Zinc-, Copper- and Cadmium-binding Protein in Ehrlich Ascites Tumour Cells

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The properties of Ehrlich ascites tumour cells exposed *in vivo* to cadmium were investigated as a function of the zinc status of the host animals. Tumour-cell growth was inhibited by cadmium in both zinc-sufficient and zinc-deficient animals. However, cells in zinc-sufficient tumours accumulate much less cadmium than those in deficient tumours. The subcellular distributions of cadmium and zinc do not depend on zinc status. Cadmium and zinc are bound to a low-molecular-weight protein with properties similar to metallothionein. Without exposure to cadmium, a zinc- and copper-binding protein is still present that behaves like a metallothionein. This protein can rapidly bind cadmium added to Ehrlich cells *in vitro*. It is shown that the zinc- and copper-binding protein contains free thiol groups. Ehrlich cells isolated from cadmium-treated animals are viable and show normal incorporation of uridine into RNA, but the cellular uptake of thymidine and its incorporation into DNA are inhibited.

Interest in metallothionein has continually expanded since Margoshes & Vallee (1957) discovered that this protein, or family of proteins, binds virtually all of the cadmium found in the cytosol of horse-kidney tissue. Concern focused initially upon the role of metallothionein in cadmium toxicity, and later upon its role in normal zinc metabolism (Kägi & Vallee, 1960; Evans *et al.*, 1970; Webb, 1972). The primary model for these inquiries has been the rat, with particular attention given to the dynamics of cadmium, zinc-metallotionein formation and degradation in liver (Chen *et al.*, 1975; Feldman & Cousins, 1976; Oh *et al.*, 1978; Andersen *et al.*, 1978). Efforts have been made to utilize cultured cells for these studies. For instance, cultured rat-liver cells can be stimulated by cadmium to produce thionein (Hidalgo *et al.*, 1978a). Other cell lines, such as lymphocytes, CHO (Chinese-hamster ovary) and HeLa cells, can also bind cadmium in metallothionein (Enger *et al.*, 1978; Hildebrand *et al.*, 1978; Rudd & Herschman, 1979). Another model, the Ehrlich-tumour-cell suspension maintained interperitoneally in female Swiss mice and

directly exposed to CdCl₂ (Petering *et al.*, 1978), is described in this paper.

In any of these simpler models, one hopes to discover properties of the metabolism of cadmium or zinc that are difficult to explore in the whole animal. However, because of problems in defining the functional roles of metallothionein, progress using animals or cell culture has been slow. The present system of the Ehrlich tumour was chosen because it provides a large population of single cells that can be readily analysed for metal contents and metal distribution, as well as for functional changes that may result from cadmium exposure. In addition, because a number of properties of the Ehrlich tumour have been studied under zinc-normal and zinc-deficient conditions, this model provides the opportunity to study cadmium–zinc interactions, which appear to be very important in the mechanism of toxicity of cadmium (Petering, 1978; Saryan *et al.*, 1979). Therefore, this work outlines basic features of the interaction of cadmium with Ehrlich cells *in vivo* under varying dietary conditions of zinc for the host mice.

Materials and Methods

Animals and animal care

Female ARS HA/ICR albino Swiss mice obtained from Sprague–Dawley Corp., Madison, WI.

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U.S.A., weighing approximately 30g and 8–11 weeks old at the onset of experiments, were housed in groups of five to seven animals in stainless steel wire cages over wood-chip bedding. A constant temperature of 25°C was maintained in the animal room. An electronic timer maintained a 12h light/12h dark cycle. Animals were fed *ad libitum* chow and drinking water with various amounts of zinc. Before the controlled diet, mice were fed Ralston Purina Laboratory Chow, which contains 70–90 µg of zinc/g, and tap water. During experiments requiring controlled levels of zinc, a zinc-deficient chow obtained from Ziegler Bros., Gardiners, PA, U.S.A. (Murthy *et al.*, 1975) with less than 1 µg of zinc/g was employed, and the zinc intake was varied by the concentration of zinc in the glass-distilled water. Animals given 80 µg of zinc/ml are designated Zn(+) animals; those given no zinc are Zn(–) animals.

Chemicals

Eagle's minimal essential medium plus Earle's salts and Trypan Blue dye were obtained from Gibco. [*methyl*-³H]Thymidine, (1 mCi/ml; 2 Ci/mmol) was purchased from Amersham-Searle, Arlington Heights, IL, U.S.A., and [³H]uridine (1 mCi/ml, 1 Ci/mmol) was received from Research Products Int. Corp., Elk Grove, IL, U.S.A. Sephadex G-75 (40–120 µm particle size) was a product of Pharmacia Fine Chemicals, Uppsala, Sweden. 5,5'-Dithiobis-(2-nitrobenzoic acid) was obtained from Sigma. All other chemicals were reagent grade.

General procedures

The biuret reagent preparation and the method for protein concentration estimation was as described by Layne (1957). Lysozyme and albumin (Sigma) were used as standards.

Cell counts were performed on an American Optical Spencer bright line phase haemocytometer (American Optical Corp., Buffalo, NY, U.S.A.).

