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Growth Inhibition and Metallothionein Induction in Cadmium-Resistant Cells by Essential and Non-Essential Metals

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SUMMARY

Essential and non-essential metal ions were compared on the basis of their growth-inhibitory potency and their mediation of metallothionein induction in a Chinese hamster ovary cell line resistant to cadmium. Cadmium-resistant cells were found to be 20-fold and 6-fold more resistant than wild-type Chinese hamster ovary cells to the non-essential metals $CdCl_2$ and $HgCl_2$, respectively. In contrast, cadmium-resistant cells showed 2-fold or less resistance to growth inhibition due to the metals with known or possible biological essentiality, $ZnCl_2$, $CuSO_4$, $CoCl_2$, and $NiCl_2$. Resistance to either cadmium or mercury was not due to decreased uptake as measured isotopically or by X-ray fluorescence. At concentrations near the threshold of growth inhibition, $CdCl_2$ and $ZnCl_2$ induced metallothionein 8- to 10-fold above background levels in cadmium-resistant cells within 8–10 hr. A 2- to 3-fold induction of this protein was produced in resistant cells by levels of $HgCl_2$, $CuSO_4$, and $CoCl_2$ near the threshold of growth inhibition whereas $NiCl_2$ produced no measurable elevations of metallothionein at concentrations below, near, and above those that inhibit cell growth. Induction of metallothionein was measured by a modified ^{203}Hg binding assay and by [^{35}S]cysteine incorporation. No measurable induction of metallothionein was evident in wild-type cells with any metal treatment using a reasonable quantity of cells consistent with our assay. These results in cadmium-resistant cells demonstrate selective induction of metallothionein by various metals and suggest that induction of this protein alone is not solely responsible for differences in the growth-inhibitory potential of these elements.

INTRODUCTION

The role of metallothionein in the development of resistance to the toxic metal cadmium has been well established in cultured cell systems (1–6). These studies have demonstrated that selection for cadmium resistance is associated with a cadmium-mediated increase in the synthesis of metallothionein. In addition, the development of resistance is associated with an amplification of the metallothionein gene as well as an increase in the rate of transcription of this gene in a variety of cell lines, including CHO,[2] hepatoma cells (Hepa 1A), murine sarcoma cells (S-180), and Friend erythroleukemia cells (7, 8). These observations provide support for a mechanism of cadmium detoxification based on the chelation of the metal ion by metallothionein. This chelation is due to the high content of cysteine (20 of 61 amino acids in metallothionein), which renders this protein with a great affinity for certain potentially toxic metals: $Hg^{2+} > Cu^{2+} > Cd^{2+} > Zn^{2+}$ (9).

Physiologcially, metallothionein is believed to function in the maintenance of zinc and copper homeostasis and to serve as a reservoir for high levels of these metals in livers of developing animals (10). In the absence of metallothionein chelator binding sites, cytotoxic responses may ensue from the high levels of these metals (10). Little information is available at the cellular level as to how general a role metallothionein plays in the amelioration of cytotoxicity due to metal ions other than cadmium. Although resistance to toxic levels of zinc has been reported in two cadmium-resistant CHO cell lines (1, 11) in most cases the development of cadmium resistance is not accompanied by a corresponding resistance to zinc (12). No information is available on the relative cytotoxic effect of other metals in cadmium-resistant cell lines. *In vivo* studies in mice have shown that cadmium

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[2] The abbreviations used are: CHO, Chinese hamster ovary; CHO^R cells, cadmium-resistant CHO cells; CHO^W cells, wild-type CHO cells.

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is one of a number of metals that can cause the induction of mRNA for metallothionein in liver and kidney (13). Zinc, copper, and mercury also induce the synthesis of metallothionein but with differing potency (13, 14). The stability of metallothionein induced by different metals may vary, since it has been shown that mercury-induced metallothionein was more unstable than the metallothionein induced by cadmium (13). These differences in half-life, together with variations in the extent of induction by certain divalent metals, may diminish the importance of metallothionein as a general detoxifying agent for metals other than cadmium. In fact, metals such as nickel, cobalt, and lead poorly induce metallothionein and surprisingly have been shown not to bind to this protein despite their affinity for sulfhydryl groups (15).

This study makes use of a CHO^R cell line whose gene for metallothionein has been amplified greater than 3-fold and additionally is capable of phenotypic expression of this gene when these cells are exposed to cadmium (3) The purposes of the present study were (a) to determine whether CHO^R cells are less sensitive to a variety of other divalent metal ions; (b) to study the metal selectivity of metallothionein induction in CHO^R and CHO^W; and (c) to correlate metal uptake with its potency[3] in causing induction of metallothionein and in attenuating cell growth.

