from hamsters by Svartengren *et al.* [41] using [$^3$H]dihydroergocryptine. This ligand binds with equal affinity to both alpha$_1$ and alpha$_2$ adrenoceptors [8]. Characterization of alpha-adrenoceptor subtypes has not yet been performed in brown fat, and species differences have to be considered.

In summary, the present results suggest the presence in brown adipocytes of alpha$_1$ adrenoceptors whose activation is responsible for the increase in the incorporation of [$^{32}$P]P$_i$ into phosphatidylinositol and phosphatidic acid. The alpha$_1$-adrenergic effect is probably related to the depolarization observed by Fink and Williams [6] in brown fat.

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* This work was supported by United States Public Health Service Research Grants AM 10149 and AM 21470 from the National Institutes of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health.

REFERENCES

1. R. E. Smith and R. J. Hock, *Science* **140**, 199 (1963).
2. R. E. Smith and B. A. Horwitz, *Physiol. Rev.* **49**, 330 (1969).
3. L. Girardier, J. Seydoux and T. Clausen, *J. gen. Physiol.* **52**, 925 (1968).
4. B. A. Horwitz and R. E. Smith, *Proc. natn. Acad. Sci. U.S.A.* **654**, 113 (1969).
5. G. Krishna, J. Moskowitz, P. Dempsey and B. B. Brodie, *Life Sci.* **9**, 1353 (1970).
6. S. A. Fink and J. A. Williams, *Am. J. Physiol.* **231**, 700 (1976).
7. S. Berthelson and W. A. Pettinger, *Life Sci.* **21**, 595 (1977).
8. B. B. Hofman, A. De Lean, C. L. Wood, D. D. Schocken and R. J. Lefkowitz, *Life Sci.* **24**, 1739 (1979).
9. J. N. Fain and J. A. García-Sáinz, *Life Sci.* **26**, 1183 (1980).
10. J. N. Fain, N. Reed and R. Saperstein, *J. biol. Chem.* **242**, 1887 (1967).
11. B. Pettersson and I. Vallin, *Eur. J. Biochem.* **62**, 383 (1976).
12. J. A. García-Sáinz and J. N. Fain, *Biochem. J.* **186**, 781 (1980).
13. G. R. Bartlett, *J. biol. Chem.* **234**, 466 (1959).
14. A. G. Gilman, *Proc. natn. Acad. Sci. U.S.A.* **67**, 305 (1970).
15. D. Allen and R. H. Michell, *Biochem J.* **148**, 471 (1975).
16. D. N. Brindley and M. Bowley, *Biochem. J.* **149**, 461 (1975).
17. J. Eichberg, L. Gates and G. Hauser, *Biochim. biophys. Acta.* **573**, 90 (1979).
18. R. H. Michell, *Biochim. biophys. Acta* **415**, 81 (1975).
19. R. H. Michell, *Trends biochem. Sci.* **4**, 128 (1979).
20. D. M. Salmon and T. W. Honeyman, *Nature, Lond.* **284**, 344 (1980).
21. J. W. Putney, S. J. Weis, C. M. Van De Walle and R. A. Haddas, *Nature, Lond.* **284**, 345 (1980).
22. T. L. Smith, J. Eichberg and G. Hauser, *Life Sci.* **24**, 2179 (1979).
23. J. A. García-Sáinz, B. B. Hoffman, S. Li, R. J. Lefkowitz and J. N. Fain, *Life Sci.* **27**, 953 (1980).
24. M. E. M. Tolbert, A. C. White, K. Aspry, J. Cutts and J. N. Fain, *J. biol. Chem.* **255**, 1938 (1980).
25. B. B. Hoffman, T. Michel, D. M. Kilpatrick, R. J. Lefkowitz, M. E. M. Tolbert, H. Gilman and J. N. Fain, *Proc. natn. Acad. Sci. U.S.A.*, **77**, 4569 (1980).
26. M. Aggerbeck, G. Guellaen and J. Hanoune, *Biochem. Pharmac.* **29**, 643 (1980).
27. N. M. Kneer, M. J. Wagner and H. A. Lardy, *J. biol. Chem.* **254**, 12160 (1979).
28. J. A. García-Sáinz and J. N. Fain, *Molec. Pharmac.* **18**, 116 (1980).
29. J. M. Horwitz, B. A. Horwitz and R. E. Smith, *Experientia* **27**, 1419 (1971).
30. J. W. Putney, *Pharmac. Rev.* **30**, 209 (1979).
31. L. Girardier and J. Seydoux, in *Non-Shivering Thermogenesis* (Ed. L. Jansky), p. 255. Academia, Prague (1971).
32. B. A. Horwitz, *Fedn. Proc.* **38**, 2170 (1979).
33. J. N. Fain, M. D. Jacobs and Y. C. Clement-Cormier, *Am. J. Physiol.* **224**, 346 (1973).
34. J. N. Fain, in *Handbook of Experimental Pharmacology Cyclic Nucleotides* (Eds. J. A. Nathanson and J. W. Kebabian), Springer, Berlin, in press.
35. N. Mohell, J. Nedergaard and B. Cannon, *Proceedings of the Symposium on Developmental and Environmental Factors in Thermoregulation* (Eds. S. Z. Donhoffer and S. Kovacs) Pecs, Hungary, July 10–12, 1980.
36. T. W. Burns, P. E. Langley and G. A. Robison, *Ann. N.Y. Acad. Sci.* **185**, 115 (1971).
37. K. J. Hittleman, C. F. Wu and R. W. Butcher, *Biochim. biophys. Acta* **304**, 188 (1973).
38. K. Itaya, *J. Pharm. Pharmac.* **30**, 632 (1978).
39. W. F. Ward and J. N. Fain, *Biochim. biophys. Acta* **237**, 387 (1971).
40. J. N. Fain, *Pharmac. Rev.* **25**, 67 (1973).
41. J. Svartengren, N. Mohell and B. Cannon, *Proceedings of the Symposium on Developmental and Environmental factors in Thermoregulation* (Eds. S. Z. Donhoffer and S. Kovacs), Pecs, Hungary, July 10–12, 1980.

