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EFFECT OF HEAVY METALS ON HUMAN RHEUMATOID SYNOVIAL CELL PROLIFERATION AND COLLAGEN SYNTHESIS

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Abstract—The dose-dependent effects of heavy metals on cell proliferation, collagen synthesis, and non-collagen protein synthesis were studied in early passage cultures of human synovial cells exposed to 1–100 μM concentration of gold, silver, mercury, cadmium or lead for 5 days. The incorporation of [^{3}H]thymidine into trichloroacetic acid insoluble material was inhibited 50% by each of the heavy metals at concentrations between 1 and 10 μM. Gold, lead and mercury (10 μM) decreased the DNA content of the cultures by less than 15%; silver (10 μM) and cadmium (10 μM) resulted in decreased DNA content, which was attributed to cytotoxicity. A dose-dependent inhibition of [^{3}H]proline incorporation into bacterial collagenase resistant (non-collagen) protein was observed after incubation with 10 μM mercury, lead and silver. During incubations with 10 μM gold and cadmium, collagenase resistant protein accumulation increased. All the heavy metals except for gold inhibited collagen accumulation to a greater extent than non-collagen protein accumulation. Gold (10 μM) stimulated the amount of collagen produced per cell, and the percentage of collagen to total protein was increased 50%. The rate of collagen accumulation in medium decreased during incubation with 10 μM silver, mercury, cadmium and lead. The stimulation of collagen synthesis may be a unique property of gold related to the therapeutic indices of gold, compared to other heavy metals, in rheumatoid arthritis.

The use of most heavy metals as drugs in medicine has been discontinued in favor of more specific and less toxic drugs. An exception is the organic gold compounds that have been used for over a half a century for the treatment of rheumatoid arthritis. The efficacy of gold therapy in treating rheumatoid arthritis has been confirmed in several double blind studies [1–4]. Theories proposed to explain the mechanism of action of gold include inhibition of lysosomal enzyme [5–8], inactivation of complement [9], alteration of immunological and inflammatory cell behavior [10–15], and alteration of collagen crosslinks [16].

Rheumatoid arthritis is characterized by the proliferation of synovial cells (inner lining cells of the joint) which then invade adjacent connective tissues. Cultured synovial cells from arthritic donors have been used to study the biochemistry, cell biology, and pathology related to rheumatoid arthritis [17–19]. Previously, we have demonstrated that gold has a direct effect on cultured synovial cell proliferation and collagen synthesis [20]. We proposed that these effects may be related to the efficacy of gold in rheumatoid arthritis and may constitute an important pharmacological mechanism of action of gold [21]. In this study, we examine the relative effects of several other heavy metals on the cultured human synovial cell culture model to determine if gold has a unique effect on synovial cell metabolism compared to other heavy metals.

METHODS

Culture conditions and exposure to heavy metals. Synovial cells were isolated and maintained as described previously [21]. Briefly, synovial tissue for explant cultures was obtained from patients undergoing reconstructive joint surgery at The Roger Williams General Hospital, Providence, RI. The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum plus gentamycin (50 μg/ml) and fungizone (2.5 μg/ml) (GIBCO, Grand Island, NY). The cultures were incubated at 37° in a humid atmosphere of 95% air–5% carbon dioxide.

Experiments were performed on synovial cells during the second to fifth passage. Monolayers were grown in 75 cm^2 flasks and subcultured into 25 cm^2 flasks which were semi-confluent when exposed to the heavy metals. Cultures derived from a single donor were exposed to heavy metals for 3 days and then the culture medium was replaced with fresh medium containing the same concentration of heavy metal for another 2 days. In each experiment, three flasks were used for control data and one flask of cells was used for each heavy metal concentration. The heavy metals silver nitrate (Ag, Baker), mercuric chloride (Hg, Baker), lead acetate (Pb, Fisher), cadmium chloride (Cd, Baker), and gold sodium

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thiomalate (Au, Merck, Sharp & Dohme) were dissolved in sterile distilled water and diluted to allow 50 μl to be added to 5 ml of culture medium.

DNA determinations. The DNA content of cultures was determined by a modified fluorimetric assay [22]. Trypsin released cells were sonicated and digested with ribonuclease (Type III-A, Sigma Chemical Co., St. Louis, MO) to degrade RNA (0.2 mg, 50°, 30 min). Sperm whale DNA (1–5 μg) was used as the standard with 10 μg ethidium bromide/tube (1 ml total assay volume). The ethidium bromide–DNA fluorescence was measured at excitation–emission wavelengths of 360–590 nm in an Aminco Bowman spectrophotofluorometer.