MATERIALS AND METHODS

Chemicals. Actinomycin D and cycloheximide were purchased from Calbiochem Behring Corporation (La Jolla, Calif.) and Sigma Chemical Company (St. Louis, Mo.), respectively. $^{203}Hg^{2+}$ (0.42 mCi/mg) and [^{35}S]cysteine (312.6 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). *Alpha* minimal essential medium was obtained from GIBCO, Inc. (Grand Island, N. Y.). All metal salts were purchased from Fisher Scientific Company (Fair Lawn, N. J.).

Cell culture techniques. CHO^W and CHO^R cells were maintained in *alpha* minimal essential medium supplemented with 10% fetal bovine serum. Cadmium-resistant cells (Cd^r 30F9) were a gracious gift from Dr. R. A. Tobey and Dr. C. E. Hildebrand, of the University of California at Los Alamos. For growth inhibition studies, 5×10^4 cells were inoculated into six-welled Costar tissue culture dishes (Cambridge, Mass.). Cells were allowed to attach overnight and were treated on the following day with increasing concentrations of metal salts. Growth was allowed to proceed for 5 days, with replacement of original medium on day 3 with fresh medium containing the indicated metals. Cells were harvested by trypsinization, and cell number was determined in each dish using a Coulter counter particle-size analyzer. Cell number present in metal-treated dishes was expressed as a function of the number of cells in untreated dishes to assess the influence of metals on cell reproduction.

Metallothionein levels were measured in confluent CHO^R and CHO^W cells treated in 60-mm culture dishes (2 to 3×10^6 cells) for 2, 4, 8, and 12 hr with varying concentrations of metal salts. Following removal of the incubation medium, monolayers were rinsed three times with 3 ml of Puck's Saline A. Cells were detached by scraping in 3 ml of Puck's Saline A and collected by centrifugation. Pellets were resuspended in ice-cold phosphate buffer (without Mg^{2+} and Ca^{2+}) and frozen in liquid N_2. Cells were stored at −70° until needed.

Determination of metallothionein levels in cultured cells. Metallothionein was measured by a modified mercury binding assay which has previously been described in detail (16) and by the rate of incorporation of [^{35}S]cysteine into the metallothionein fraction. For the mercury binding assay, frozen cell samples were thawed and lysed by sonication. Mercury binding was determined by allowing 100 μl (corresponding to 4×10^5 cells) of each sample lysate to react with 50 μl of ^{203}Hg (1–2 nmoles of ^{203}Hg, 6×10^6 dpm) for 10 min at room temperature. Non-metallothionein protein-bound ^{203}Hg was precipitated by addition of trichloroacetic acid to a final concentration of 2.2%. Free ^{203}Hg was removed by centrifugation of the trichloroacetic acid supernatant through Sephadex G-10 minicolumns. The ^{203}Hg bound to metallothionein in the filtrates was counted directly in a Beckman Gamma 4000. The ^{203}Hg binding assay was verified for specificity and sensitivity using a homogeneous metallothionein standard from rat liver (cadmium/zinc metallothionein II) and other potential mercury-binding ligands such as glutathione (16). Alternatively, [^{35}S]cysteine-labeled metallothionein can also be measured in the same filtrates processed to detect ^{203}Hg binding (*vide supra*). Monolayer CHO^R cells in 10-cm tissue culture dishes were exposed to various concentrations of metal salts for 8 hr in cysteine-free Dulbecco's medium supplemented with 50 μCi of [^{35}S] cysteine. Cells were then rinsed twice with Puck's Saline A, collected, and processed as described above.

Determination of metal uptake. The uptake of mercury was determined in confluent cultures of CHO^R cells using $^{203}HgCl_2$. Cells were incubated with 10 μM $^{203}Hg^{2+}$ (7 mCi/mmole, approximately 1.5×10^6 cpm/100-mm dish) for varying intervals of time. Following treatment, cells were rinsed once with Puck's Saline A containing 1 mM EDTA and twice with Puck's Saline A alone. The monolayers were allowed to drain and then were dissolved in 3 ml of 0.2 M NaOH overnight. The 3-ml samples were transferred to glass tubes and counted directly in a Beckman Gamma 4000.