Biochemical Pharmacology, Vol. 29, pp. 3333–3336.

0006-2952/80/1215-3333 $02.00/0

## Sulphydryl dependence of the inhibition of mitogen-induced human lymphocyte proliferation by sodium aurothiomalate

(*Received* 6 *November* 1979; *accepted* 21 *August* 1980)

Gold mercaptides have been used effectively in the treatment of rheumatoid arthritis for several decades [1]. Although the nature of their anti-inflammatory activity *in vivo* is not known, gold compounds have been shown to inhibit lysosomal enzymes [2, 3], prostaglandin synthesis [4], γ-globulin aggregation [5], macrophage phagocytosis [6] and lymphocyte blastogenesis [7–9] among other biological processes *in vitro*. The activity of such compounds,

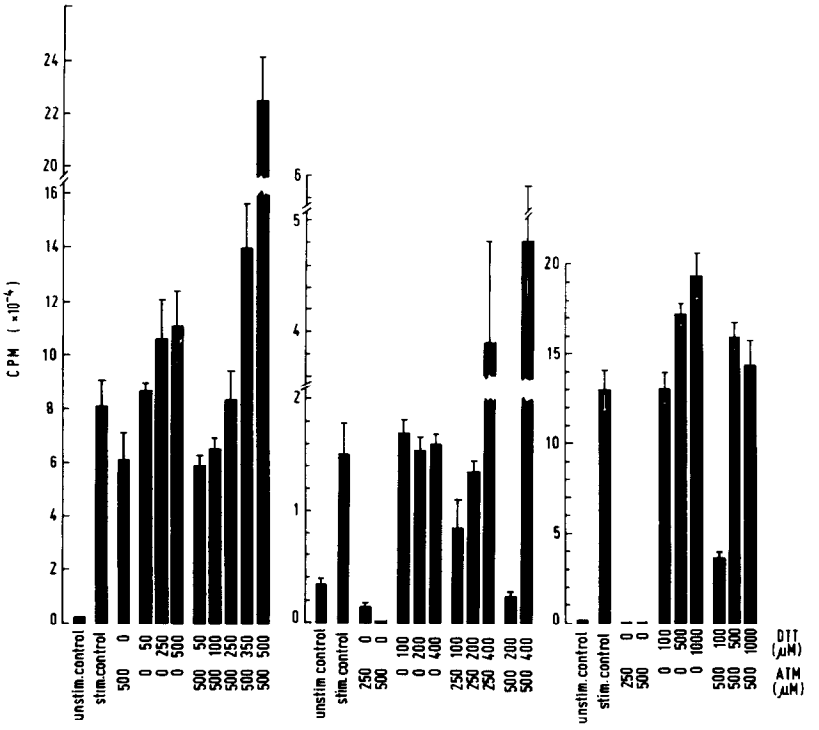


Fig. 1. Effect of aurothiomalate (ATM) on [$^3$H]thymidine incorporation by PHA-stimulated peripheral blood lymphocyte (PBL) cultures in the presence and absence of dithiothreitol (DTT). PHA, ATM and/or DTT were added to culture at zero hour; [$^3$H]thymidine was added 20 hr prior to harvest at 72 hr. (Results from three representative donors shown).

may reside in a single biochemical property of the drug. Although sulphydryl reactivity has been shown to play a role in several of the observed activities of gold salts [2, 5, 10, 11], the importance of this chemical property has not been generally accepted. This study was undertaken to assess the role of the sulphydryl reactivity of ATM in inhibiting PHA-induced blast transformation of human peripheral blood lymphocytes (PBL) [12, 13].

