

Inhibition of Polyclonally Induced Immunoglobulin Secretion by Aurothiomalate

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The influence of sodium aurothiomalate on the secretion of immunoglobulins by normal human lymphocytes *in vitro* was investigated by means of a reverse hemolytic plaque forming cell (PFC) assay. Aurothiomalate inhibited the PFC response induced by pokeweed mitogen (PWM) and by Epstein-Barr virus (EBV) in a dose dependent manner. The inhibition was irreversible, as pre-incubation for 2 h with the drug followed by extensive washing and further culture in gold salt-free medium still caused an inhibition of the PFC response to PWM and to EBV. Cell proliferation was not significantly affected, suggesting that the inhibition of PFC formation was not due to cytotoxicity. Pre-incubation of monocytes/macrophages (Mø's), T lymphocytes and B lymphocytes with the gold compound prior to culture with PWM showed that Mø's and B cells were highly sensitive, whereas T lymphocytes were resistant to the drug. The findings indicate that aurothiomalate inhibits the polyclonally induced PFC response by interfering with accessory Mø function and by affecting the B lymphocyte itself.

Key words: immunoglobulin-secreting cells; polyclonal B cell activation; sodium aurothiomalate.

Accepted for publication 27 July 1983

In rheumatoid arthritis (RA) excessive B lymphocyte activity is important for the pathogenesis of the disease. Thus cells synthesizing and secreting immunoglobulins including auto-antibodies are numerous within rheumatoid synovial tissue (20, 30). Recent studies indicate that cell-mediated immune mechanisms also are important for the pathogenesis (1, 4).

Gold salts are widely used in the treatment of RA, and the efficacy of these drugs to induce remissions for months or even years has been demonstrated in numerous reports. The precise mechanisms of action of the gold compounds are not known. A number of investigations have shown that gold salts interfere with functional capabilities of monocytes/macrophages (Mø's) including inhibition of the release of lysosomal enzymes (25) and suppression of phagocytosis (13).

An alternative explanation for the mode of action is based on the clinical observations that treatment with gold salts frequently results in decrease in anti-immunoglobulin titers (5) and serum immunoglobulins (22). These findings suggest that gold compounds may be immunosuppressive drugs, and this assumption is supported by the observation of Lipsky & Ziff (18) that gold salts strongly inhibit mitogen- and antigen-induced lymphocyte proliferation. Further evidence for this assumption has been provided by the inhibitory effect of gold salts on the secretion of immunoglobulins as induced by the polyclonal B lymphocyte activator, pokeweed mitogen (PWM) (19).

The purpose of this study is to investigate mechanisms of the inhibitory action of aurothiomalate on the B lymphocyte activation in response to PWM and to Epstein-Barr virus (EBV).

MATERIAL AND METHODS

Isolation of mononuclear cells

Mononuclear cells were isolated from heparinized blood of healthy adult volunteers by centrifugation on Lymphoprep® (Nyegaard, Oslo, Norway), washed and resuspended in RPMI 1640 (GIBCO, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS) (Flow, Irvine, Scotland), L-glutamine (0.08 mM), penicillin (500 IU/ml), streptomycin (500 µg/ml) and nystatin (20 U/ml).

Preparation of Mø-depleted and Mø-enriched suspension

Mononuclear cells (4×10^6 /ml) were incubated in Petri dishes (diameter 60 mm, No. 3002, Falcon, Oxnard, CA, USA) in a volume of 1.5 ml for 90 min at 37°C as described previously (26). Non-adherent cells were decanted, and the dishes were washed 5 times with 37°C RPMI 1640 with 1% FCS. Adherent Mø's were detached by incubation with EDTA (10 mM in phosphate buffered saline) at 4°C for 20 min, washed and kept at 4°C for a maximum of 16 h.

Mø's were quantified by staining cytocentrifuged preparations for α -naphthyl acetate esterase activity according to Horwitz et al. (10). Mø-depleted cells contained $1.1 \pm 0.3\%$ Mø's (mean $\pm$ SEM), whereas Mø-enriched cells comprised $81 \pm 4\%$ Mø's.

