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METALLOTHIONEIN IN CULTURED HUMAN EPITHELIAL CELLS AND SYNOVIAL RHEUMATOID FIBROBLASTS AFTER *IN VITRO* TREATMENT WITH AURANOFIN

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Abstract—Radioimmunoassay (RIA) and reversed-phase high-pressure liquid chromatography (HPLC) were used to investigate gold-binding proteins of possible metallothionein (MT) nature occurring upon auranofin exposure of cultured human cells. An epithelial cell line (HE) and two sub-strains were examined. The HE_{AF} sub-strain had been made resistant to 2 μmole auranofin/l culture medium. The resistance was associated with the appearance of gold-binding substances with gel filtration characteristics like MT. The HE_{100} sub-strain had been made resistant to 100 μmole $CdCl_2$/l and contained high amounts of cytosolic Cd-induced MT. In addition, cultured synovial fibroblasts, derived from normal (S_N) and rheumatoid (S_{RA}) synovial tissues, were investigated. Evidence was obtained by RIA that the low molecular weight (mol.wt. 6000–7000) gold-binding proteins occurring in the HE_{AF} cells and S_{RA} cells following auranofin exposure, were of MT nature. The relative amounts of MT in the epithelial cell lines were: $HE:HE_{AF}:HE_{100} = 1:18:100$. The relative amounts in the synovial fibroblasts were: $S_N:S_{RA}:S_{RA}$ treated with auranofin = 1:3:10. The HPLC methods used were found suitable for isolation of Cd-MT in the HE_{100} cells, but not for the Au-MT in the HE_{AF} cells. By HPLC, the Cd-MT in the HE_{100} cells was resolved into 3 MT-1 and 1 MT-2 iso-proteins exhibiting the amino acid composition typical of MT. Judged by HPLC, the MT in these cells constituted 0.4% of the cytosolic proteins.

The question of whether or not gold compounds have the capacity to induce the synthesis of metallothionein (MT) has been raised by several authors. MT is a group of low molecular weight (mol.wt. 6000–7000) iso-proteins with high metal binding capacity, and with a unique amino acid composition in that 30% of the 61 amino acid residues are cysteine [1]. One of the functions suggested for MT is metal detoxification (Cd, Zn), by binding the metals in metal-thiolate clusters [1, 2]. The binding of gold to proteins of assumed MT-nature has been observed, e.g. in the kidneys following treatment of rats with gold–chloride [3], triethylphosphine–gold–chloride [4] and the anti-arthritic drugs sodium aurothiomalate [5, 6] and auranofin [7]. Repeated injections of gold(III)–chloride into rats combined with [^{35}S]cysteine incorporation studies [8] have pointed to an induced biosynthesis of the MT-like proteins under the influence of gold–chloride. During sodium aurothiomalate treatment of rats, observations of increased uptake of Au into the MT-like fractions in kidney cytosols may indicate that the Au(I)-compound similarly leads to the synthesis of these proteins [5, 6]. These earlier experiments have not shown that the gold-binding proteins meet all the requirements for being MT [1], and have not unequivocally established that the synthesis of the proteins is induced by gold in rats injected with the gold compounds.

We have previously observed low molecular weight gold-binding proteins of possible MT-nature occurring in the kidneys and in the small intestine of rats treated with auranofin [7]. These proteins were not demonstrated in untreated animals. We have also shown that cultured human epithelial cells can develop resistance to the antiproliferative effect of 2 μmole auranofin/l culture medium, and that the resistance is associated with the accumulation of gold with proteins of possible MT-nature [9].

The present study was undertaken to characterize further the gold-binding proteins eluting from gel filtration columns in fractions characteristic of MT. The nature and functions of these proteins, occurring upon auranofin treatment of cultured human cells, may have relevance to the effects and side effects observed when the drug is used clinically. As synovial tissue may be one target for gold compounds in rheumatoid arthritis, cultured rheumatoid synovial fibroblasts, exposed to auranofin *in vitro*, were also investigated. The nature of the proteins was compared with cadmium-induced MT obtained from a cadmium-resistant sub-strain of the human epithelial cells [10]. The methods used included reversed-phase high-pressure liquid chromatography and a radioimmunoassay, the most sensitive and specific methods for MT-detection currently available.

MATERIALS AND METHODS

Cell culturing and preparation. The human epithelial cells (HE) were derived from normal skin (NCTC 2544) and obtained from the American

Tissue Type Culture Collection, Maryland, U.S.A. They have been characterized as human and epithelial by antigen typing, determination of isoenzyme patterns and by transmission electron microscopy [11]. One sub-strain (HE_{AF}) had developed resistance to the otherwise lethal concentration of 2 μmole auranofin/l [9]. The possible mechanisms of resistance in the HE_{AF} cells include the occurrence of cytosolic low molecular weight gold binding proteins, i.e. candidate-MT proteins. In addition, the cells had the capacity to maintain the cellular gold concentration at a low level. Another sub-strain (HE_{100}) contains high amounts of cytosolic Cd-induced MT [10]. These cells had previously been made resistant to the lethal effect of 100 μmole cadmium chloride/l culture medium [12]. Their resistance can probably be ascribed to sequestering of Cd by MT.