Estimation of DNA synthesis. The rate of DNA synthesis was measured by the incorporation of [^{3}H]thymidine (TdR) into trichloroacetic acid (TCA) insoluble material [19]. Three hours before harvesting cells, 0.5 μCi [^{3}H]TdR (20 Ci/mmole; New England Nuclear Corp., Boston, MA) was added to each culture. The trypsin released cell suspension was precipitated twice with 10% TCA, dissolved in 0.05 N sodium hydroxide, counted, and adjusted for DNA content of the cell layers.

Estimation of collagen and non-collagen protein synthesis. The rate of ^{3}H-labeled collagen and non-collagen protein accumulation in the synovial cell culture medium was determined by the susceptibility of labeled TCA-precipitable material to purified bacterial collagenase digestion [23]. Forty-eight hours before harvesting the cells, [2,3-^{3}H] proline (10 μCi/ml, 20.7 Ci/mmole; New England Nuclear), β-aminopropionitrite (BAPN, 100 μg/ml), and ascorbate (100 μM) were added to the cultures. Ascorbate was added again 24 hr before harvesting the cells. Harvested medium was dialyzed at 4° against 0.1 M Tris–HCl, pH 7.5, and then digested for 3 hr at 37° with 100 units of protease free bacterial collagenase [21]. The synthesis of non-collagenase digestible protein was determined from the cpm in the TCA precipitate of the digested sample. The synthesis of collagenous protein was determined by the difference in the cpm of the TCA precipitants of the non-digested sample and the digested sample. Isotope incorporation into the TCA insoluble material was determined by liquid scintillation counting, and the values were adjusted for the DNA content of the cell layers. The percentage of collagen to total protein was calculated, as described by Diegelmann *et al.* [24], which corrects for the 5.4-fold enrichment of imino acids in collagen compared to non-collagen proteins.

RESULTS

Antiproliferative effect of heavy metals. To determine the relative effect of heavy metals on synovial cell proliferation, the [^{3}H]TdR incorporation into DNA and the total DNA content of the cultured cells were measured after 5 days of exposure to heavy metals. We previously found that this was the minimum amount of time to affect proliferation and protein synthesis [20, 21]. Heavy metals were added to the cell cultures when the cells reached late log phase of growth. Cultures became confluent after 3 days of exposure to control medium and to medium containing low concentrations of heavy metals. Cells at late log phase were used so sufficient collagen would be produced for reliable measurements. Exposure to higher concentrations of heavy metals resulted in a loss of cells by microscopic observation which was confirmed by the dose-related decrease in DNA content of the cultures after 5 days (Fig. 1A). The incorporation of [^{3}H]TdR was inhibited by all concentrations of heavy metals tested (Fig. 1B). Table 1 shows that all the heavy metals tested inhibited [^{3}H]TdR incorporation into DNA with IC_{50} values between 1 and 5 μM. The IC_{50}s values for reduction in DNA content ranged from 12 to > 100 μM. At 1 μM concentrations all cultures treated with heavy metals were confluent, but at 10 μM only the mercury, gold- and lead-treated cell cultures were confluent. A medium change 48 hr before harvesting did not stimulate cell division in the cultures exposed to a low concentration of heavy metals to the same extent as observed in the control cultures (Fig. 1B).

