suggested the possibility that the effect of GST on mitogen and antigen responsiveness might result from interference with monocyte or macrophage function. First, the initiation of antigen- and mitogen-induced T-lymphocyte DNA synthesis requires the active participation of an ac-

TABLE V
GST Inhibition of Con A Responsiveness: Lack of Effect
when Present only during the Initial 24 H

GST concn.* during Con A activation	$[^3\text{H}]$ Thymidine incorporation† by Con A-activated PBM
$\mu\text{g/ml}$	Δcpm
0	23,233 \pm 2,824
25	20,828 \pm 1,797
100	21,881 \pm 7,829

* PBM were incubated in test tubes ($1 \times 10^6/\text{ml}$ in medium containing 10% FBS) with 5 $\mu\text{g/ml}$ Con A or an equal volume of medium as control and GST at the indicated final concentration. After 24 h incubation at 37°C, the cells were exposed to 40 mM α -methyl-D-mannoside, washed four times, suspended in fresh medium containing 10% FBS, and aliquoted into the wells of microtiter plates ($1 \times 10^5/\text{well}$). After an additional 48 h incubation at 37°C without GST or additional mitogen, [^3H]thymidine incorporation was assayed. Δcpm indicates the difference in [^3H]thymidine incorporation between Con A-activated and control PBM. Mean [^3H]thymidine incorporation of control PBM was 980 cpm. This was not significantly affected by presence of GST during the initial 24 h of incubation.

† Each term represents the mean \pm SEM of three separate experiments. When GST was present during the entire 72 h incubation, 25 $\mu\text{g/ml}$ led to 25.8 \pm 5.4% inhibition and 100 $\mu\text{g/ml}$ led to 65.1 \pm 3.9% inhibition of Con A responsiveness.

cessory cell (17–22). In populations of human PBM, the monocyte subserves this function (18, 21, 22). Second, GST has been demonstrated to be actively internalized by macrophages *in vivo* (29–31) and to inhibit certain enzyme systems (32), as well as functional capabilities of these cells (33). Thus, the action of gold on mitogen- and antigen-induced lymphocyte proliferation might not result from inhibition of lymphocyte responsiveness *per se*, but rather from interference with the requisite function of the monocyte population in the initiation or support of such responses.

The magnitude of the proliferative response of T lymphocytes induced by mitogens is dependent on the number of accessory cells supporting that response (15, 20, 22). This accessory cell requirement is most easily observed in cultures of T lymphocytes which have been partially depleted of adherent cells. In such cultures, the degree of mitogen-induced lymphocyte [³H]thymidine incorporation varies directly with the number of accessory cells added. We reasoned, therefore, that if gold inhibited lymphocyte proliferation by causing a partial interference with a requisite monocyte function, this inhibitory effect would be accentuated in cultures in which the number of monocytes was decreased. Conversely, a GST-induced monocyte dysfunction might be overcome by supplementation of the cultures with excess monocytes. Fig. 4 depicts the relationship between the number of monocytes supporting a mitogen-induced proliferative response and the degree of GST inhibition of that response. GST inhibition of the Con A response observed in a standard population of PBM is shown. This population of PBM contained between 25 and 30% monocytes. When the PBM were depleted of adherent cells by sequential Petri dish plating and nylon column passage, the resultant population contained 0.5% monocytes. Two differences were observed in the Con A response of the monocyte-depleted peripheral blood lymphocytes (PBL) compared to PBM. First, the magnitude of the response in terms of Δcpm (A) was diminished (from 72,606 to 18,396) and second, the degree of inhibition produced by all concentrations of GST was markedly greater (B). When the PBL were supplemented with purified autologous mitomycin-C-treated monocytes to levels equivalent to that found in unfractionated PBM (2.5×10^4 monocytes/ 1×10^5 PBL), the magnitude of the Con A response was restored to that observed with PBM. Likewise, the degree of GST inhibition was decreased and seen to approximate that observed with PBM. When twice as many monocytes were added to the cultures, GST-mediated inhibition of the Con A response was further decreased, although there was no further change in the magnitude of [³H]thymidine incorporation observed in cultures without added GST. The