The uptake of the other metals was determined by measurement of cellular metal levels with X-ray fluorescence spectroscopy (17). Cells were not dissolved in NaOH but were dislodged in Puck's Saline A with a rubber policeman, collected by centrifugation, and frozen at −70° for analysis of metal content. Protein determinations were performed using the commercial Coomassie blue reaction kit available from Bio-Rad Laboratories (Richmond, Calif.). Bovine serum albumin was used as the standard for determination of protein concentration.

RESULTS

Effect of essential and non-essential elements on the growth of CHO^R and CHO^W cells. The growth of CHO^W and CHO^R cells was inhibited in a concentration-dependent manner by the addition of the various metal salts (Fig. 1). Statistical analysis indicated that CHO^R cells were significantly ($p \ll 0.001$) more tolerant to higher concentrations of the non-essential metals, cadmium and mercury, than were CHO^W cells (Table 1). The IC_{50} values (concentration that inhibits cell growth by 50% relative to untreated cells) for $CdCl_2$ and $HgCl_2$ were increased from 0.57 μM to 11 μM and 3.6 μM to 23 μM, respectively, in the resistant cells (Table 1). These increases in IC_{50} values represent a 20-fold decrease in the sensitivity of CHO^R cells for $CdCl_2$ and a 6-fold decrease in sensitivity toward $HgCl_2$. When the CHO^R cells were maintained in suspension culture rather than in monolayer they exhibited even greater resistance to $CdCl_2$ (e.g., 40 μM) (3).

Figure 1 shows the sensitivity of CHO^R and CHO^W to the essential elements zinc, copper, cobalt, and nickel. Relative to CHO^W, CHO^R cells were 2-fold or less resistant to growth inhibition by these essential metals as assessed by comparison of IC_{50} values (Table 1). Although in some instances the CHO^R had statistically significant enhancement of resistance relative to CHO^W, the differences in IC_{50} values were clearly less than those obtained with $HgCl_2$ and $CdCl_2$. In terms of its relative

[3] The term "maximally tolerated concentrations" refers to those concentrations that are near the threshold of growth inhibition determined as described under Materials and Methods. Both "threshold of growth inhibition" and "maximally tolerated concentrations" are used interchangeably in this report.