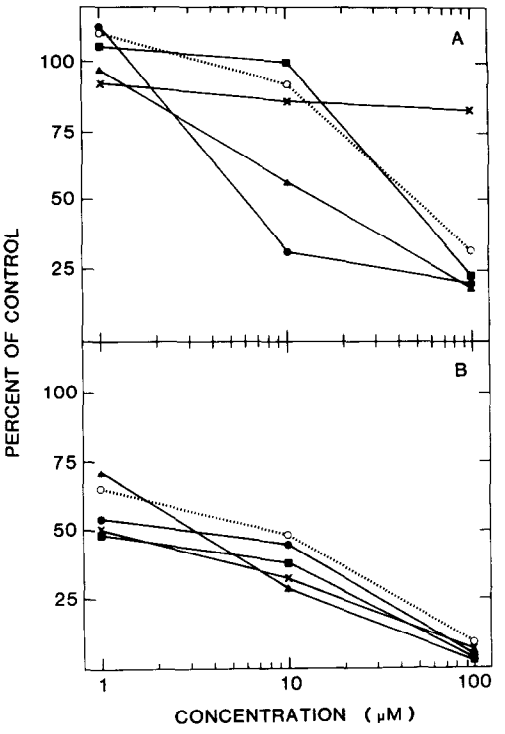

Fig. 1. Dose-dependent effect of a 5-day exposure of heavy metals on synovial cell proliferation. (A) DNA content of cultures as measured by ethidium bromide fluorescence of trypsin-released cell layers. Values are the average from two experiments in which three flasks were used for control data and one flask of cells for each heavy metal concentration. Control values were 6.8 ± 0.2 and 6.0 ± 0.1 μg DNA (mean ± S.E.). (B) DNA synthesis of cultures as measured by [^{3}H]TdR incorporation into TCA precipitable material in the trypsin-released cell layer. Values are the average from duplicate determinations of one flask of cells for each heavy metal concentration from one experiment. The control value was 7500 ± 400 cpm/g DNA (mean ± S.E. from triplicate cultures). Symbols: cadmium (●), mercury (■), silver (▲), lead (×), and gold (○).

Table 1. IC_{50} Values for heavy metals on human synovial cell proliferation in culture*

Heavy metals	IC_{50} (μM) DNA synthesis	DNA content
Cadmium	2.4	12
Silver	3.5	15
Mercury	1.2	37
Lead	1.0	> 100
Gold	4.7	57

* Synovial cells were exposed to heavy metals for 5 days. After the first 3 days of exposure, the culture medium was changed, and the exposures were continued for another 2 days. For the last 3 hr ([^{3}H]TdR was added. DNA content was determined by ethidium bromide fluorescence and DNA synthesis by [^{3}H]TdR in TCA insoluble material in the trypsin released cell layer. Concentration for 50% inhibition (IC_{50}) was determined from the log linear regression analysis of the least squares fit of the data in Fig. 1.

Table 2. Effect of heavy metals on collagen synthesized as a percentage of total medium protein by human synovial cells*

	% Collagen Heavy metal concn (μM) 1	10	100
Cadmium	2.8	1.5	<0.1
Silver	8.2	0.8	0.2
Mercury	8.1	4.3	0.1
Lead	3.9	3.2	2.1
Gold	9.0	9.1	0.6

* The exposure to heavy metals was the same as in Table 1. During the last 2 days of exposure, [^{3}H]proline, BAPN and ascorbate were added to the cultures with fresh medium. Values are the percentage of collagen synthesized to total medium protein from the data in Fig. 2 calculated according to Diegelmann *et al.* [24]. The control value was 6.2 ± 0.6% (mean ± S.E. for triplicate cultures).

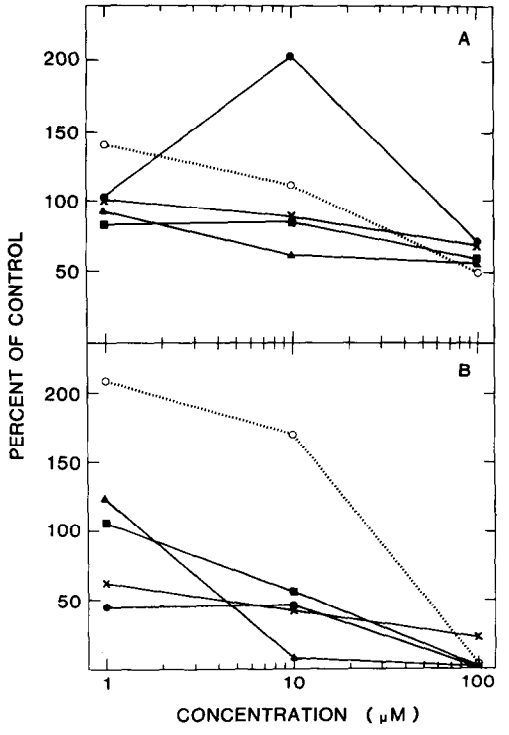

Fig. 2. Dose-dependent effect of a 5-day exposure of heavy metals on synovial cell protein synthesis. (A) Medium non-collagen protein/μg DNA as measured by 48-hr incorporation of [^{3}H]proline (in the presence of BAPN and ascorbate) into non-bacterial collagenase digestible TCA insoluble material. Values are the average of duplicate determinations of one flask of cells for each heavy metal concentration from one experiment. The control value was 5800 ± 120 cpm/μg DNA (mean ± S.E. from triplicate cultures). (B) Medium collagen protein/μg DNA. The control value was 2100 ± 200 cpm/μg DNA (mean ± S.E from triplicate cultures). Values are the average of duplicate determinations of one flask from one experiment. Symbols: cadmium (●), mercury (■), silver (▲), lead (×), and gold (○).