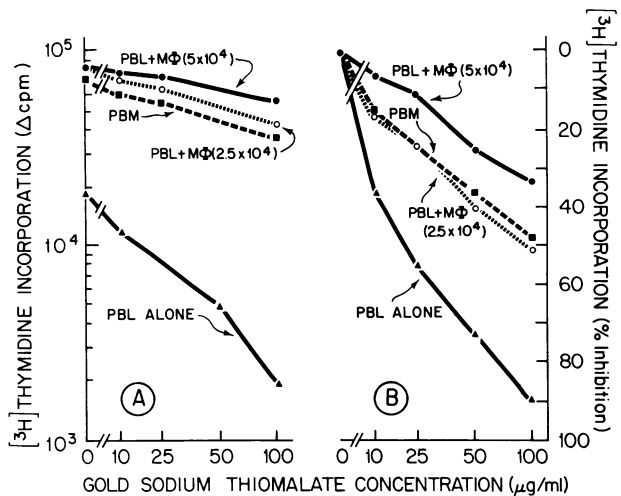


FIGURE 4 Con A-induced [³H]thymidine incorporation as a function of GST concentration. PBM or PBL (1×10^5 /well) were cultured in flat-bottomed microtiter wells with or without $5 \mu\text{g/ml}$ Con A. PBL were cultured either alone or supplemented with mitomycin-C-treated monocytes (2.5×10^4 or 5×10^4 /well). Cultures also contained GST at final concentration indicated on the abscissa. After 72 h incubation, [³H]thymidine incorporation was determined and expressed as Δcpm (A) and percent inhibition of control stimulation (B). Each point represents the mean of triplicate determinations. When cultured alone, the mitomycin-C-treated monocyte population manifested no significant degree of Con A-induced [³H]thymidine incorporation.

inverse relationship between monocyte number and the degree of GST inhibition of lymphocyte proliferation supports the contention that gold acts not by inhibiting the potential responsiveness of the lymphocytes *per se*, but rather by interfering with a critical function subserved by monocytes in these responses.

Effect of prolonged GST preincubation on subsequent lymphocyte proliferation. Since previous observations (Fig. 1) had suggested that decreased lymphocyte proliferation might result from lengthy GST exposure, experiments were undertaken to determine whether preincubating PBM with GST would alter their mitogen responsiveness. PBM were incubated with GST for various lengths of time at 37°C. The cells were then washed extensively, aliquoted into the wells of microtiter plates, and incubated, with or without mitogen, for 72 h at 37°C in fresh medium containing no gold. These latter cultures were carried out at a variety of cell densities (1.25×10^4 – 1.0×10^5 /well) in microtiter plates with flat-bottomed wells. This modified limiting dilution culture technique has been described previously (20) as a way to accentuate small differences in accessory cell number or function which might be missed in standard higher density cultures.

PBM were cultured with varying concentrations of

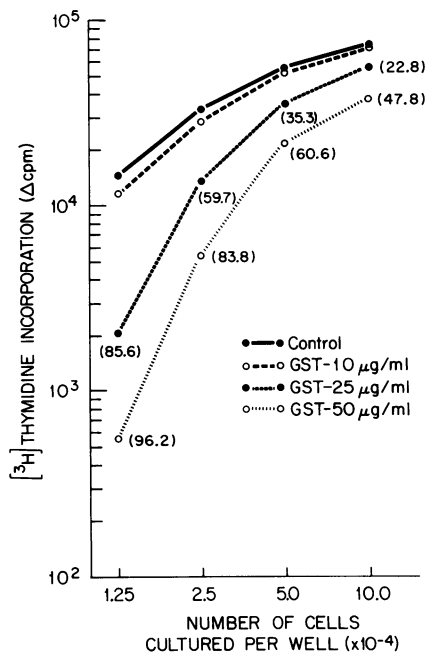


FIGURE 5 Effect of 72 h GST preincubation on subsequent Con A-induced lymphocyte proliferation. PBM were incubated with GST at the indicated concentration for 72 h as previously described. The cells were then washed, aliquoted into flat-bottomed microtiter wells at various cell densities, incubated for 72 h with or without 5 $\mu\text{g/ml}$ Con A, and assayed for [^3H]thymidine incorporation. Each point represents the mean of triplicate determinations. Numbers in parentheses indicate percent inhibition compared to control.