Preparation of T-enriched and T-depleted suspensions

Mø-depleted cells (3×10^6 /ml) in volumes of 3 ml were incubated with SRBC (1% in RPMI 1640 with 40% FCS) treated with 2-aminoethyl-iso-thiouonium (AET) (Sigma, St. Louis, Mo., USA) for 30 min at 37°C, centrifuged at 72 g for 5 min, resuspended and centrifuged on Lymphoprep. Interphase cells (non-T cells, non-Mø's) were harvested and washed; these B-enriched cells were stored at 4°C for a maximum of 16 h. The cell pellet was exposed to NH_4Cl , washed and re-rosetted with untreated SRBC overnight at 4°C. Non-rosetting cells

were removed after an additional centrifugation on Lymphoprep. The residual cell pellet was exposed to NH_4Cl and washed; these T-enriched cells were resuspended in RPMI 1640 with 10% FCS.

B-enriched cells contained $1.9 \pm 0.3\%$ Mø's, $1.2 \pm 0.2\%$ E-rosetting cells and $69 \pm 9\%$ cells with membrane immunoglobulin as judged by use of FITC-conjugated anti-human μ , γ , α , κ , and λ chains (DAKO, Copenhagen, Denmark). T-enriched cells contained $1.0 \pm 0.3\%$ Mø's, $88 \pm 3\%$ E-rosetting cells and $1.0 \pm 0.3\%$ cells with membrane immunoglobulin.

Drugs

Aurothiomalate was purchased from Rhone-Poulenc, France (Myocrisin®) and diluted in RPMI 1640. A stock solution (1 mg/ml) was stored at 4°C and used within 1 week. In most experiments, aurothiomalate was added at concentrations corresponding to those found in serum of patients receiving weekly chrysotherapy ($0.25 - 2.5 \times 10^{-5}$ M) (14, 21, 28). In some experiments, cells were pulsed at 37°C for 2 h with aurothiomalate, washed 3 times and resuspended in RPMI 1640 with 10% FCS.

Thiomalic acid (Sigma) was used in the same manner as aurothiomalate.

Generation of the PFC response

Mononuclear cells, or mixtures of subpopulations of mononuclear cells, were cultured at 1.0×10^6 cells/ml in RPMI 1640 with 10% FCS and antibiotics. Mixtures of cells were always done on the basis of viable cells as determined by nigrosin exclusion. Cultures were carried out in flat-bottomed microtiter plates (Nunc, Roskilde, Denmark), 0.25 ml per well as described previously (26). At least triplicate experiments were performed. Pokeweed mitogen (PWM) (GIBCO) was added at a concentration of 0.8 µg/ml. In the case of stimulation with Epstein-Barr virus (EBV), 2×10^6 mononuclear cells were exposed to 1 ml of B95-8 supernatant from a marmoset lymphoblastoid cell line for 2 h at 37°C and washed twice prior to culture as described elsewhere (17). Cells

were incubated for 6 days at 37°C in a humidified atmosphere of 95% air and 5% CO₂. At harvest, cells were washed in minimum essential medium (MEM) (Flow) prior to plating.

PFC assay

The PFC assay used is modified from Hammarström et al. (8) and is described further elsewhere (2). In brief, *S. aureus* protein A (SPA) (Pharmacia, Uppsala, Sweden) was conjugated to sheep red blood cells (SRBC) by the chromium chloride method (7).

All reagents were diluted in MEM. 100 µl cultured cells in a dilution suitable for plaque counting, 50 µl SPA-SRBC diluted 1:6, 25 µl anti-human IgM, IgA or IgG (DAKO) diluted 1:30, 25 µl guinea pig complement (Flow) absorbed twice with SRBC and diluted 1:4 were mixed with 800 µl 0.5% Noble agar (DIFCO, Detroit, Mich., USA) supplemented with DEAE-dextran (0.45 mg/ml) (Pharmacia) at 45°C. Three drops of 200 µl were placed in 9 cm Petri dishes (Nunc) and covered with 50 × 25 mm cover slips. The preparations were incubated for 4 h at 37°C and plaques were read at 10 × magnification in indirect light. The number of PFC was calculated per 10⁶ originally cultured cells.