Effect of heavy metals on protein synthesis. The effect of heavy metals on protein synthesis in the synovial cell cultures was measured as the rate of collagen and non-collagen protein synthesis per μg DNA (Fig. 2). The addition of cadmium (10 μM) resulted in a 2-fold increase in non-collagen protein synthesis per μg DNA. Gold (1 μM) caused a 40% increase in non-collagen protein per μg of DNA and a 12% increase at a concentration of 10 μM. Exposure of cells to mercury, silver or lead resulted in a dose-dependent decrease of non-collagen protein production.

At a 10 μM concentration of silver, cadmium or mercury, collagen synthesis was inhibited more than non-collagen protein synthesis (Fig. 2B and Table 2). In gold-treated cells, the incorporation of [^{3}H]proline into collagen per μg of DNA increased over 2-fold at 1 μM gold and increased 70% at 10 μM gold. At 1 μM gold, silver and mercury, there was an increase in the percentage of collagen to total protein synthesized but, at 10 μM, only gold resulted in an increase (50%) above the control. At heavy metal concentrations of 100 μM, collagen synthesis was inhibited to a greater extent than non-collagen protein synthesis by all heavy metals tested (Fig. 2 and Table 2).

DISCUSSION

The *in vitro* cytotoxicity of heavy metals has been demonstrated using a wide variety of cultured cells [25, 26]. This work was conducted to determine the relative effect of various heavy metals on proliferation and protein synthesis of synovial cells from rheumatoid arthritic donors.

Previously, we reported that decreased [^{3}H]TdR incorporation was correlated with gold deposition in the synovial cell layer and that DNA content subsequently decreased [21]. All heavy metals used in the current study had similar dose–response curves for inhibition of DNA synthesis with IC_{50}s values between 1 and 3 μM. The heavy metal effects on DNA content of the cultured synovial cells were more varied. Using DNA content as a marker, silver

and cadmium were found to be more cytotoxic with IC_{50}s values of 15 and 12 μM respectively. Lead, mercury and gold had IC_{50}s values of greater than 35 μM for the 5-day exposures.

During gold therapy, serum concentrations of 5–15 μM are achieved and tissue concentrations of 100–150 μM have been reported [27–34]. Therefore, gold serum levels would decrease the rate of synovial cell proliferation, but cell death would not occur until there is an accumulation of gold in the synovial tissue.

All heavy metals tested except for cadmium and gold caused a dose-dependent inhibition of non-collagen protein synthesis. Cadmium (10 μM) caused a 2-fold increase in secreted non-collagen protein per μg DNA, but, because the number of cells was less than the controls, the total non-collagen protein found in medium was lower in the cadmium-treated cells than in the control. Since human synovial cells have a doubling time of approximately 5 days and since the exposure of the cells to the heavy metals began in late log phase, the decrease in cells to 20–50% of control is due to cytotoxicity and the detachment of cells from the flasks. The resulting cell debris would contribute to the increased non-collagen protein content seen with cadmium. Gold (10 μM) was the only heavy metal that did not decrease non-collagen protein per cell culture and per μg DNA.

The effect of gold on collagen synthesis relative to non-collagen protein synthesis was greater than the effect observed with the other heavy metals. Gold (1–10 μM) caused an approximate 2-fold increase in collagen secreted into the medium and a 50% increase in the percentage of collagen to total medium protein. All the other heavy metals (10 μM) were more potent in depressing collagen synthesis than non-collagen synthesis. At 100 μM all the heavy metals depressed collagen more than non-collagen protein synthesis.

We have reported previously [20] that gold (7.5 to 30 μM) increased both the rate of synthesis and amount of collagen secreted per cell. The increase was predominantly type I collagen. We proposed that a change in the type III/I ratio may be related to the elaboration of a matrix that is not conducive for the development of a hyperplastic synovium. Although the collagen types were not measured in the present experiments, none of the heavy metals tested except gold increased the rate of total collagen synthesis. In the case of gold, concentrations similar to those found in serum (5–15 μM) result in increased *de novo* synthesis of matrix, while concentrations consistent with those found in tissue (50–150 μM) mimic the general heavy metal toxic effect of decreasing collagen and non-collagen protein synthesis. Therefore, gold has a unique bifunctional effect on collagen synthesis. This effect of gold at high concentration is similar to the effect of penicillamine, another anti-rheumatic drug, which has also been shown to preferentially decrease collagen production [35].