Synovial fibroblasts were obtained from explant cultures of synovial tissues [13]. The tissue specimens were obtained from the wrist joint of one patient admitted for synovectomy because of a classical seropositive rheumatoid arthritis [14] (rheumatoid fibroblasts, S_{RA}) and from the knee joint of one patient being operated upon for ruptured meniscus (normal fibroblasts, S_N). The arthritic patient had never been treated with gold-containing drugs.

All cells were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with L-glutamine (0.6 g/l), 2.5% fetal calf serum, 15% horse serum (Flow Laboratories, Irwine, Scotland), penicillin (10^5 U/l), steptomycin (100 mg/l) and nystatin (5×10^4 U/l) (Gibco Laboratories, NY). Incubation was at 37° in the presence of 5% carbon dioxide in air and in a 100% humidified atmosphere.

The human epithelial cell lines HE (3.4×10^8 cells), HE_{AF} (1.1×10^8 cells) and HE_{100} (2.7×10^7 cells) were prepared for the HPLC examinations. The HE_{AF} cells, which usually were grown with 2 μmole auranofin/l, were grown with 2 μmole [^{195}Au]auranofin/l for one week prior to harvest.

The following cell lines were prepared for the RIA: (1) HE cells (1.0×10^8); (2) HE_{AF} cells (7.6×10^7) usually grown with 2 μmole auranofin/l and grown with the auranofin labelled with [^{195}Au] for 24 hr prior to harvest; (3) HE_{100} cells (3.4×10^7) continuously grown with 100 μmole $CdCl_2$/l culture medium; (4) S_N cells (8.8×10^6); (5) S_{RA} cells (2.8×10^6); and (6) S_{RA} cells (3.0×10^6) grown with 2 μmole auranofin/l for 5 days and the auranofin labelled with [^{195}Au] for 24 hr prior to harvest. The S_N cells were investigated at the 9th passage and the S_{RA} cells at the 7th passage of cultures.

Exponentially growing cells were harvested by trypsinization (0.05% (w/v) trypsin (1:250) and 0.02% (w/v) EDTA in salt solution, Flow Laboratories). The cells were counted using a Coulter Counter. Cytosols were obtained by ultrasound sonication (15 sec × 4, intervals 1 min, on ice) and subsequent ultracentrifugation (105,000 *g*, 1 hr, 4°). The protein contents were determined by an Abbott Bichromatic Analyser, using human albumin as standard. The cytosols were gel filtrated (G75 Sephadex column (1.6 × 90 cm), 5 mmole/l Tris–HCl buffer, pH 8, tp 4°, elution rate 18 ml/hr, fraction volume 3 ml, or a G50 Sephadex column (1.5 × 56 cm), 10 mmole/l Tris–HCl buffer, pH 8.6, tp 4°, elution rate 13.6 ml/hr, 2.3 ml/fraction). To determine the distribution of metals with the cytosolic proteins, a flame atomic absorbtion spectrophotometer (Cd, Zn, Cu) and a gamma scintillation spectrometer (^{195}Au) were used. The metal-containing fractions eluting with substances of presumed MT-nature ($V_e/V_0 \approx 2$, mol.wt. 5–10,000) were pooled and stored at −70° for HPLC and RIA examinations. The analytical procedure is outlined in Fig. 1.

Agents. Auranofin, (2,3,4,6-tetra-*O*-acetyl-1-thio-β-*D*-glucopyranosato)-triethylphosphine-gold, and [^{195}Au]-auranofin (specific activity 34.73 μCi/mg, radiochemical purity 98.9%) were kindly supplied by the Smith Kline & French Laboratories (Philadelphia, PA). The drug was stored as powder, protected from light, and dissolved in ethanol and subsequently in culture medium to the desired concentration. Fresh drug solutions were prepared for each medium change. Cadmium chloride purum was purchased from Fluka AG (Switzerland).

Reversed-phase high-pressure liquid chromatography (HPLC). The HPLC instrument used is described elsewhere [15]. Water twice distilled from a quartz apparatus was used for the preparation of buffers. Acetonitrile was purchased from J. T. Baker (Deventer, The Netherlands), Tris from Sigma Chemical Co. (St. Louis, MO), and trifluoroacetic acid ("zur Sequenzanalyse") from Fluka AG (Buchs, Switzerland). Separations were achieved on a Hyperchrom column (LiChrosorb RP-18, 10 μm particle size, from Bischoff Analysentechnik, Leonberg, F.R.G.), and on an aquapore RP-300 column (RP-8 support, 10 μm particle size, from Brownlee Laboratories, Santa Clara, U.S.A.).

Amino acid analysis. Amino acid analysis following performic acid oxidation [16] and hydrolysis for 22 hr at 110°, were carried out on a Durrum D-500 analyser.

Radioimmunoassay (RIA). Following the determination by HPLC and amino acid analysis of the significant MT-content in the HE_{100} cells, the relative MT content of the various cell lines was determined by a double-antibody, competitive binding RIA, according to protocols previously described [17].

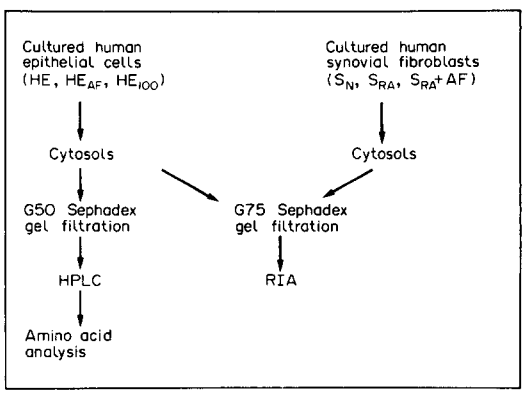

Fig. 1. Analytical procedure for MT detection in various lines of cultured human cells.