Proliferative capacity

Mononuclear cells were incubated for 6 h at 37°C in a volume of 100 µl with 0.05 µCi [¹⁴C]-thymidine (Sp.act. > 50 mCi/mmol) in order to determine the extent of DNA synthesis after culture for 6 days. The cells were collected on glass fiber filters in a multiple cell harvester, and thymidine incorporation was measured by liquid scintillation counting. The results are medians of cpm measured in triplicate determinations, and the cpm are given per 10⁵ originally cultured cells.

Statistics

Data are given as mean ± 1 SEM. Results were analyzed by means of Student's two-tailed t test. *P* values < 0.05 were regarded as significant.

RESULTS

Aurothiomalate inhibits the PWM-generated PFC formation

PWM-stimulated mononuclear cells generated a large number of immunoglobulin-secreting cells as opposed to the unstimulated controls. Addition of aurothiomalate markedly inhibited the formation of IgM-PFC (Fig. 1) in a dose-dependent manner; a similar inhibition of IgG- and IgA-PFC was found (data not shown). Thiomalic acid also inhibited PFC formation, although to a significantly lesser degree (Fig. 1).

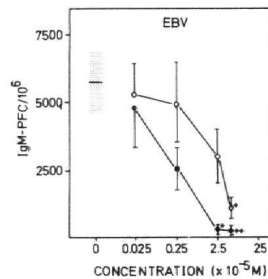


Fig. 1. Effect of aurothiomalate and thiomalic acid on the PWM-induced IgM secretion. Means of 7 experiments are shown; shaded area or vertical bars represent 1 SEM. Abscissa: concentration of the drugs. Ordinate: IgM-PFC/10⁶ originally cultured cells. Closed symbols: aurothiomalate. Open symbols: thiomalic acid.
+ : *P* < 0.05; ++ : *P* < 0.01.

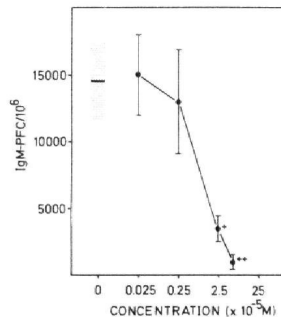


Fig. 2. Effect of a 2-h pulse with aurothiomalate on the PWM-induced IgM secretion. Means ± 1 SEM of 6 experiments are shown. Ordinate and abscissa as in Fig. 1.
+ : *P* < 0.05; ++ : *P* < 0.01.

Table 1

Effect of aurothiomalate on the PWM-induced PFC responses: influence of aurothiomalate-pulsed M ϕ -enriched cells and M ϕ -depleted cells

Pulsed cells ¹	Aurothio- malate pulse	IgM-PFC ²	IgG-PFC	IgA-PFC
M ϕ -enriched cells	—	6435 $\pm$ 2278	9163 $\pm$ 1909	5872 $\pm$ 1761
M ϕ -enriched cells	+	943 $\pm$ 414 ³	1478 $\pm$ 512 ³	618 $\pm$ 215 ³
M ϕ -depleted cells	—	5880 $\pm$ 1660	7614 $\pm$ 1093	4820 $\pm$ 1320
M ϕ -depleted cells	+	129 $\pm$ 86 ⁴	201 $\pm$ 80 ⁴	119 $\pm$ 39 ⁴

¹ M ϕ -enriched cells or M ϕ -depleted cells were incubated for 2 h at 37°C with aurothiomalate (2.5×10^{-5} M) or with medium alone, and washed 3 times. 80% M ϕ -depleted cells were then co-cultured with 20% M ϕ -enriched cells in the presence of PWM for 6 days prior to assay for PFC.

² PFC are calculated per 10^6 originally cultured cells.

³ $P < 0.05$ when compared to experiments using cells pulsed with medium alone ($n = 5$).