*Materials and Methods.* Human peripheral blood was obtained by venepuncture from healthy donors and was anti-coagulated with 10 Units/ml of preservative-free Heparin B.P. (Weddel Pharmaceuticals Ltd). The mononuclear cell population was separated on Ficoll-sodium diatrizoate density gradients by a modified Boyum technique [14, 15]. The separated mononuclear cells were washed three times in phosphate-buffered saline supplemented with 5% foetal calf serum (FCS; Gibco) and then resuspended at a final cell density of $10^6$/ml in Dulbecco's modified Eagle's MEM (Flow Laboratories) supplemented with 10% FCS, 2 mm L-glutamine, 20 mM HEPES, 100 U/ml penicillin G and 100 μg/ml streptomycin sulphate. The normal recovery of lymphocytes was 80–85 per cent while the final lymphocyte preparations were 90–95 per cent pure.

Lymphocytes were cultured for 72 hr at 37° in round-bottomed microplates (Cooke Microtitre) using 0.2 ml of cell suspension per well, in a humidified incubator with 5% $CO_2$ in air as the gas phase. At the initiation of culture, thiol reagents were added to the appropriate wells in 5 μl volumes and incubated for 30 min prior to the addition of disodium aurothiomalate (ATM; Aldrich Chemical Co, Milwaukee, Wisconsin, U.S.A.) and 5 μg/ml phytohaemagglutinin (PHA; HA16, Wellcome Reagents Ltd, England) each in 5 μl volumes. Cultures were performed in triplicate or quadruplicate.

In preincubation experiments, larger volumes (1–3 ml) of the cell suspension were treated with the thiol reagents in glass culture tubes for 30 min at 37°, then centrifuged and resuspended in fresh medium. These treated cells were also cultured in 0.2 ml aliquots with PHA and ATM. Control cultures were treated similarly, except that thiol reagents were omitted.

Twenty hours before harvesting 1 μCi of methyl-[$^3$H]thymidine in 5 μl (sp. act. 18–21 Ci/mmole: Amersham) was added to each culture. Cultures were harvested at 72 hr onto glass fibre filters using a semi-automated cell harvester (Dynatech, Micro Mash AM77) with a distilled water wash of 40 volumes. The filters were dried and the radioactivity (cpm of [$^3$H]thymidine incorporated) determined by liquid scintillation counting in toluene–POPOP–PPO.

The gold compounds and sulphydryl reagents were prepared fresh by dissolving in half the final desired volume of medium, adjusted to pH 7.00, made up to the final volume with medium, sterile filtered and the appropriate dilutions prepared.

The values expressed are the mean cpm ± standard deviation of triplicate or quadruplicate cultures. Owing to large variations in the response of individual donors' lymphocytes to ATM and the thiol reagents, each histogram represents the results of experiments from only one donor.

*Results.* When the thiol reducing agent, dithiothreitol, was present throughout the culture period it enhanced the blastogenic response of the lymphocytes to PHA and protected the cells in a dose-related manner, against the inhibitory activity of aurothiomalate (Fig. 1). When cultures were merely preincubated with dithiothreitol for 30 min, the PHA response was still elevated above control levels. However, such brief exposure to dithiothreitol did not

prevent the inhibition of blastogenesis by ATM (Fig. 2).

The other thiol reducing agents tested (L-cysteine, 2-mercaptoethanol, cysteamine, *N*-acetyl-L-cysteine) had little effect except for L-cysteine which reduced the inhibitory effects of aurothiomalate.

In contrast to the thiol reducing agents, thiol binding (blocking) reagents mimicked the action of ATM in inhibiting PHA-induced blast transformation. Iodoacetamide, *N*-ethylmaleimide, and mersalyl all reduced the level of DNA synthetic activity to below that of the unstimulated control cultures (not illustrated). Total inhibition was seen even when the cultures were exposed to the thiol binding agents for the initial 30 min of culture only.