RESULTS

Establishment by HPLC of the MT nature of Cd-*binding proteins in* HE_{100} *cells*

The occurrence in the HE_{100} cells of Cd-binding proteins in the low molecular weight region ($V_e/V_0 \approx 2$, mol.wt. 5–10,000) is demonstrated by the G75 Sephadex elution profiles of Cd (Fig. 2B, peak II). When the corresponding peak, eluting from a G50 Sephadex gel filtration of HE_{100} cytosols (profile not shown) was analysed by HPLC (at neutral pH), the proteins were separated into four Cd-containing fractions (Fig. 3A, peaks 1–4). The recoveries of the applied amount of Cd were as follows: peak 1: 56%, peak 2: 18%, peak 3: 9% and peak 4: 3%. The four peaks had retention times identical with the human liver MT-2 and with three of the five MT-1 iso-forms reported elsewhere [18]. Rechromatography of MT-2 (Fig. 3A, peak 1) at pH 2.1 revealed one main protein-containing peak (Fig. 3B). The amino acid composition of the protein fraction thus isolated, is shown in Table 1. The

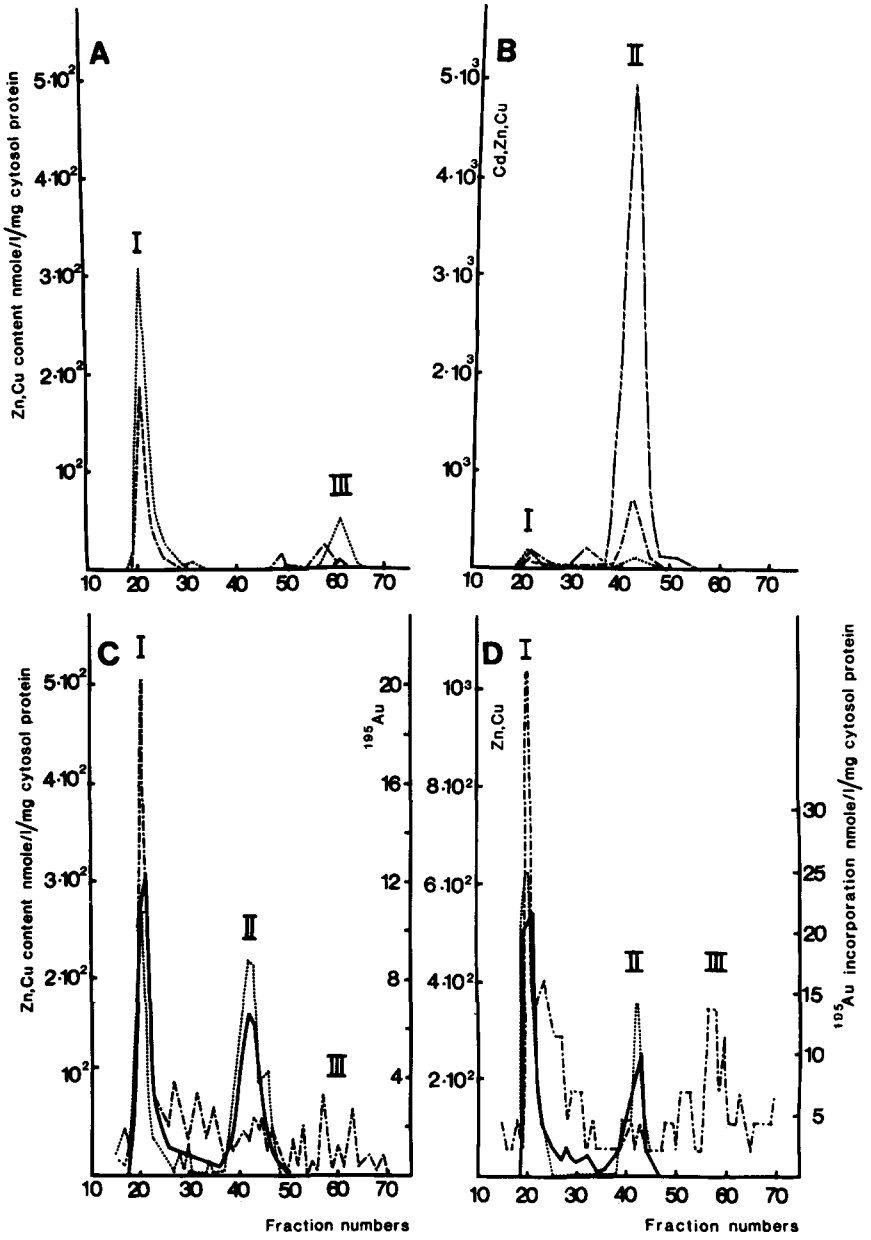

Fig. 2. Elution profiles of ^{195}Au(——), Cd(– – –), Zn(— — —) and Cu(– – – –) following G75 Sephadex gel filtration of HE (A), HE_{100} (B), HE_{AF} (C) and S_{RA} + AF (D) cytosols. The HE_{100} cells were continuously grown with 100 μmole $CdCl_2$/l culture medium, and contain high amounts of cytosolic Cd-MT (peak II). The HE_{AF} cells were continuously grown with 2 μmole auranofin/l, the S_{RA} + AF fibroblasts were grown with 2 μmole auranofin/l for 5 days, and both lines with 2 μmole [^{195}Au]-auranofin/l for 24 hr before harvest. During auranofin treatment, gold accumulates with low molecular weight proteins of possible MT nature (C and D, peak II).