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REFERENCES

1. Research Subcommittee of the Empire Rheumatism Council, *Ann. rheum. Dis.* **19**, 95 (1960).
2. Research Subcommittee of the Empire Rheumatism Council, *Ann. rheum. Dis.* **20**, 315 (1961).
3. Cooperative Clinics Committee of the American Rheumatism Association, *Arthritis Rheum.* **16**, 353 (1973).
4. J. W. Sigler, G. B. Bluhm, H. Duncan, J. T. Sharp, D. C. Enirgin and W. R. McCrum, *Ann. intern. Med.* **80**, 21 (1974).
5. R. H. Persellin and M. Ziff, *Arthritis Rheum.* **9**, 57 (1966).
6. R. S. Ennis, J. L. Granda and A. S. Posner, *Arthritis Rheum.* **11**, 756 (1968).
7. M. J. DiMartino and D. T. Walz, *Inflammation* **2**, 131 (1977).
8. A. E. Finkelstein, F. Roisman and D. T. Walz, *Inflammation* **2**, 143 (1977).
9. D. R. Schultz, J. E. Volanokis, I. Arnold, N. L. Gottlieb, K. Sakai and R. M. Stroud, *Clin. exp. Immun.* **17**, 395 (1974).
10. A. E. Finkelstein, D. R. Burrone, D. T. Walz and A. Misher, *J. Rheumatol.* **4**, 245 (1977).
11. A. G. Mowat, *Ann. rheum. Dis.* **37**, 1 (1978).
12. J. D. Jessop, B. Vernon-Roberts and J. Harris, *Ann. rheum. Dis.* **32**, 294 (1973).
13. P. E. Lipsly and M. Ziff, *J. clin. Invest.* **59**, 455 (1977).
14. B. Vernon-Roberts, J. D. Jessop and J. Dore, *Ann. rheum. Dis.* **32**, 301 (1973).
15. K. Ugai, M. Ziff and P. E. Lipsky, *Arthritis Rheum.* **22**, 1352 (1979).
16. M. Adam, P. Fietzek and K. Kuhn, *Eur. J. Biochem.* **3**, 411 (1968).
17. C. W. Castor and E. L. Dorstewitz, *J. Lab. clin. Med.* **68**, 300 (1966).
18. J. Dayer, R. Graham, G. Russel and S. M. Krane, *Science* **195**, 181 (1977).
19. E. Vuorio, *Scand. J. clin. Lab. Invest.* **37** (Suppl. 149), 1 (1977).
20. R. L. Goldberg, D. P. Parrott, S. R. Kaplan and G. C. Fuller, *Biochem. Pharmac.* **29**, 869 (1980).
21. R. L. Goldberg, D. P. Parrott, S. R. Kaplan and G. C. Fuller, *J. Pharmac. exp. Ther.* **218**, 295 (1981).
22. A. S. Prasad, E. Du Mouchelle, D. Koniuch and D. Oberlas, *J. Lab. clin. Med.* **80**, 598 (1972).
23. B. Peterkofsky and R. Diegelmann, *Biochemistry* **10**, 988 (1971).
24. R. I. Diegelmann, L. C. Rothkope and I. K. Cohen, *J. surg. Res.* **19**, 239 (1975).
25. S. Horvath, *Toxicology* **16**, 59 (1980).
26. A. P. Stammati, V. Silano and F. Zucco, *Toxicology* **20**, 91 (1981).
27. R. C. Gerber, H. E. Paulus, R. Bluestone and C. M. Pearson, *Ann. rheum. Dis.* **31**, 308 (1972).
28. B. R. Mascarehas, J. L. Granda and R. H. Freyberg, *Arthritis Rheum.* **15**, 391 (1972).
29. J. D. Jessop and R. G. S. Johns, *Ann. rheum. Dis.* **32**, 228 (1973).
30. H. M. Rubinstein and A. A. Dietz, *Ann. rheum. Dis.* **32**, 2 (1973).
31. N. L. Gottlieb, P. M. Smith and E. M. Smith, *Arthritis Rheum.* **15**, 16 (1972).
32. R. Graham, R. Billings, M. Laurence, V. Marks and P. J. Wood, *Ann. rheum. Dis.* **33**, 536 (1974).
33. B. Vernon-Roberts, J. L. Dore, J. D. Jessop and W. J. Henderson, *Ann. rheum. Dis.* **35**, 477 (1976).
34. A. Lorber, C. J. Atkins, C. C. Chang, Y. Lee, J. Straus and R. A. Bovy, *Ann. rheum. Dis.* **32**, 133 (1973).
35. D. Schorn, M. J. O. Francis, M. Loudon and A. G. Mowat, *J. Rheumatol.* **8**, 124 (1979).