⁴ $P < 0.02$ when compared to experiments using cells pulsed with medium alone ($n = 5$).

As seen in Fig. 2, incubation for 2 h with aurothiomalate followed by washing of the cells had a significant, dose dependent inhibitory effect on IgM-PFC generation. Similar results were obtained with regard to IgG- and IgA-PFC (data not shown). However, the inhibition was less pronounced as compared to experiments with the drug present during the entire culture period. The inhibitory action of aurothiomalate pulse was not due to decreased viability of cells.

A 2 h pulse with thiomalic acid had no influence on PFC formation, even when used in a concentration of 2.5×10^{-4} M (data not shown).

Effect of pulse treatment of M ϕ -depleted and M ϕ -enriched suspensions with aurothiomalate

As B cell activation induced by PWM is highly dependent on M ϕ 's (27), experiments were carried out to determine whether the inhibitory effect of aurothiomalate was due to reduced accessory function of M ϕ 's. Mononuclear cells deprived of M ϕ 's generated few or no PFC in the presence of PWM, but reconstitution with 20% M ϕ -enriched cells re-established the response (for details, see ref. 26); after incubation for 2 h and washing, M ϕ -enriched cells still reconstituted the PWM response (Table 1). Pulse treatment of M ϕ 's with aurothiomalate reduced the capacity of these cells to support

the PFC responses of M ϕ -depleted cells after stimulation with PWM (Table 1). However, M ϕ -depleted cells subjected to pulse treatment with aurothiomalate completely failed to respond to PWM after addition of fresh M ϕ 's (Table 1).

Viability of M ϕ -enriched and M ϕ -depleted cells was not affected by the 2 h pulse with aurothiomalate.

Effect of pulse treatment of T-enriched and B-enriched cells with aurothiomalate

PWM is a T cell-dependent polyclonal B lymphocyte activator (11). Therefore, the following experiments were performed to elucidate whether the inhibitory influence of aurothiomalate could be ascribed also to altered function of T cells or to action at the B cell level. B-enriched cells cultured alone did not respond to PWM. The response was fully reconstituted by co-culture of 40% T-enriched cells and 20% M ϕ -enriched cells (Table 2); incubation of T-enriched cells for 2 h with subsequent washings did not affect the response. As shown in Table 2, incubation of T-enriched cells with aurothiomalate prior to co-culture with B cells and M ϕ 's had no influence on the PWM-induced PFC response, even when aurothiomalate was used in a concentration of 12.5×10^{-5} M.

Table 2

Effect of aurothiomalate on the PWM-induced PFC responses: influence of aurothiomalate-pulsed T- and B-enriched cells

Pulsed cells ¹	Aurothio- malate pulse	IgM-PFC ²	IgG-PFC	IgA-PFC
T-enriched cells	—	16523 ± 4684	34149 ± 8311	8074 ± 1250
T-enriched cells	+	17860 ± 5653 ³	36073 ± 9244 ³	8191 ± 1080 ³
T-enriched cells	+ ⁴	15290 ± 4306 ³	30848 ± 9116 ³	7447 ± 1003 ³
B-enriched cells	—	11405 ± 3449	29120 ± 7370	9231 ± 2952
B-enriched cells	+	264 ± 138 ⁵	221 ± 95 ⁵	343 ± 109 ⁶

¹ T-enriched cells or B-enriched cells were incubated for 2 h at 37°C with aurothiomalate (2.5×10^{-5} M) or with medium alone, and washed 3 times. 40% T-enriched cells, 40% B-enriched cells and 20% Mφ-enriched cells were then co-cultured in the presence of PWM for 6 days prior to assay for PFC.

² PFC are calculated per 10^6 originally cultured cells.

³ Not significantly different from experiments using cells pulsed with medium alone ($n = 5$).

⁴ T-enriched cells were pulsed with aurothiomalate at 12.5×10^{-5} M.

⁵ $P < 0.01$ when compared to experiments with cells pulsed with medium alone ($n = 5$).