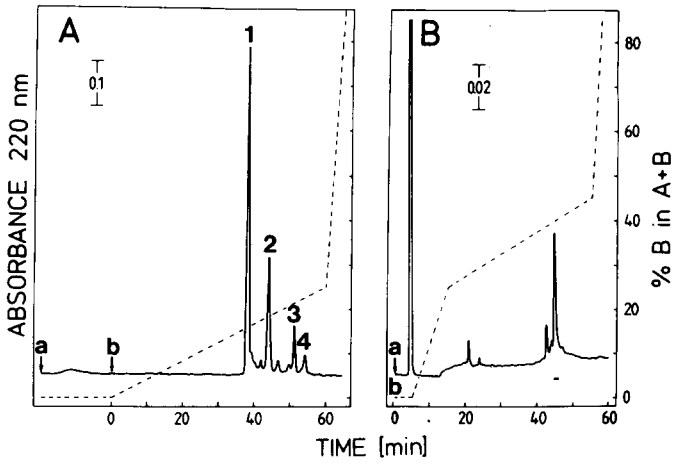

Fig. 3. HPLC-profiles of the MT fraction of HE_{100} cells after G50 Sephadex gel filtration chromatography. (A) HPLC was performed on an RP-18 column, flow-rate 1 ml/min, using a gradient (dashed line) formed between buffer A (25 mmole/l Tris–HCl, pH 7.5) and buffer B (same as A, containing 60% (v/v) acetonitrile). Peak 1 corresponds to MT-2, peaks 2, 3 and 4 correspond to iso-forms of MT-1. (B) Rechromatography of MT-2 from panel A performed on an RP-300 column, flow-rate 1 ml/min, using a gradient formed between buffer A (0.1% (v/v) trifluoroacetic acid, pH 2.1) and buffer B (same as A, containing 60% (v/v) acetonitrile). For amino acid analysis, fractions were collected as indicated by the horizontal bar. In both panels, ↓ a) indicates sample injection, ↓ b) indicates start of the gradient.

identity with MT is demonstrated by a 30% content of cysteine and by the absence of aromatic amino acids. Amino acid determination of the peaks 2–4 from Fig. 3A, revealed the same results with regard to cystein and aromatic amino acids and a slight heterogeneity with respect to other amino acid residues.

No detectable amounts of MT eluted from the HPLC of low molecular weight fractions from HE cytosols.

Table 1. Amino acid analysis of Cd-induced MT-2 from HE_{100} cells corresponding to the indicated fraction (horizontal bar) in Fig. 3B

Amino acid	MT-2 from HE_{100} cells
Cys	*34.08 (32.79)
Asp	6.58 (6.56)
Thr	3.47 (3.28)
Ser	12.96 (13.11)
Glu	2.73 (3.28)
Pro	2.66 (3.28)
Gly	8.48 (8.20)
Ala	11.35 (11.48)
Val	1.73 (1.64)
Met	†0.90 (1.64)
Ile	1.71 (1.64)
Leu	0
Tyr	n.d.
Phe	0
His	0
Lys	13.40 (13.11)
Arg	0

The amino acid contents are given in percent of total and following normalization to 8 lysine residues. Numbers in parentheses are obtained in the same way and refer to the known amino acid sequence of human hepatic MT-2 [32].

* Determined as cysteic acid.

† Determined as methionine sulfone.

n.d. = not determined.

Search for MT in human epithelial cells exposed to auranofin (RIA and HPLC)

The occurrence of metal-binding proteins in the HE_{AF} cytosols is demonstrated by the G75 Sephadex elution profiles of [^{195}Au], Zn and Cu (Fig. 2C). Peak I ($V_e/V_0 = 1$, mol.wt. > 75,000) contains the void volume proteins, peak III ($V_e/V_0 \approx 3$) substances of mol.wt. $\leq$ 1000. Peak II of the HE_{AF} profile contains low molecular weight proteins of possible MT-nature, appearing upon auranofin-treatment [9]. The V_e/V_0 of the peak II is similar to that of the MT-peak eluting from the HE_{100} cytosols (Fig. 2B). No such peak could be demonstrated in cytosols from the parent HE cells (Fig. 3A). The results of the RIA analysis of the peak II fractions from HE_{AF} and HE_{100} cytosols, and of the corresponding fractions following gel filtration of HE cytosols, are shown in Table 2. The similarity of the slopes of the standard curves of HE_{100} and HE_{AF} cytosol fractions and the similarity of both slopes to that of the reference antigen ([^{125}I]-labelled rat MT-1) indicate that the reacting protein from the HE_{AF} cells is MT. In addition, the known MT character of the reference antigen and of the proteins from HE_{100} cells, and the slight difference (a factor of 2.4) in the intercepts of HE_{AF} and HE_{100} cells at Y = 0.5 (see legend, Table 2), are evidences of the MT nature of the HE_{AF} cell proteins. An estimate of the MT content in the HE_{100} cells was obtained using the respective intercept values for the HE_{100} standard curve and that of the reference rat-MT. The HE_{100} cells contained about 800 pg MT/μl eluate, corresponding to 0.4% of the total cytosol proteins. The relative MT contents in the HE:HE_{AF}:HE_{100} cells were 1:18:100 (per cell basis). Only the slope of the HE standard curve differed significantly ($P < 0.05$) from that of the reference standard curve (developed using [^{125}I]-labelled rat MT-1 as the reference antigen and a 50/50 mix of rat MT-1 and MT-2 as