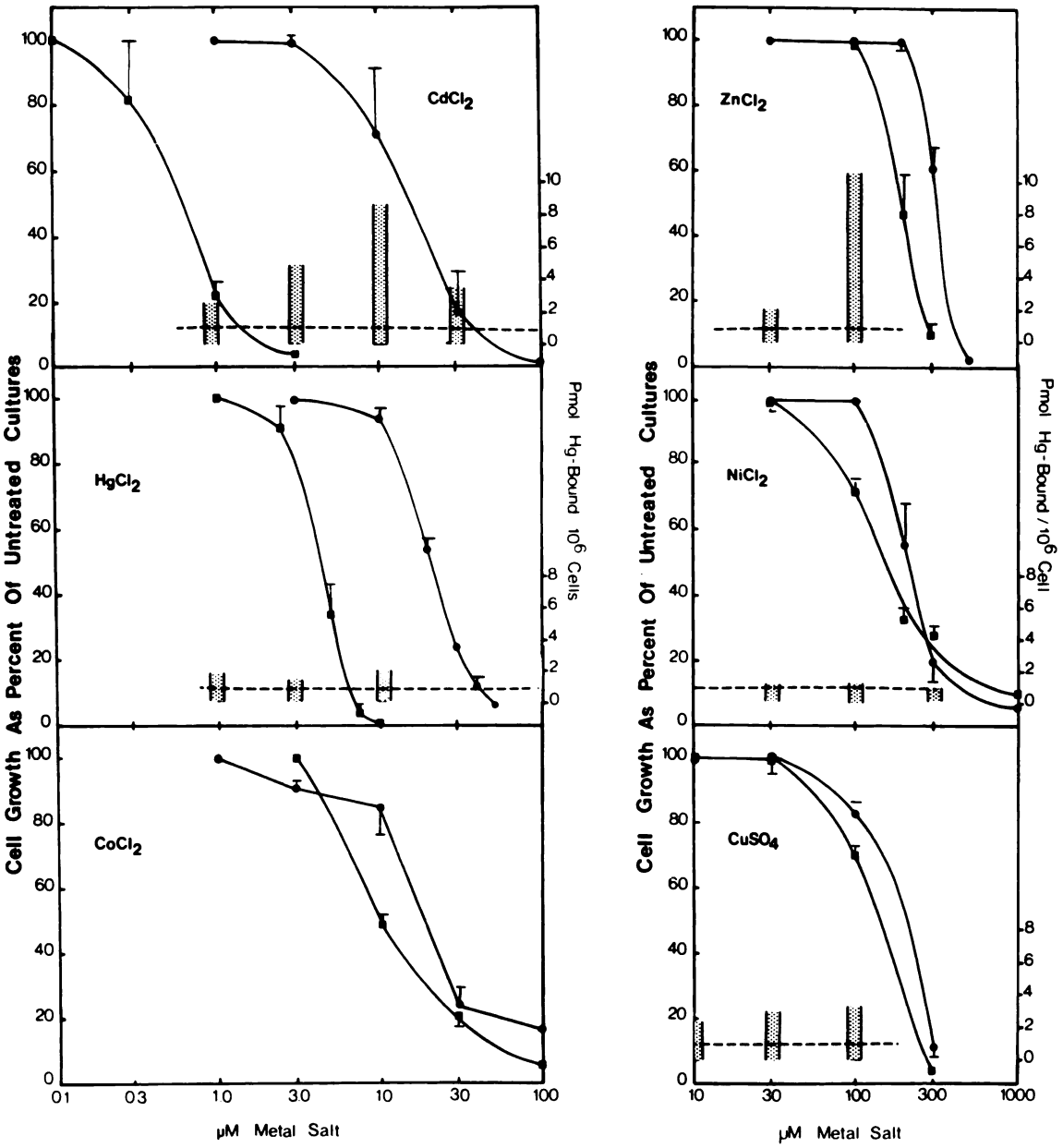

FIG. 1. *Effect of essential and non-essential metals on cell growth and metallothionein levels*

CHO^W and CHO^R cells were inoculated into six-well Costar tissue culture plates (50,000 cells/well) and allowed to attach overnight. The following day, the original medium was removed and replaced with medium containing increasing concentrations of metal salt; cells were then allowed to grow for 5 days in the continuous presence of the metal. At the end of the incubation, the cell number present in each treated dish of cells was expressed as percentage of untreated cultures of CHO^R (●) and CHO^W (■). Each point represents the mean ± standard error of the mean for at least four separate plates. The *bars* indicate the induction of metallothionein (picomoles of mercury bound per 10^6 cells) following an 8-hr exposure of CHO^R cells to the indicated concentration of metal. The *broken lines* represent the background picomoles of mercury bound in untreated cultures.

potency toward CHO^W cells, $CoCl_2$ was found to be as effective as either $CdCl_2$ or $HgCl_2$ in impairing cell replication. $ZnCl_2$, $CuSO_4$, and $NiCl_2$ were much less potent than the non-essential metals, requiring 5- to 10-fold higher concentrations to inhibit growth by 50% in both CHO^W and CHO^R (Table 1).

The greater growth-inhibitory potency of $CdCl_2$ and $HgCl_2$ is in agreement with the physiochemical reactivity of these two non-essential elements. Both metals have the lowest values of σ_p (chemical "softness") of the six metals examined, which indicates a high propensity to form coordinate covalent bonds and correlates directly with their lower LD_{50} values (18).

Induction of metallothionein in CHO^R cells. The CHO^R variant used in this study has previously been well characterized with respect to cadmium-mediated induction of metallothionein (3). This cell line, which is capable of expressing the gene for metallothionein when exposed to either cadmium or zinc, was clonally selected from a population of CHO cells made resistant to cadmium by long-term culturing in low, marginally toxic, levels of the metal (3). Such cells are therefore suitable for determining which metals may substitute for cadmium and zinc in the process of metallothionein induction.

In addition to showing the effect of metals on the growth of CHO^W and CHO^R cells, Fig. 1 also illustrates

TABLE 1

Comparison of IC_{50} values for various metal compounds in CHO^R and CHO^W

Data from Fig. 1 were fitted on a log plot and the IC_{50} values were determined. Linear correlation coefficients obtained from the growth curve analysis for CHO^W ranged from 0.920 to 0.972 and for CHO^R, they ranged from 0.920 to 0.997. Statistical analysis using Student's *t*-test were performed comparing the CHO^W and CHO^R cells for significant differences in IC_{50} values. The IC_{50} values were determined following 5 days of metal treatment (see Materials and Methods); shown is the mean IC_{50} value ± standard error of the mean for at least three separate plates for each of four metal concentrations.