GST for 72 h. Such preincubation of PBM in 10 $\mu\text{g/ml}$ of GST had no significant effect on the ability of these cells to respond to Con A when subsequently cultured in the absence of the gold compound (Fig. 5). However, a 72-h preincubation with higher concentrations of GST (25 or 50 $\mu\text{g/ml}$) rendered PBM deficient in their ability to respond to Con A. Differences in Con A responsiveness between these cells and control cells became markedly more pronounced when cultured at lower cell densities. These cells, likewise, were deficient in their responses to PHA, PWM, and SK/SD (data not shown). These findings could not be explained by GST-induced cell death since the absolute number of cells surviving control and GST-containing cultures was not significantly different and all cultures manifested similar viability as assessed by trypan blue exclusion after the 72 h incubation.

Development of GST-induced depression of mitogen responsiveness required prolonged incubation. Thus, culture of PBM for 24 h with varying concentrations of GST had no significant effect on the ability of these cells to respond to mitogens when subsequently cultured in the absence of gold (Table VI). Furthermore, limiting dilution analysis did not reveal

subtle differences resulting from GST preincubation (data not shown).

The data obtained from limiting dilution analysis (Fig. 5), suggested that gold-induced monocyte dysfunction might explain the depressed responsiveness of PBM observed after prolonged GST exposure. For this reason, the potential ability of fresh monocytes to restore mitogen responsiveness to PBM which had been preincubated with GST was tested (Fig. 6). PBM were preincubated for 5 days in varying concentrations of GST. The cells were then extensively washed, aliquoted into the wells of microtiter plates, cultured for 72 h with or without Con A, and assayed for [^3H]thymidine incorporation. These latter cultures were supplemented with either fresh medium as control, 2-ME, or fresh autologous monocytes. Those cells which had been preincubated with 10 $\mu\text{g/ml}$ of GST exhibited responses to Con A on subsequent culture which were not dissimilar from control cells which had been preincubated in medium alone. However, cells which had been preincubated with higher concentrations of GST (25 or 50 $\mu\text{g/ml}$) showed a marked depression in Con A responsiveness when recultured alone. Addition of 2-ME to these cultures only partially restored responsiveness. However, supplementation of these cultures with fresh autologous monocytes resulted in a complete restoration of lymphocyte DNA synthesis to control levels. These data suggest that prolonged exposure to GST had resulted in monocyte dysfunction and not an alteration in potential lymphocyte responsiveness.

To confirm these observations, purified monocytes were incubated for 72 h at 37°C with 10 or 25 $\mu\text{g/ml}$ GST or an equal volume of medium as control. The cells were then washed extensively and evaluated

TABLE VI
Effect of 24 H GST Preincubation on Subsequent Mitogen Responsiveness

GST concn. during 24 h preincubation*	Lymphocyte DNA synthesis			
	0	PHA	Con A	PWM
$\mu\text{g/ml}$	[^3H]Thymidine incorporation-cpm $\times 10^{-3}$			
0	0.6 \pm 0.2	82.2 \pm 1.5	72.7 \pm 1.4	21.9 \pm 0.3
10	0.6 \pm 0.1	74.5 \pm 5.5	68.4 \pm 1.1	22.5 \pm 0.4
25	0.6 \pm 0.1	85.1 \pm 1.4	73.5 \pm 1.2	22.1 \pm 0.8
50	0.5 \pm 0.1	77.5 \pm 2.5	65.6 \pm 2.6	20.8 \pm 0.3

* PBM were incubated in test tubes ($1 \times 10^6/\text{ml}$) with GST at various concentrations for 24 h at 37°C. The cells were then washed four times to remove GST, suspended in fresh medium, and aliquoted into microtiter wells ($1 \times 10^5/\text{well}$). The cells were then incubated with 0.5 $\mu\text{g/ml}$ PHA, 5 $\mu\text{g/ml}$ Con A, or PWM (1:100) or an equal volume of medium as control for 72 h, and assayed for [^3H]Thymidine incorporation. † Each term represents the mean \pm SEM of triplicate determinations.