Table 2. RIA* for MT obtained from cytosol fractions of human epithelial cells

Cell ratios†	Ratio of cells per unit volume	Ratio of μl values at Y = 0.5‡	Relative MT content per cell §
$HE_{100}/HE_{AF}/HE$	1/2.24/3.07	1/2.4 (0.2)/32.3 (2.8)	99.1 (8.7)/18.3 (1.4)/1

* Standard curves (SC, linear logit-log regressions) were developed following protocols previously described [17]. The [^{125}I]-labelled reference antigen was rat MT-1. Data analysis followed the methodology outlined in [21]. For each cell line the responses Y (Y = fraction of labelled antigen bound) were obtained for sextuplet samples of a series of doubling dilutions covering the range 200 μl to 0.78 μl of cytosol fractions. A reference SC was also developed using a 50/50 mix of the iso-forms rat MT-1 and rat MT-2 as competitor antigen; the responses were obtained for sextuplet samples of a series of doubling dilutions over the range 20000–100 pg competitor MT. The responses were transformed to logit form ($Z = \log(100\ Y)/(1 - Y)$), with Z expressed as a function of Q, the latter being the log of MT concentration (in pg) for the known rat MT and the log of 100 × μl cytosol fraction for the three cell lines tested. Emphasis in developing the regressions was on the region Y = 0.3 to 0.7. This characteristically is associated with maximum accuracy in the RIA. Similar slopes of various competitor antigens over this region indicate similar antigens; the intercepts at Y = 0.5 provide maximum accuracy in comparing MT concentrations of various competitor antigens. The standard errors of the mean Z values of the various sets of sextuplets were in the range 1.5–1.7% of the mean value over the mentioned response range.

† HE: parent cells; HE_{100}: Cd-resistant sub-strain grown with 100 μmole $CdCl_2$/l; HE_{AF}: auranofin-resistant sub-strain, grown with 2 μmole auranofin/l culture medium.

‡ Ratios determined from the results of the RIAs; standard errors (parentheses) determined by the statistical protocol for ratios. The parameters of the various SCs were as follows: b = slope; r^2 = square of correlation coefficient; intercept at Y = 0.5 (in μl for cytosol fractions, in pg for the reference MT SCs), standard errors in parentheses:
HE: $b = -0.7567$ (0.0385); $r^2 = 0.9923$; intercept 50.0 (3.2)μl
HE_{AF}: $b = -0.8018$ (0.0341); $r^2 = 0.9946$; intercept 3.75 (0.16)μl
HE_{100}: $b = -0.8578$ (0.0627); $r^2 = 0.9894$; intercept 1.54 (0.10)μl
Rat MT: $b = -0.8819$ (0.0316); $r^2 = 0.9949$; intercept 1250 (77)pg MT.
An estimate of the MT content in the HE_{100} cells was obtained using the respective intercept values for HE_{100} and rat MT SCs.

§ Combining the data of the first two numerical columns yields the relative MT content per cell, standard errors in parentheses.

Table 3. RIA* for MT obtained from cytosol fractions of synovial fibroblasts

Cell ratios†	Ratio of cells per unit volume	Ratio of μl values at Y = 0.5‡	Relative MT content per cell§
$S_{RA+AF}/S_{RA}/S_N$	1/0.94/2.90	1/3.33 (0.30)/3.62 (0.30)	10.51 (0.87)/3.13 (0.28)/1

* Procedure as described in legend, Table 2. The [^{125}I]-labelled rat MT-1 used in developing the reference standard curves (SC) in this experiment was the product of a separate isolation/purification/labelling procedure, and was not identical to the labelled MT-1 used in the experiment reported in Table 2. The reference SC will not be identical to that of Table 2.

† S_N: synovial fibroblasts derived from a non-rheumatoid patient; S_{RA}: synovial fibroblasts derived from a patient with rheumatoid arthritis; S_{RA+AF}: S_{RA} fibroblasts treated with 2 μmole auranofin/l culture medium for 5 days before harvest.

‡ Ratios determined from the results of the RIAs, standard errors in parentheses. The parameters (see ‡, legend, Table 2) of the various SCs were as follows:
S_N: $b = -0.9257$ (0.0201); $r^2 = 0.9991$; intercept 28.5 (0.5)μl
S_{RA}: $b = -0.9692$ (0.0478); $r^2 = 0.9952$; intercept 26.3 (1.1)μl
S_{RA+AF}: $b = -0.9228$ (0.0915); $r^2 = 0.9807$; intercept 7.89 (0.63)μl
Rat MT: $b = -1.0412$ (0.1140); $r^2 = 0.9766$; intercept 1636 (146)pg MT

§ Combining the data of the first two numerical columns yields the relative MT content per cell, standard errors in parentheses.