Metal compound	Cell line IC_{50}[a] values		Fold resistance
	CHO^W	CHO^R	
	μM		
$CdCl_2$	0.57 ± 0.02	11.21 ± 0.72[b]	20.0
$HgCl_2$	3.60 ± 0.10	23.49 ± 0.17[b]	6.0
$CoCl_2$	12.07 ± 0.43	21.00 ± 6.40[c]	1.7
$ZnCl_2$	199.50 ± 3.40	318.00 ± 9.40[d]	1.6
$CuSO_4$	115.90 ± 2.4	136.10 ± 3.7[e]	1.2
$NiCl_2$	117.30 ± 13.5	254.00 ± 26.0[e]	2.2

[a] IC_{50} = concentration that inhibits cell growth by 50% relative to untreated cells.

[b] $p \ll 0.001$ (CHO^R versus CHO^W).

[c] Not statistically significant.

[d] $p < 0.01$.

[e] $p < 0.05$.

the effect of an 8-hr exposure to increasing concentrations of the metal on metallothionein induction in CHO^R. At concentrations around those maximally tolerated by CHO^R cells, zinc and cadmium caused a 10-fold induction in measurable ^{203}Hg-bound metallothionein (Fig. 1; Table 2). Figure 2 shows the time course of this induction using several different metal concentrations and also demonstrates the sensitivity of this induction to either actinomycin D or cycloheximide. However, it should be noted that the effects of actinomycin D and cycloheximide on metallothionein induction are complicated, involving processes such as superinduction; therefore their effects depend upon time of addition and are subject to considerable variations in replicate experiments. The decrease in the level of induction observed at 30 μM $CdCl_2$ in Fig. 1 may be ascribable to the general inhibitor effect of this metal on protein synthesis specifically or alternatively to overt cytotoxicity. $HgCl_2$ apparently did not induce metallothionein at maximally tolerated concentrations (Figs. 1 and 2; Table 2) because of the problem involved with using a ^{203}Hg binding assay to measure mercury-induced metallothionein (i.e., lack of isotopic exchange). Therefore, the rate of [^{35}S]cysteine incorporation was used as an alternative index of induction. Table 2 demonstrates that $HgCl_2$ exposure leads to approximately a 2-fold increase in the [^{35}S]cysteine incorporation rate without an actual increase in mercury-binding activity. Nickel, at all concentrations tested (10 μM to 1 mM), was an ineffective inducer of metallothionein as measured by both ^{203}Hg binding and [^{35}S]cysteine incorporation. A comparison of Table 2 and Fig. 2 shows that measurement of copper-mediated metallothionein induction was variable using the ^{203}Hg binding assay; however, measurement of [^{35}S]cysteine incorporation consistently demonstrated approximately 2-fold induction by copper.

TABLE 2

Induction of metallothionein by various metals

Metal	[^{35}S]Cysteine[a]	Mercury bound[b]
	cpm/mg protein	*pmoles/10^6 cells*
Control	1024 (1)	0.95
Cadmium (10 μM)	3097 (3)	9.69
Cadmium + actinomycin D	1954 (1.9)	4.87
Zinc (10 μM)	3091 (3)	7.88
Mercury (10 μM)	1950 (1.9)	0.69
Copper (100 μM)	2314 (2.2)	0.75
Cobalt (10 μM)	2153 (2.1)	2.88
Nickel (100 μM)	1042 (1)	1.67

[a] The degree of [^{35}S]cysteine incorporation into the metallothionein was measured following an 8-hr incubation of CHO^R cells in cysteine-free medium supplemented with the indicated concentration of metal salt and [^{35}S]cysteine (10.0 μCi/ml). Results are expressed as counts per minute [^{35}S]cysteine found in gel filtrates per milligram of protein cell extract. The numbers in parentheses represent the fold increase over untreated cultures.

[b] Confluent CHO^R cells were treated with metal salts for 8 hr, following which cells were collected and the level of metallothionein induction was determined as described under Materials and Methods. Results are expressed as picomoles of ^{203}Hg in gel filtrates per 10^6 cells. The approximate conversion factor for cell number to milligrams of protein was 10^6 cells = 0.5 mg of protein. For details of the assay procedure see Materials and Methods.

Both the essential and non-essential elements failed to induce a measureable increase in metallothionein in CHO^W cells at concentrations below, near, or above those that inhibit cell growth, using reasonable cell numbers consistent with our modified ^{203}Hg binding assay (data not shown; see ref. 16).