To determine the extent of cell viability, 0.5 ml of the cell suspension used for the cell counts was used for a Trypan Blue exclusion test. Finally, acid digestion of cells and particulate fractions of cells was done so that cadmium and zinc analyses could be performed by atomic absorption spectroscopy. An aliquot of the tumour suspension was centrifuged, the supernatant was decanted, and the cell pellet was treated with 1.0 ml of an acid digestion solution composed of 50% (v/v) HClO₄, 30% (v/v) HNO₃, and 20% (v/v) glass-distilled water. The mixture was heated in a water bath at 100°C overnight. In this and all other procedures described here glassware had been washed in 10% (v/v) HNO₃ and then in 1 mM-EDTA to remove traces of contaminant metals.

Ehrlich ascites tumours, and cadmium and zinc treatment

Ehrlich tumour cells were grown in female Swiss mice. Cells were introduced into animals by interperitoneal injection (0.1 ml injection/animal; approx. 10⁷ cells). The tumour was allowed to grow *in vivo* in the mouse peritoneal cavity for 14 days. On days 5, 7, 9, 11, and 13, each animal received 0.1 ml of 0.5 mM-CdCl₂ or 10 mM-ZnSO₄ by intraperitoneal injection. On day 14, the animals were killed by cervical dislocation. This amount of cadmium was toxic to the animals, but was tolerated for the course of the experiment.

Tumour cells in ascites fluid were harvested by quantitative aspiration of the peritoneal contents into sterile 10 ml syringes and deposited into disposable culture tubes. A drop of heparin (1000 units/ml) was added to the culture tube to prevent agglutination. Cells and fluid were separated by centrifugation. Cells were freed of erythrocytes before being used for experiments. The tumour cells were resuspended in minimal essential medium of Ringer solution (0.9% NaCl/0.03% KCl/0.025% CaCl₂/0.02% NaHCO₃), depending on the particular experiment. Cell counts and biuret tests for protein were routinely performed (see above).

The mice were individually weighed and the average weight change per mouse for that particular group was calculated. Changes in body weight are an index of tumour growth rate.

Uptake of cadmium by Ehrlich ascites cells in vitro

Tumour cells from mice fed a normal zinc-sufficient diet and not injected with CdCl₂ were studied for reaction with cadmium *in vitro*. Cells were harvested as above. From earlier experiments, it was decided to incubate the cells with 80 ng-atoms of cadmium/mg of protein, which should result in an internal cell concentration of about 6 ng-atoms of cadmium/mg of protein (Solaiman *et al.*, 1979). Suspensions of cells and CdCl₂ were always incubated for 20 min.

After incubation, the solution was centrifuged and the supernatant was removed. The cells were then resuspended in minimal medium or Ringer solution using a Supermixer. Cells were washed free of excess cadmium by repeating this procedure three more times before the cell contents were analysed for cadmium.

Preparation of cell fractions

Differential centrifugation was used to determine the relative amounts of cadmium and zinc in the subcellular fractions. Six fractions were examined: 480 × g pellet (nuclei and unbroken cells), 480 × g nuclear supernatant, 12 350 × g supernatant (mitochondrial supernatant), 149 000 × g supernatant (soluble fraction), and 149 000 × g pellet (micro-

somal fraction). Initial centrifugations were carried out in a Sorvall RC2B centrifuge (SS-34 rotor, 0–5°C). Ultracentrifugation was done in a Beckman L5-75 preparative ultracentrifuge (fixed angle Ti-75 rotor). All samples of cells were sonicated gently on a Branson Sonifier Cell Disruptor 200 under pulsed mode, which allows some relaxation time for the cell. Output was set at 4, with a 30% duty cycle. The sample was sonicated for 30–90 s, depending on the volume and concentration of the sample, in an ethanol/ice-slush bath to absorb excess shock and provide uniform temperature. The volume of sample homogenates was usually 9–12 ml.

The sonicated sample was transferred to a cold centrifuge tube and centrifuged at $480 \times g$ for 10 min. The supernatant solution was carefully decanted into another centrifuge tube. The $480 \times g$ pellet was resuspended in Ringer solution to a volume approx. equal to the volume of the original homogenate. This suspension was centrifuged at $270 \times g$ in the refrigerated Sorvall centrifuge for 10 min. The supernatant (nuclear supernatant) was decanted off and saved. The pellet was resuspended in Ringer solution and was designated the nuclear fraction. The initial supernatant from the above procedure was centrifuged at $12350 \times g$ (10000 rev./min) for 10 min. The supernatant was decanted into a chilled centrifuge tube and saved for ultracentrifugation. The $12350 \times g$ pellet was resuspended in Ringer solution and centrifuged at $4430 \times g$ (6000 rev./min) for 10 min. The supernatant was decanted and designated mitochondrial supernatant. The pellet, containing the mitochondria, was resuspended in Ringer solution. The $12350 \times g$ supernatant was subjected to ultracentrifugation at $100000 \times g$ (60000 rev./min) for 60 min. Finally, the nuclear, mitochondrial, and microsomal fractions were each resuspended in Ringer solution by using a small Dounce hand-held homogenizer. A sample of each of the six fractions (nuclei, nuclear supernatant, mitochondria, mitochondrial supernatant, soluble fraction, and microsomal fraction) was digested in acid and the metal contents were determined by atomic absorption spectrophotometry. The protein content of cellular fractions was determined by the biuret method.