Added note: The assay of the HE cytosol fractions (see Table 2) was repeated in this experiment. The parameters were: $b = -0.9729$ (0.0435); $r^2 = 0.9972$; intercept 32.1 (1.1)μl. These parameters are not identical to those obtained in the epithelial cell experiment (Table 2). The two assays used differently prepared and labelled reference antigens (both were rat MT-1) and the responses are not identical in reactions with the same competitor antigen. In this repeated assay there was no statistical significance in the difference of the slopes of the rat MT and the HE cytosol fraction SCs.

competitor antigen). This is not unexpected in view of the relatively massive volumes (as much as 100–200 μl) of the HE cytosol fractions required to obtain responses in the region of interest (50% of the labelled antigen bound). Non-MT proteins and other agents in the cytosol can interfere with the principal reaction and influence the slope of the regression. That the reacting protein in the HE cytosol fractions is also a MT (although of very low concentration) is supported by the associated RIA on the synovial

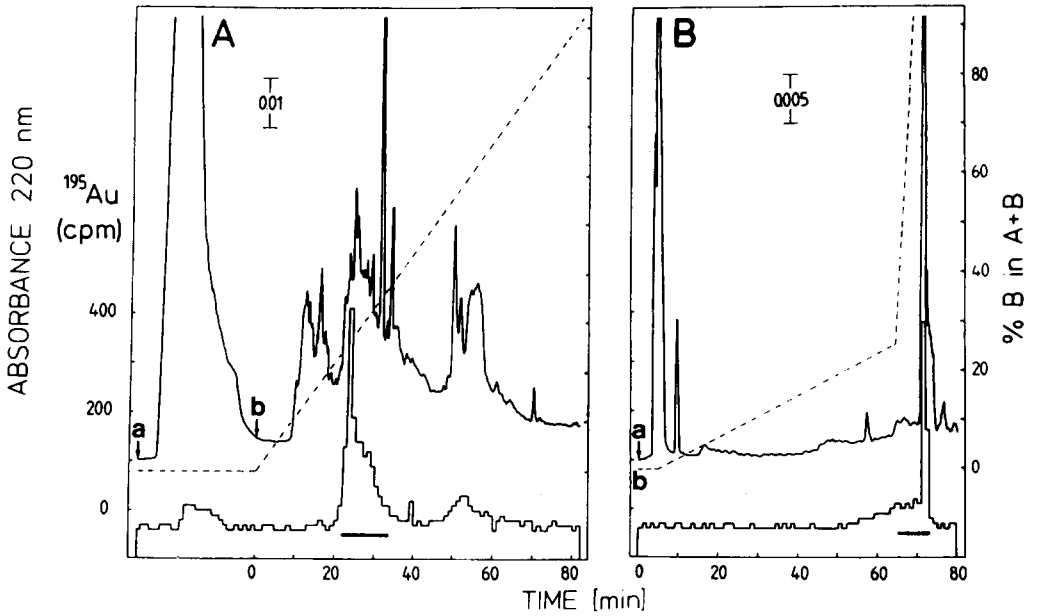

Fig. 4. HPLC-profiles of the low molecular weight gold-containing fractions of HE_{AF} cytosols following G50 Sephadex gel filtration chromatography. HPLC was performed using the same system as in Fig. 3A. (A) One broad peak of gold (histogram plot) co-eluted with several overlapping proteins. The gold-containing fractions (horizontal bar) were collected, dried by evaporation and redissolved in water. (B) Rechromatography of the gold-containing pool from panel A. One gold-containing peak eluted at the end of the gradient. In both panels, ↓ a) indicates sample injection, ↓ b) indicates start of the gradient.

cytosol fractions (Table 3) where a standard curve of the HE cytosol fractions was also developed to permit relating the results of the epithelial cells and synovial fibroblast experiments. The slope difference of the HE cytosol fractions and reference antigen standard curves in that assay was not significant.

The HPLC at neutral pH of the low molecular weight gold-containing pool after G50 gel filtration of the HE_{AF} cytosols resolved several overlapping gold-containing proteins (Fig. 4A). [^{195}Au] eluted as a broad peak with one predominant fraction (Fig. 4A, histogram plot). The total recovery of radioactive gold in the pooled fractions was only 23% of the applied amount. Rechromatography at neutral pH of the gold-containing fractions (Fig. 4A, horizontal bar), using the gradient that proved suitable for iso-metallothionein separation from the HE_{100} cells, resulted in resolution of one gold-containing peak (Fig. 4B) which eluted with a retention time different from Cd-MT in the HE_{100} cells (Fig. 3A). Rechromatography of the gold containing fraction (Fig. 4B, horizontal bar) at pH 2.1 exhibited a complex mixture of several proteins (profiles not shown). Amino acid analysis supported the non-identity of these proteins with MT by showing a much lower content of cystein and the presence of aromatic amino acids (data not shown).

Search for MT in synovial fibroblasts (RIA)

The G75 Sephadex gel filtration profile of cytosols obtained from S_{RA} cells exposed to auranofin, shows that gold accumulates with low molecular weight proteins of possible MT nature (Fig. 2D, peak II). The results of the RIA analysis of peak II from the profiles of S_{RA} fibroblasts treated with auranofin (Fig. 2D) and of the corresponding fractions eluting by gel filtration of S_N and S_{RA} cytosols, are shown in Table 3. There are no significant differences between any of the slopes of the standard curves nor between any of the slopes of the cytosol fraction standard curves and that of the reference MT. The relative MT contents in the fibroblasts S_N:S_{RA}:S_{RA} treated with auranofin were 1:3:10 (per cell basis).