Comparison of the uptake of mercury ions in CHO^W and CHO^R cells. A possible explanation for differences in metal resistance in cultured cells may reside at the level of metal uptake into the cells. Table 3 serves to illustrate that there were no striking differences in uptake of various metal ions and in fact, with the exception of nickel, the resistant cells tended to accumulate more metal ions as compared with the wild-type cells. Figure 3 shows a more extensive examination of the uptake of $HgCl_2$ in CHO^W and CHO^R. A significantly greater uptake of $HgCl_2$ was found in CHO^R cells as compared with CHO^W cells. These results suggest that reduced uptake of $HgCl_2$ cannot account for the greater resistance to this metal displayed by CHO^R (Fig. 1; Table 1).

DISCUSSION

The present study has examined the effect of various divalent metals on the reproductive capacity of wild-type and cadmium-resistant CHO cells and correlates this response with the intracellular induction of metallothionein. Previous studies have examined the uptake, cytotoxicity, and mechanisms of metallothionein induction in this same resistant cell line (Cd^r 30F9) following treatment with $CdCl_2$ (3). These studies have demonstrated that cadmium binds initially to a number of non-metallothionein proteins and during early time intervals permeates into the nucleus. Subsequent induction of metallothionein was accompanied by the disappearance of cadmium from the nuclear compartment and the concurrent association of the metal with cytosolic metallothi-

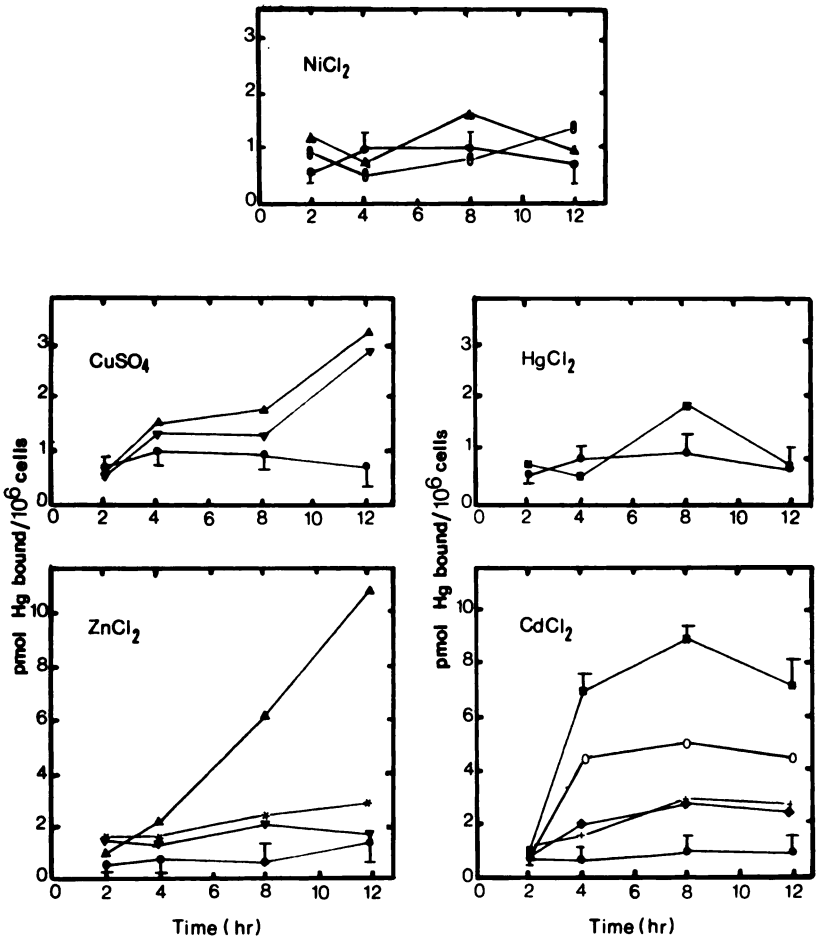

FIG. 2. *Time course of metallothionein induction*

Confluent CHO^R cells in 60-mm tissue culture dishes were treated with different concentrations of metal salt for the indicated intervals of time. Induction of metallothionein was determined as described under Materials and Methods. Results are expressed as picomoles of mercury bound per 10^6 cells for the following experimental conditions: untreated cultures (●), 1 μM metal (◆, CdCl only); 3 μM metal (○, $CdCl_2$ only), 10 μM metal (■, $HgCl_2$ and $CdCl_2$), 10 μM metal + 3 μM actinomycin D (+, $CdCl_2$ only), 30 μM metal (▼, $CuSO_4$ and $ZnCl_2$), 100 μM metal (▲, $NiCl_2$, $CuSO_4$, and $ZnCl_2$), 100 μM metal with 25 μM cycloheximide (*, $ZnCl_2$ only), and 1 mM metal (◓, $NiCl_2$ only). Each point represents the mean ± standard error of the mean for at least four separate determinations using separate culture dishes for each determination.