Co-incubation of the thiol binding reagents with ATM resulted in greater inhibition than that observed with either compound alone.

The importance of the gold moiety to the inhibitory activity of the intact aurothiomalate molecule, was assessed by comparing the activity of ATM with that of the thiol ligand, thiomalic acid, as well as two inorganic gold compounds, sodium tetrachloroaurate (III) and gold trichloride. The same weight equivalent of gold atom or equimolar thiomalate levels were used. Thiomalate produced a dose-related inhibition of lymphocyte activation similar to that observed with ATM (Fig. 3). The addition of thiomalate to cultures incubated with ATM resulted in additive inhibition. Gold trichloride and sodium tetrachloroaurate (III) were found to have a far greater inhibitory activity than either ATM, or thiomalate. However, unlike ATM or thiomalate, the two inorganic gold compounds reduced the cell viability, determined by trypan blue dye exclusion, to 30–50 per cent at the higher dose levels.

*Discussion.* Various investigators have demonstrated that the sulphydryl reactivity of gold compounds appears to be important in several of the effects gold compounds have been demonstrated to have *in vitro* [2, 5, 11]. Subnormal levels of serum sulphydryl groups have been observed in rheumatoid arthritis [16, 17]. This has been attributed to an accelerated formation of disulphide bonds, which could result in protein denaturation and globulin aggregation. ATM administration, both *in vitro* and *in vivo* has been shown to inhibit the sulphydryl–disulphide interchange reaction between rat serum sulphydryl groups and dithiobisnitrobenzoic acid [18]. Furthermore, the sulphydryl-dependent aggregation of human γ-globulin, which is a stimulus to the formation of rheumatoid factors, has been shown to be inhibited by both ATM and other sulphydryl binding reagents [5].

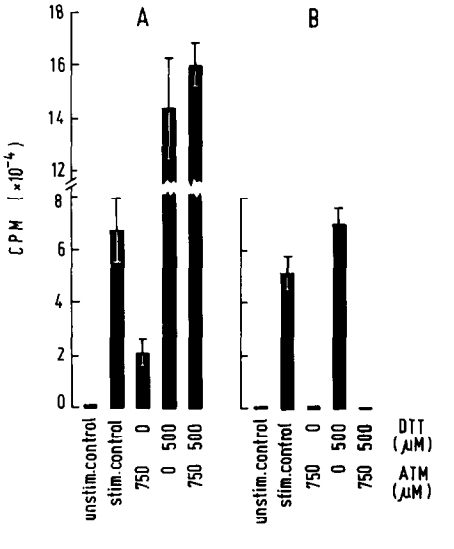


Fig. 2. Effect of preincubation with dithiothreitol (DTT) compared with continuous presence of DTT on subsequent ATM inhibition of [$^3$H]thymidine incorporation by PHA-stimulated PBL cultures. (A) Continuous presence of ATM and DTT in culture. (B) 30 min exposure to DTT, then washed and cultured for 72 hr with PHA and ATM.

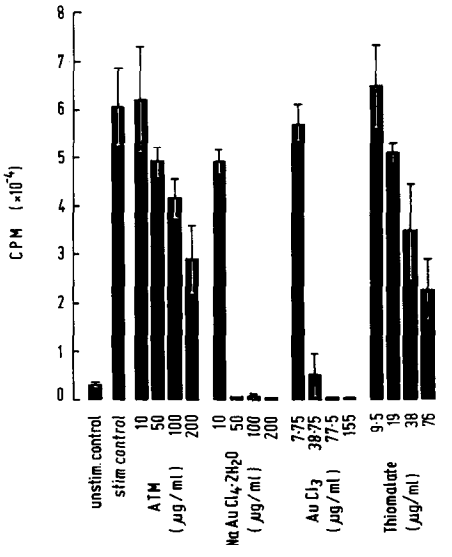


Fig. 3. Comparison of the effect of ATM with those of sodium tetrachloroaurate (III), gold trichloride and thiomalate on [$^3$H]thymidine incorporation by 72 hr PHA-stimulated PBL cultures.

Our findings confirm the similar inhibitory properties of ATM and sulphydryl binding agents on an *in vitro* model of lymphocyte activation. The thiol binding compounds were approximately 10-fold more effective than ATM and enhanced the inhibition observed with ATM.

In constrast, the thiol reducing agents enhanced the PHA response when added alone and partially protected the cells from the inhibitory action of ATM.