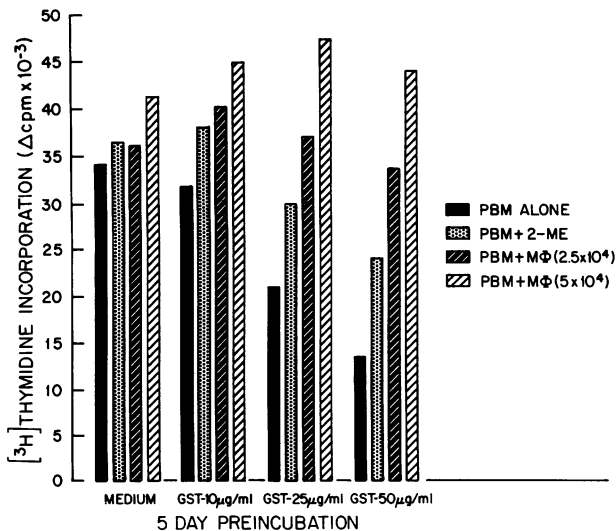


FIGURE 6 Monocyte rescue of GST-induced depression of Con A responsiveness. PBM were preincubated with various concentrations of GST for 5 days at 37°C. The cells were then washed four times, suspended in fresh medium containing 10% FBS, aliquoted into flat-bottomed microtiter wells (2.5×10^4 /well), incubated with or without 5 μ g/ml Con A for 72 h at 37°C, and assayed for [³H]thymidine incorporation. These latter cultures were supplemented with either medium as control, 25 μ M 2-ME or fresh autologous monocytes (MΦ). The monocytes had been treated with mitomycin-C and manifested no significant [³H]thymidine incorporation in response to Con A when cultured alone. Each bar represents the mean of triplicate determinations.

for their ability to support mitogen responsiveness in populations of fresh PBL which had been depleted of adherent cells (Fig. 7). When fresh adherent cell-depleted PBL were cultured alone, no significant degree of DNA synthesis was triggered by Con A. Supplementation of these cultures with aged control monocyte or macrophage (MΦ) led to the development of a vigorous proliferative response to Con A. By contrast, MΦ, which had been preincubated for 72 h with GST, were markedly deficient in their ability to support Con A-induced T-lymphocyte proliferation. This deficiency could not be explained by cell death, since cell yields, both in terms of absolute number and viability as gauged by trypan blue exclusion, were similar in all three monocyte populations. Furthermore, it is unlikely that these observations resulted from carry over of GST or induction of a suppressor cell since GST-preincubated monocytes did not inhibit Con A-induced DNA synthesis of PBL supported by control monocytes (data not shown).

DISCUSSION

A number of clinical trials have established that chrysotherapy can lead not only to amelioration of the symptoms of rheumatoid arthritis but also to actual remissions of disease activity (10–13). Since the patho-

genesis of chronic rheumatoid synovitis is thought to involve immunologic mechanisms (1), it seems reasonable to suggest that the therapeutic efficacy of gold compounds may relate to their ability to modify immune responsiveness. Although a number of studies have indicated that gold compounds may inhibit non-specific inflammatory responses in a variety of experimental models (33–35), evidence as to whether these agents exert a direct effect on lymphocyte responsiveness remains conflicting (36).

To conclude that the effects of gold compounds observed *in vitro* have significance *in vivo*, such effects should be observed 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. Although blood concentration of gold has been shown to depend on variables such as dosage schedule and excretion rate, serum levels attained in patients treated with gold tend to be in the range of 2–5 μ g/ml (37–42), equivalent to 4–10 μ g/ml of GST.