onein (3). Although these results suggest that metallothionein is important in the chelation and redistribution of cadmium in the cell, the existence of additional cadmium-binding proteins in Cd^r 30F9 cells indicates that the induction of metallothionein may not be exclusively responsible for resistance to cadmium (3). Thus, as previously shown (3), resistance to cadmium is probably inherently more complex than the regulation of the cellular levels of metallothionein. Our results further support the importance of metallothionein as a detoxifying agent against non-essential metals but reveal certain limitations with regard to its protection against growth-inhibitory levels of the essential metals. We demonstrated the induction of metallothionein with $ZnCl_2$ in cadmium-resistant cells to a level nearly equivalent to the induction attributable to $CdCl_2$, yet there was only a 2-fold enhancement of resistance to zinc as compared with a 20-fold increased resistance to cadmium. Conversely, CHO^R cells were significantly more resistant (2.2-fold) to $NiCl_2$, without a notable nickel-mediated induction of metallothionein in these cells (Fig. 3). It appears, however, that an alteration in the uptake of nickel by CHO^R cells may provide the basis for resistance. These results further illustrate the complexity involved with cellular resistance to metals. These findings also suggest that the inhibition of cellular reproduction by metal ions occurs by unique mechanisms which cannot be attenuated even in cells with a high level of metallothionein induction. However, since metallothionein was induced in CHO^R cells not only following challenge with cadmium

TABLE 3

Comparison of metal ion uptake in CHO^W and CHO^R cells

Metal ion	Uptake by cell line[a]	
	CHO^W	Cd^r 30F9
	nmoles metal/mg protein	
$CuSO_4$ (0.1 mM)	0.40	0.56
$ZnCl_2$ (0.1 mM)	4.80	3.80
$NiCl_2$ (0.1 mM)	0.30	0.16
$HgCl_2$ (10 mM)	0.70	0.86
$CdCl_2$ (10 mM)	0.92	0.92

[a] Each value represents the average of single determinations in two separate cultures following 8 hr of treatment with the indicated metal salt. Additional determinations using varied metal concentrations and incubation times demonstrated no notable difference in uptake between these two cell lines.

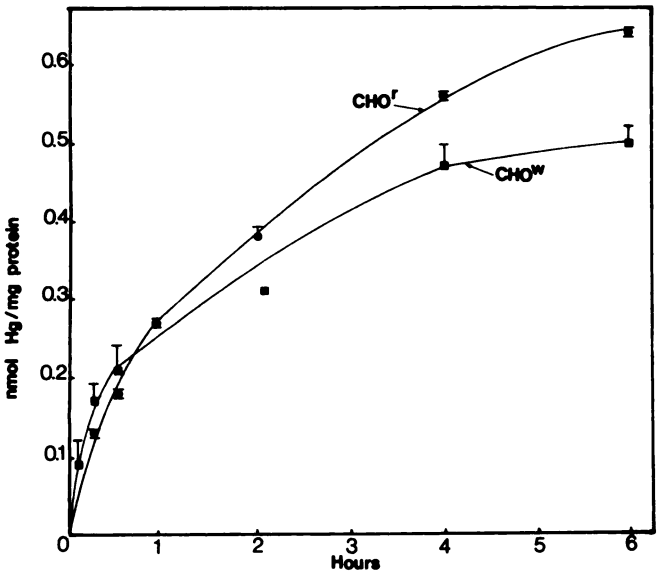

FIG. 3. *Mercury uptake in CHO^W and CHO^R cells*

Confluent cells in 100-mm dishes were incubated with 10 μM $^{203}HgCl_2$ (approximately 1.5 × 10^6 cpm/dish) for the various time intervals indicated. Cells were processed as described under Materials and Methods. Where error bars are shown the points represent the mean ± standard error of the mean for at least four determinations. These representative error bars demonstrate the low variability of the assay.

but also with other metals, these observations suggest that its induction lacks elemental specificity with respect to zinc, cadmium, mercury, copper, and cobalt, but not nickel. At metal concentrations near the threshold of growth inhibition, the magnitude of metallothionein induction in CHO^R cells was greater with $CdCl_2$ and $ZnCl_2$ than with $CuSO_4$, $CoCl_2$ and $HgCl_2$, suggesting a degree of metal specificity in regulating the quantity of induction. These findings are in agreement with those of Piotrowski and Szymanska (19). These investigators demonstrated induction of metallothionein in rat livers by cadmium (+++), zinc, copper (++), or cobalt (++) and in kidney by cadmium (+++), zinc, copper (+), mercury (+++), or bismuth (++). No metallothionein induction was observed by parenteral administration of magnesium, strontium, barium, tin, lead, arsenic, vanadium, selenium, chromium, manganese, iron, or nickel to rats (19).