DISCUSSION

The present RIA examinations provide strong evidence that the low molecular weight gold binding proteins occurring in the auranofin resistant HE_{AF} cells and after auranofin treatment of synovial rheumatoid fibroblasts are MT. The polyclonal rabbit anti-rat antibody produced against rat MT [19] is specific for the two immunodominant determinants of vertebrate MT [20], both on the amino-terminal half (residues 1–29) of the 61 residue molecule, and both determinants (residues 1–5 and 20–26) prominent reverse turns [21]. Cross-reactivity is exhibited by all vertebrate MT thus far examined, including MT containing different metals, such as Cd and Zn [19, 22], Cu [23], Hg [24], Pt [25] and Au [26]. Cross-reactivity with vertebrate low molecular weight proteins other than MT has not been demonstrated [17, 22]. This also applies to various non-vertebrate metal-binding proteins.*†

* M. Nordberg, I. Nuottaniemi, M. G. Cherian, G. Nordberg, T. Kjellstrom and J. S. Garvey, *Environ. Health Perspect.* in press.

† D. J. Gingrich, D. Weber, C. F. Shaw III, J. S. Garvey and D. H. Petering, *Environ. Health Perspect.* in press.

Judged from the RIA examinations, all the cell lines studied contained MT in various amounts. G75 Sephadex gel filtration profiles of metals did not reveal any peaks in the low molecular weight regions of the HE, S_N and S_{RA} cells not exposed to auranofin (data not shown), nor did the HPLC reveal the MT in the HE cells. This probably reflects the superior sensitivity of the RIA-method, and is compatible with reports on the widespread occurrence of MT in various vertebrate and invertebrate species and also in microorganisms [1].

As regards the HE_{100} cells, the presence of a significant MT content was confirmed by HPLC and subsequent amino acid analysis. In these cells, the amount of cytosolic MT estimated from the HPLC examinations was 5 times higher than the amount calculated from previous ion-exchange chromatography studies of the same cells [10] and was also higher than the RIA estimates in the present study. These discrepancies may in part be due to the variable amounts of proteins in the samples prepared for RIA, rendering the ratios of intercepts less accurate. Another explanation may be the observation made by Dieter *et al.** that the recoveries of MT obviously varies between different methods. In a comparative study, they have demonstrated a difference in recovery by a factor up to 3.6 between different analytical methods. Results obtained by G75 gel chromatography and subsequent Cd-detection by atomic absorbtion spectrophotometry were found to be too low (up to 45%) compared to the known amounts of purified MT added to the samples. The recoveries of the RIA-method fitted best, but were most sensitive to variations in dilution. The HPLC-method was not included in their study.

The amount of MT in the cells exposed to auranofin (HE_{AF} and S_{RA} + auranofin) exceeded that in their respective parent cells, indicating an auranofin-induced MT synthesis. Auranofin seems to be a significant MT-inducer substance, since the MT content of the HE_{AF} cells relative to that of HE cells, has increased by a factor of 18, that is up to 1/5 of the amount of MT in the HE_{100} cells, as calculated from the RIA results. The finding that auranofin induces MT synthesis is in agreement with results obtained by Butt *et al.* [27] during exposure of Chinese hamster ovary cells to auranofin. In an auranofin-resistant substrain (IC_{50} of 17.5 μmole auranofin/l), they found that the metallothionein genes were actively transcribed in the presence of auranofin, resulting in mRNA accumulation and increased MT synthesis.

The HPLC-system proved to be well suited for the resolution of human liver Zn-MT [18] and the Cd-MT in the HE_{100} cells, but was found to be inappropriate for the resolution and detection of the auranofin-induced gold-containing MT in the HE_{AF} cells. The recovery of gold was remarkably low, and the MT did not elute at the expected retention time. Similar difficulties have also been reported by Piotrowski *et al.* [28] and Mogilnicka and Webb [6] using ion-exchange chromatography. Gold losses and probable aggregation of the MT-like protein during purification, prevented its characterization. Laib *et al.* [26] have recently shown that native horse kidney Cd,Zn-MT undergoes changes when, *in vitro*, the metals are displaced by gold from aurothiomalate. Loss of native conformation and an increase in Stokes radius were observed. These findings demonstrate that the auro-thioneins in certain respects are different from the Cd,Zn-MT, and may also be an indication that chromatography methods developed for Cd,Zn-MT are not suitable for auro-thioneins.

The results also indicate a higher MT content in the rheumatoid synovial fibroblasts than in the normals. The S_{RA} fibroblasts were derived from an arthritic patient who was treated with penicillamine (250 mg s.i.d.), prednisolone (5 mg s.i.d.) and non-steroidal anti-inflammatory drugs at the time of surgery. She had never received any treatment with gold-containing drugs. Thus, the possibility exists that one of the drugs given, or the disease itself, may stimulate the synthesis of MT. Prednisolone and dexamethasone [29], various stress-like conditions [30] and certain mediators involved in the acute phase response of inflammation [31] are reported to lead to induction of MT. This may be relevant to rheumatoid arthritis. We suggest that our findings, obtained by examination of synovial tissues from one healthy and one arthritic patient only, merit further investigation.