⁶ $P < 0.05$ (see foot-note ⁵).

Table 3

Effect of aurothiomalate in the PWM-induced proliferation¹

	Concentration of aurothiomalate ($\times 10^{-5}$ M)		
	0	2.5	12.5
Direct culture ²	1559 ± 419	617 ± 219 ³	174 ± 75 ⁴
Pulsed cells ⁵	2188 ± 727	1432 ± 301 ³	1235 ± 223 ³

¹ Results are given as cpm/ 10^5 originally cultured cells.

² Mononuclear cells were cultured for 6 days in the presence of PWM or without aurothiomalate.

³ Not significantly different ($n = 5$).

⁴ $P < 0.05$ ($n = 5$).

⁵ Mononuclear cells were incubated for 2 h at 37°C with aurothiomalate or with medium alone, and washed 3 times. The cells were then cultured for 6 days in the presence of PWM prior to assay for cell proliferation.

By contrast, incubation of B-enriched cells with aurothiomalate prior to mixture with T cells and Mφ's had a marked inhibitory effect on the PWM-generated PFC response as compared to the responses obtained with B-enriched cells incubated with medium alone (Table 2).

Incubation of T-enriched cells as well as B-enriched cells for 2 h with aurothiomalate had no effect on viability of the cells.

Effect of aurothiomalate on the PWM-induced cell proliferation

Table 3 summarizes the effect of aurothiomalate on thymidine incorporation as induced by PWM. As is apparent from the table, the presence of aurothiomalate during the entire culture period markedly inhibited proliferation. Pulse treatment with the drug had a less pronounced inhibitory effect on the cellular division in contrast to the marked effect on PFC formation.

Aurothiomalate inhibits EBV-induced PFC responses

Because aurothiomalate was found to inhibit PWM-PFC formation also at the B cell level, it was of interest to analyze the effects of the gold salt on B cell activation achieved by the direct stimulator EBV (3). As demonstrated in Fig. 3, EBV-induced PFC generation was inhibited by the gold salt in a dose-dependent manner, similar to the findings obtained with PWM-stimulated cells. Again, thiomatic acid inhibited PFC less efficiently.

In order to further analyze the inhibitory effect of aurothiomalate, experiments were carried out as summarized in Table 4. A 2-h pulse with the drug after exposure to EBV had no

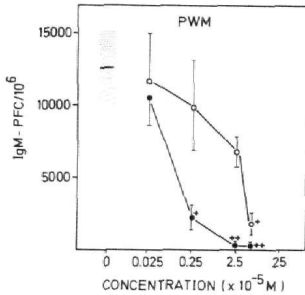


Fig. 3. Effect of aurothiomalate and thiomalic acid on the EBV-induced IgM secretion. Means $\pm$ 1 SEM of 5 experiments are shown. Symbols, ordinate and abscissa as in Fig. 1. +: $P < 0.05$; ++: $P < 0.01$.

effect on IgM-, IgG or IgA-PFC responses. By contrast, a 2 h pulse prior to exposure to EBV significantly reduced the PFC responses.

DISCUSSION

The experiments shown in this study have confirmed that aurothiomalate strongly inhibits immunoglobulin secretion *in vitro* as induced by PWM (19). The inhibition was obtained with concentrations of the gold salt comparable to those found in serum of patients receiving weekly gold administration (21, 28). Other reports have indicated that even higher concentrations of gold salts are found in the inflamed synovium (6). The observed inhibition of the PWM response was irreversible: an incubation for 2 h with the drug, extensive washing and further culture in gold-free medium still

caused significant inhibition. The observed reduction of the PWM-driven immunoglobulin secretion does not seem to be due to unspecific cytotoxicity, as proliferation of aurothiomalate-pulsed cells was affected to a lesser degree.