CHO^R cells exhibit more sensitivity to cadmium when grown in suspension culture than when grown in monolayer (3), as was done in our study. Additionally, metal uptake and cytoxocity are highly dependent upon the levels of metal-binding amino acids such as cysteine and histidine present in the medium (20). Thus considerable variation in cytotoxic levels of metals may be explained on the basis of these parameters alone. It should be noted that all assays of metallothionein at the present time measure induction relative to a basal level. The assessment of ^{203}Hg binding requires precise knowledge of stoichiometric relationships within the conditions of the assay to obtain a quantitative value of metallothionein protein. By using a pure metallothionein standard, we were able to determine that the assay system is able to detect 0.1 pmole of metallothionein and that the upper limit of detection was greater than the levels of metallothionein present in our samples. The degree of induction measured with [^{35}S]cysteine incorporation as compared with ^{203}Hg binding was in general agreement with regard to the relative levels of induction produced by each metal, although the magnitude of induction was higher with the ^{203}Hg binding assay (Table 2). However, this probably reflects differences in relative stoichiometry of cysteine incorporation and ^{203}Hg binding to this protein.

CHO^R displayed a reduced sensitivity to $HgCl_2$, suggesting some overlap with the mechanism of cadmium resistance. Both of these metals have relatively low σ_p values, indicating a high degree of chemical softness and a significant propensity to form coordinate covalent bonds. Additionally, unlike the other metal ions tested, neither of these elements has implicated essentiality for biological systems. Hg^{2+} and (to a lesser extent) Cd^{2+} rarely exist as free cations extracellularly or intracellularly, owing to their high degree of reactivity. For example, in the presence of 0.1 M Cl^-, complexes such as $[HgCl_2]$, $[HgCl_3]^{1-}$, and $[HgCl_4]^{2-}$ exist in equal amounts which far exceed those of Hg^{2+} (21). An un-ionized complex such as $[HgCl_2]$ would tend to be more lipid-soluble and readily enter the cell. Uptake studies support this prediction by demonstrating the rapid and pronounced entry of $HgCl_2$ into cells relative to the uptake of other metal compounds (i.e., $ZnCl_2$, $CoCl_2$, $CuSO_4$, $NiCl_2$, and $CdCl_2$) (22). At acutely cytotoxic levels, this rapid uptake of $HgCl_2$ leads to X-ray like effects in cells, including depletion of glutathione and induction of DNA damage (22). Enhanced resistance of CHO^R to $HgCl_2$ cannot be accounted for by reduced uptake relative to CHO^W (Fig. 3). Although the level of metallothionein induction by $HgCl_2$ was enhanced during the development of resistance to $CdCl_2$, its inducibility was less than that attributable to zinc (Fig. 2; Table 3). However, even small increases in the inducible levels of metallothionein following mercury treatment may be more effective in reducing mercury cytotoxic responses owing to a more efficient exchange of mercury with endogenous zinc-bound metallothionein (3).

Although metallothionein is clearly implicated in the cellular detoxification of heavy metals and its induction correlates with toxic metal exposure, it is not operating alone or without a similar regulatory scheme in response to different metal ions. Hildebrand *et al.* (3) have shown that CHO^R cells synthesize at least four additional proteins in response to cadmium and have elevated levels of a non-metallothionein cadmium-binding protein. Identification of these additional proteins and studies of their induction by specific metal ions may indirectly lead to an understanding of the mechanisms of cytotoxicity of selected metal ions. It is apparent from our study that the non-essential metals, mercury and cadmium, are much more potent growth inhibitors than are the essential metals. The potency of the former most likely is based on their high degree of chemical reactivity, making it possible for them to exert a disruptive effect at any number of critical sites. As a detoxifying mechanism against high levels of both non-essential and essential metals, the induction of metallothionein (or lack of induction) does not necessarily correlate the resistance of

CHO^R cells to metals other than cadmium. Further characterization of the interaction of mercury with other proteins as well as its subcellular distribution in the CHO^R cells should be useful in understanding the mechanism of mercury-mediated growth inhibition. Additionally, selection of CHO cells resistant to other metals may allow for the isolation and identification of binding proteins other than metallothionein which are responsible for regulating metal ion levels and distribution.

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