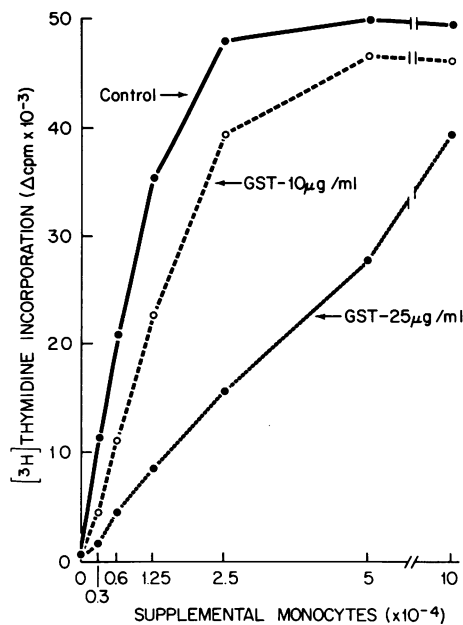


FIGURE 7 The effect of GST preincubation on the ability of monocytes to support Con A-induced proliferative responses. Purified monocytes were suspended in medium containing 10% FBS (0.5×10^6 /ml), and cultured in test tubes with or without GST. After a 72-h incubation at 37°C, the cells were washed three times, exposed to 40 μ g/ml mitomycin-C for 60 min at 37°C, and washed four more times. They were then mixed with fresh, autologous, adherent-cell depleted PBL (2.5×10^4 /well) in flat-bottomed microtiter wells and incubated with 5 μ g/ml Con A or an equal volume of medium as control. After a 72-h incubation at 37°C, [³H]thymidine incorporation was determined. Each point represents the mean of triplicate determinations. Con A-induced [³H]thymidine incorporation manifested by PBL cultured alone resulted in Δ cpm = 248. The monocytes, when cultured alone, made no significant response to Con A.

However, the concentration of gold in the blood does not appear to correlate with either the therapeutic or toxic effects of gold compounds in man (38–42), suggesting that the anti-inflammatory action of gold compounds is not related to serum gold level. The administration of gold compounds leads to rapid distribution of gold throughout the body with a tendency for it to concentrate in areas of inflammation as well as in organs rich in reticuloendothelial elements (43,44). Thus, tissue gold levels may provide a more meaningful estimation of both pharmacologically attainable and therapeutically effective gold concentrations. Mean synovial tissue gold concentrations of 21.1 $\mu\text{g/g}$ 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 (45). These findings are consistent with those reported in a patient who had received a total gold dose of 2,530 mg over a 5-yr period and at postmortem examination had a mean synovial tissue gold concentration of 25 $\mu\text{g/g}$ wet weight (46). Although such estimations of tissue concentrations obtained by atomic absorption spectroscopy give no information about the physical state of the gold or its availability to cellular elements in the synovium, these data suggest that the maximal concentration of gold attainable at the relevant tissue site is equivalent to 42–50 $\mu\text{g/ml}$ of GST.

GST was found to reversibly inhibit antigen- and mitogen-induced human lymphocyte proliferation. These findings are similar to results reported by others using PHA (47, 48) and allogeneic lymphocytes (48) 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. Inhibition of lymphocyte proliferation was dependent on the gold ion itself and not the sulfur-containing ligand inasmuch as GST and gold chloride were both inhibitory while TMA was not. Suppression of responsiveness could not be ascribed to cell death or an alteration in the kinetics of the *in vitro* response. Moreover, gold inhibition of responsiveness did not result from an interference with the binding or handling of mitogen by the cells. This was indicated by the observation that GST present only during an initial 24-h period of mitogen activation, but not during the subsequent 48 h of incubation, had no effect on the magnitude of the resultant proliferative response. This was supported by data obtained when the binding of radioiodinated mitogens to human PBM was studied. 100 $\mu\text{g/ml}$ GST had no effect on the binding of either ^{125}I -PHA or ^{125}I -Con A measured after a 30-min incubation at 4°C (unpublished observation).