In conclusion, the presence of MT was demonstrated in cytosols of cultured human epithelial cells and in synovial fibroblasts. The MT content was enhanced over the control level following *in vitro* auranofin exposure to the epithelial cells as well as to the rheumatoid synovial fibroblasts. The MT level was enhanced over the normal level in the rheumatoid synovial fibroblasts.

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REFERENCES

1. J. H. R. Kägi and M. Nordberg, *Experientia* **35** (Suppl. 34), 1 (1979).
2. Y. Boulanger, I. M. Armitage, K. A. Miklossy and D. R. Winge, *J. biol. Chem.* **257**, 13717 (1982).
3. E. M. Mogilnicka and J. K. Piotrowski, *Biochem. Pharmac.* **26**, 1819 (1977).
4. H. O. Thompson, J. Blaszak, C. J. Knudtson and C. F. Shaw III, *Bioinorg. Chem.* **9**, 375 (1978).
5. R. P. Sharma and E. G. McQueen, *Biochem. Pharmac.* **29**, 2017 (1980).

* H. H. Dieter, L. Müller, J. Abel and K. H. Summer, in *Proceedings of the 2nd International Meeting on Metallothionein and other Low Molecular Weight Metal-Binding Proteins*, Zürich, 21–24 August 1985 (Eds. J. H. R. Kägi and Y. Kojima). Birkhäuser Verlag, Basel, in press.

6. E. M. Mogilnicka and M. Webb, *J. appl. Toxic.* **1**, 287 (1981).
7. A. Glennås, *Acta pharmac. Toxic.* **56**, 404 (1985).
8. E. M. Mogilnicka and J. K. Piotrowski, *Biochem. Pharmac.* **28**, 2625 (1979).
9. A. Glennås and H. E. Rugstad, *Scand. J. Rheumatol.* **14**, 230 (1985).
10. A. Bakka, L. Endresen, A. B. S. Johnsen, P. D. Edminson and H. E. Rugstad, *Toxic. appl. Pharmac.* **61**, 215 (1981).
11. K. Ree, A. S. Johnsen, H. E. Rugstad, A. Bakka and T Hovig, *Acta Pathol. Microbiol. Immunol. Scand. Sect. A* **89**, 73 (1981).
12. H. E. Rugstad and T. Norseth, *Nature, Lond.* **257**, 136 (1975).
13. A. Glennås, A. K. Thorsrud, H. E. Rugstad and E. Jellum, *Ann. Rheum. Dis.* **44**, 302 (1985).
14. A committee of the American Rheumatism Association 1958, Revision of diagnostic criteria for rheumatoid arthritis. *Arthritis Rheum.* **2**, 16 (1959).
15. S. Klauser, J. H. R. Kägi and K. J. Wilson, *Biochem. J.* **209**, 71 (1983).
16. C. H. W. Hirs, *Methods Enzymol.* **11**, 197 (1967).
17. J. S. Garvey, R. J. Vander-Mallie and C. C. Chang, *Methods Enzymol.* **84** PtD, 121 (1982).
18. P. E. Hunziker and J. H. R. Kägi, *Biochem. J.* **231**, 375 (1985).
19. R. J. Vander-Mallie and J. S. Garvey, *Immunochemistry* **15**, 875 (1978).
20. D. R. Winge and J. S. Garvey, *Proc. natn. Acad. Sci. U.S.A.* **80**, 2472 (1983).
21. J. S. Garvey, *Environ. Health Perspect.* **54**, 117 (1984).
22. R. J. Vander-Mallie and J. S. Garvey, *J. biol. Chem.* **254**, 8416 (1979).
23. D. R. Winge, B. L. Geller and J. S. Garvey, *Archs. Biochem. Biophys.* **208**, 150 (1981).
24. J. S. Garvey, in *Nephrotoxic Mechanisms of Drugs and Environmental Toxins* (Ed. G. A. Porter), p. 437. Plenum Press, New York (1982).
25. A. J. Żelazowski, J. S. Garvey and J. D. Hoeschele, *Archs Biochem. Biophys.* **229**, 246 (1984).
26. J. E. Laib, C. F. Shaw III, D. H. Petering, M. K. Eidsness, R. C. Elder and J. S. Garvey, *Biochemistry* **24**, 1977 (1985).
27. T. R. Butt, E. J. Sternberg, C. K. Mirabelli and S. T. Crooke, *Molec. Pharmac.* **29**, 204 (1986).
28. J. K. Piotrowski, J. A. Szymańska, E. M. Mogilnicka and A. J. Żelazowski, *Experientia* **35** (Suppl. 34), 363 (1979).
29. M. L. Failla and R. J. Cousins, *Biochim. biophys. Acta* **543**, 293 (1978).
30. S. H. Oh, J. T. Deagen, P. D. Whanger and P. H. Weswig, *Am. J. Physiol.* **234**, E282 (1978).
31. D. M. Durnam, J. S. Hoffman, C. J. Quaife, E. P. Benditt, H. Y. Chen, R. L. Brinster and R. D. Palmiter, *Proc. natn. Acad. Sci. U.S.A.* **81**, 1053 (1984).
32. M. M. Kissling and J. H. R. Kägi, *Febs. Lett.* **82**, 247 (1977).