It would seem that the sulphydryl reactivity of ATM plays an integral role in inhibiting PHA-induced blast transformation of human lymphocytes but it is still uncertain whether the biological activity of aurothiomalate can be entirely attributed to either the thiol ligand or the gold moiety. In contrast to the results of others [19, 20], thiomalate under the present experimental conditions was found to cause a dose-related inhibition of PHA responsiveness in much the same manner as ATM. When added in conjunction with ATM an additive effect was demonstrated. Although the two inorganic gold compounds exhibited far greater inhibitory activity than either ATM or thiomalate this was accompanied by a cytotoxic effect.

The observation that the thiol reagents affected the PHA response, after only a brief initial exposure, suggested that an interaction of the thiol compounds with sulphydryl groups present in the medium was not a determining factor, as has been suggested [21].

Inhibition of lymphocyte activation might be dependent upon inhibition of lysosomal enzymes since lyosomal enzyme accumulation and release appear to be involved in "derepression" of the genome and a prerequisite for lymphocyte mitosis and cell division [23, 24].

A direct action of ATM on macrophages cannot be discounted, as gold salts accumulate in macrophage lysosomes [22] and may inhibit functions associated with the induction of transformation [20]. Thiol compounds have been shown to substitute for this macrophage function, possibly by preserving lymphocyte viability.

*Summary.* The thiol reducing agent dithiothreitol

enhanced the PHA response and opposed or reversed the inhibitory activity of aurothiomalate on PHA-induced blastogenesis of human peripheral blood lymphocytes. In contrast, the thiol binding reagents iodoacetamide, *N*-ethylmaleimide and mersalyl were found to be inhibitory. Thiomalate produced a dose-related inhibition of blastogenesis similar to that observed with aurothiomalate. Two inorganic gold salts were more inhibitory than either thiomalate or aurothiomalate, but were also cytotoxic. These observations suggest that the sulphydryl reactivity of aurothiomalate could be the active principle in its activity *in vitro*.

*Acknowledgement*—The financial support of the Medical Research Council of New Zealand is gratefully acknowledged.

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REFERENCES

1. Empire Rheumatism Council, *Ann. rheum. Dis.* **20**, 315 (1961).
2. R. S. Ennis, J. L. Granda and A. S. Posner, *Arthritis Rheum.* **11**, 756 (1968).
3. R. H. Persellin and M. Ziff, *Arthritis Rheum.* **9**, 75 (1966).
4. N. S. Penneys, V. Ziboh and N. L. Gottlieb, *J. Invest. Dermat.* **63**, 356 (1974).
5. D. A. Gerber, *Arthritis Rheum.* **17**, 85 (1974).
6. K. E. Viken and J. O. Lamvik, *Acta path. microbiol. Scand., Sect. C* **84**, 395 (1976).
7. P. L. McCormack and D. G. Palmer, *Proc. Univ. Otago Med. Sch.* **54**, 50 (1976).
8. M. Harth, *Clin. exp. Immun.* **27**, 357 (1977).
9. R. S. Panush, *Arthritis Rheum.* **19**, 907 (1976).
10. D. A. Gerber, *J. Pharmac. exp. Ther.* **143**, 137 (1964).
11. M. Davidson and L. Thomas, *Antimicrob. Ag. Chemother.* **312**, (1966).
12. N. J. Zvaifler, *Arthritis Rheum,* **17**, 297 (1974).
13. M. Ziff, *Arthritis Rheum.* **17**, 313 (1974).
14. A. Bøyum, *Scand. J. clin. Lab. Invest.* **21**, Suppl. 97, 77 (1968).
15. W.H.O./I.A.R.C. Technical Report, *Scand. J. Immun.* **3**, 521 (1974).
16. A. Lorber, C. M. Pearson, W. L. Meredith and L. E. Gantz-Mandell, *Ann. intern. Med.* **61**, 423 (1964).
17. A. Lorber, R. A. Bovy and C. C. Chang, *Metab. clin. exp.* **20**, 446 (1971).
18. D. T. Walz and M. J. DiMartino, *Proc. Soc. exp. biol. Med.* **140**, 263 (1972).
19. R. B. Lies, C. Cardin and H. E. Paulus, *Ann. rheum. Dis.* **36**, 216 (1977).
20. P. E. Lipsky and M. Ziff, *J. clin. Invest.* **59**, 455 (1977).
21. P. A. Kendall, *Lancet* **ii**, 862 (1976).
22. N. S. Penneys, S. McCreary and N. L. Gottlieb, *Arthritis Rheum.* **19**, 927 (1976).
23. A. C. Allison and L. Malluci, *Lancet* **ii**, 1371 (1974).
24. K. Hirschhorn and R. Hirschhorn, *Lancet* **i**, 1046 (1964).