Gold compounds were found to act by specifically interfering with an early step in the inductive phase of lymphocyte activation, even before DNA synthesis

began. Initiation of T-lymphocyte proliferation by the stimulating agents used requires the active participation of an accessory cell, which itself does not undergo a DNA synthetic response (17–22). In populations of human PBM, the monocyte subserves the requisite accessory cell function (15, 18, 19, 21, 22). Therefore, experiments were carried out to determine whether one or the other participant in this two-cell responding unit was differentially affected by gold compounds. This question was first approached by investigating the inhibitory capacity of GST in cultures with various ratios of responding to accessory cells. An inverse relationship was noted between the degree of GST inhibition of the proliferative response to mitogens and the number of monocytes supporting that response. The data suggested that the action of GST on lymphocyte activation resulted not from inhibition of the potential responsiveness of the T-lymphocyte population, but rather from interference with the ability of the monocytes to serve as effective accessory cells in the initiation of such responses. Alternatively, monocytes merely could have acted in a protective capacity in these experiments, perhaps by internalizing GST and thus preventing its inhibitory effect on lymphocytes. To investigate this possibility, 100 $\mu\text{g/ml}$ GST was incubated with PBM (containing 30% monocytes) or with medium alone for 24 h at 37°C, and then tested at various concentrations for its capacity to inhibit lymphocyte proliferation. Preincubation of GST with PBM ($1 \times 10^6/\text{ml}$) did not diminish its subsequent inhibitory action on Con-A-induced lymphocyte DNA synthesis compared to GST similarly incubated in medium alone (unpublished observation). These data militate against the possibility that the function of monocytes in the cultures was to protect lymphocytes from the effects of gold by internalizing or otherwise inactivating GST.

Another indication of the cellular site of action of GST became apparent only after prolonged incubation of PBM with this gold compound. Thus, preincubation of PBM with GST for 72 h resulted in diminished mitogen responsiveness of these cells on subsequent challenge. This effect could be induced by prolonged exposure to concentrations of GST (25 or 50 $\mu\text{g/ml}$) equivalent to those found in the synovial tissues of patients treated with gold compounds. Furthermore, this effect was irreversible inasmuch as the PBM exhibited diminished mitogen responsiveness even after removal of GST. The ability of fresh autologous monocytes to restore mitogen responsiveness to these populations indicated that prolonged exposure of this mixed PBM population to GST had not affected the intrinsic ability of the lymphocytes to respond but rather had altered the functional capacity of the monocytes to support such responses. This interpretation was confirmed by the observation that preincubation of purified monocytes with GST

for 72 h rendered them deficient in their ability to support mitogen-induced T-lymphocyte proliferation on subsequent culture.

These studies indicate that a major action of gold compounds involves interference with the functional capability of MΦ. This conclusion is consonant with a number of previous observations. First, microscope examination of rheumatoid synovial tissue obtained from patients treated with gold compounds has revealed a selective accumulation of gold particles in the lysosomes of the type A synovial cells and other macrophages of the synovium (29–31). Second, GST has been shown to be actively endocytosed in vitro by guinea pig peritoneal macrophages with resulting inhibition of their lysosomal enzyme activity (32). Finally, the phagocytic activity of macrophages in experimentally induced inflammatory exudates has been found to be suppressed in patients with rheumatoid arthritis receiving gold therapy as compared to untreated rheumatoid patients (33).

Mononuclear phagocytes play a central role in the initiation and maintenance of chronic inflammatory reactions. They not only function as effector cells in such responses (49), but also possess the unique capacity to interact with both B and T lymphocytes in the induction of cellular and humoral immune responses. Thus, antigen-induced proliferation and mediator synthesis by T lymphocytes require the active participation of MΦ (17, 50). These accessory cells initially take up antigen and act to facilitate its functionally effective presentation to primed T lymphocytes (51). Similarly, macrophages play a critical role in the humoral response to many antigens by supporting the induction of antigen-specific helper T cells (52) and providing a focus for collaboration between helper T cells and B cells whose progeny secrete antibody (53, 54). In view of the critical role of mononuclear phagocytes in the induction of both cellular and humoral immune responses, pharmacologically induced interference with their function by gold compounds may, in part, explain the efficacy of these agents in reducing the chronic immunologically mediated inflammation of rheumatoid arthritis.

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