The PWM-induced PFC response is highly dependent on M ϕ 's (27). As gold salts have been shown to interfere with various functional activities of M ϕ 's (13, 15, 18, 25), experiments were carried out to determine whether the observed decrease of the PWM response was due to loss of accessory M ϕ function. The results indicated that M ϕ 's exposed to the drug prior to mixture with M ϕ -depleted cells supported PFC formation poorly as compared to M ϕ 's exposed to medium alone. Since aurothiomalate has been shown to reduce the expression of Fc and C3 receptor sites on M ϕ 's (29), one explanation for the gold-induced alteration of M ϕ function might be interference also with receptors for polyclonal activators on M ϕ 's. Subsequent experiments do not indicate that gold-treated M ϕ 's are suppressor cells, and the gold-induced loss of M ϕ assisting function is not reversed by addition of M ϕ supernatants (Petersen, unpublished observations).

The data of the present study have revealed that the gold compound also affected subpopulation(s) among M ϕ -depleted cells. As the *in vitro* effect of aurothiomalate on the functions of human blood lymphocytes has been claimed to be directed at T cells (9, 16), experiments were first carried out using highly purified T cells pulsed with aurothiomalate. It was clearly shown that T lymphocytes were resistant to the

Table 4
Effect of aurothiomalate on the EBV-induced PFC response¹

0-2 h	2-4 h	4-6 h	IgM-PFC ²	IgG-PFC	IgA-PFC
Medium	EBV	Medium	6349 $\pm$ 1328	4624 $\pm$ 984	2285 $\pm$ 501
Medium	EBV	Aurothiomalate	5364 $\pm$ 986 ³	3160 $\pm$ 584 ³	1599 $\pm$ 649 ³
Aurothiomalate	EBV	Medium	1919 $\pm$ 603 ⁴	1196 $\pm$ 313 ⁴	780 $\pm$ 258 ⁴

¹ Mononuclear cells were incubated for 3 $\times$ 2 h with either EBV, medium of aurothiomalate-containing medium (12.5×10^{-5} M); after each 2-h incubation, cells were washed 3 times.

² Results are given as PFC/10⁶ originally cultured cells ($n = 5$).

³ Not significant when compared to cells pulsed with gold-free medium.

⁴ $P < 0.05$ when compared to cells pulsed with gold-free medium.

gold salt. These results are in analogy with those of Lipsky & Ziff (18), indicating that the effect of aurothiomalate on the polyclonally induced lymphocyte proliferation does not imply the T cell itself but rather accessory Mø's. B lymphocytes pulsed with aurothiomalate failed to respond to PWM after addition of fresh T cells and Mø's. The sensitivity of B cells to aurothiomalate was not restricted to PWM-stimulated cells, but was also visualized using cells activated by EBV. The experiments indicated that pre-treatment of mononuclear cells with the gold salt reduced the response to EBV, whereas the drug had no influence on cells once activated by EBV. Thus, aurothiomalate, which binds to lymphocyte cell membranes (23), may disturb the interaction between EBV and B cell receptors (3).

In the present study, thiomalic acid also inhibited the PWM-induced secretion of immunoglobulins. However, the effect differed from that of the gold salt in two ways; 1) thiomalic acid inhibited PFC formation to a significantly lesser degree, and 2) the inhibition was reversible when thiomalic acid was removed from the cultures. Thiomalic acid inhibited the EBV response in a similar manner. The findings agree with those of Jennings et al. (12), and with those of McCormark & Palmar (24), but disagree with the observations of Lipsky & Rosenberg (19). The latter discrepancy remains to be clarified. Probably the sulphhydryl group of aurothiomalate is of importance for the actions of the gold salt. Thus it was shown that the thiol-reducing agents dithiotreitol and L-cysteine partially reduced the inhibitory effect of aurothiomalate on polyclonally induced T lymphocyte proliferation (24). However, the precise impact of the sulphhydryl part of the gold salt on B lymphocyte function *in vitro* remains to be established.

ACKNOWLEDGEMENTS

This work was supported by grants from The National Danish Association against Rheumatic Diseases. The skilful technical assistance of Ms. Anne Ambjørnsen and Ms. Vita Weibull is gratefully acknowledged. EBV was a generous gift from Jette Hesse, M.D.

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