Preparation and analysis of cell supernatant

In many experiments, after initial cell sonication, centrifugation was carried out at $48000 \times g$ (20000 rev./min) for 30 min. The supernatant was decanted and saved for separation of its constituents on a Sephadex column. The pellet was also saved for digestion and subsequent zinc and cadmium analyses. The supernatant was applied to a column (2.5 cm $\times$ 60 cm) of Sephadex G-75 equilibrated with 0.01 M-phosphate buffer, pH 7.0. The column was then eluted with the same buffer at a

rate of 20 ml/h. A series of molecular weight standards was used to calibrate the column. Fractions (4.5 ml) were collected and analysed as follows. First, absorbance was measured at 250 and 280 nm with the Beckman Acta V spectrophotometer. Secondly, cadmium and zinc analyses on the fractions were performed by using an atomic absorption spectrophotometer. In a few cases, copper analyses were also done. Assays of the thiol content of the binding protein were done by adding 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959). As is described elsewhere, colour development is time-dependent, and the increase in absorbance at 412 nm must be followed until the reaction is complete in order to obtain valid results (Li *et al.*, 1979).

Utilization of [^3H]thymidine and [^3H]uridine by ascites cells

The utilization of [^3H]thymidine (a DNA precursor) and [^3H]uridine (an RNA precursor) by Ehrlich ascites cells was measured by using a previously published procedure (Saryan *et al.*, 1979). Incubations were performed concurrently using cells from animal hosts on different regimes, each suspended at the same cell concentration to ensure valid comparison. Data are reported as a percentage of the total radioactivity recovered from extracellular, intracellular acid-soluble, and intracellular acid-precipitable compartments. Uncertainty in the reported value of the percentage of radioactivity recovered is less than $\pm 10\%$ of the reported value. Incorporation of [^3H]thymidine or [^3H]uridine into the HClO_4 -insoluble compartment of cells was interpreted as a measure of DNA synthesis or RNA synthesis respectively. Radioisotope remaining in the extracellular medium after the incubation was used to evaluate the uptake of the precursor by the cells.

Results

Growth of tumour

Typical effects of cadmium, as CdCl_2 , on the growth of the Ehrlich tumour in mice given optimal and deficient levels of zinc in their diets are shown in Fig. 1. Injections of 50 ng-atoms of cadmium were given on days 7, 9, 11, and 13. This amount and interval of exposure of cells and animal to cadmium permitted the animals to survive until they were killed on day 14 and also allowed the growth of sufficient numbers of Ehrlich cells for the chemical and biochemical analyses described below. Fig. 1 shows that CdCl_2 effectively inhibited the growth of the tumour cells with respect to control populations under both $\text{Zn}(+)$ and $\text{Zn}(-)$ conditions.

Cadmium and zinc in Ehrlich cells and ascites fluid

The amounts of cadmium and zinc in the Ehrlich cells and the fluid bathing them were assessed by

atomic absorption spectroscopy. The summary of a number of experiments is given in Table 1. In experiments involving only variations in the exposure of mice to zinc, the zinc content of ascites fluid of animals on zinc-deficient regimens was markedly depressed compared with levels in animals given diets containing zinc. Cellular zinc concentrations are also

somewhat depressed in zinc-deficient tumours in comparison with tumours in mice fed zinc-normal diets. Interestingly, when tumour-bearing mice are given four injections of $1 \mu\text{mol}$ of ZnCl_2 on days 7, 9, 11, and 13 after tumour implantation, the cellular concentration of zinc, 3.17 nmol/mg of protein, did not rise above control concentrations despite the nearly 2.5-fold higher zinc concentration of the fluid a day after the last injection.

In the presence of CdCl_2 , cellular and fluid zinc concentrations of tumours in the $\text{Zn}(+)$ and $\text{Zn}(-)$ animals did not change. The small variations are not statistically significant, as shown by the limits of measurement (Table 1). There was a dose dependence for the accumulation of cadmium by the Ehrlich cells. Over the range $100\text{--}200 \text{ ng-atoms}$ of cadmium, the cellular cadmium rose from 0.29 to 0.62 ng-atoms/mg of protein. However, most striking is the finding that the total cadmium uptake is dependent on the zinc status of the tumour. Thus, $\text{Zn}(+)$ tumours exposed to 200 ng-atoms of cadmium had a cellular concentration of 0.35 ng-atoms of cadmium/mg of protein, much less than the 0.62 ng-atoms/mg seen in $\text{Zn}(-)$ tumours, but similar to the amount in the tumour after 100 ng-atoms of cadmium in the zinc-deficient tumours. These differences are statistically significant as shown by the error limits (Table 1). The zinc-deficient and zinc-sufficient tumours accumulate similar total amounts of cadmium (24 versus 34% of the total injected cadmium).

Cadmium and zinc distributions in cells

The distribution of cadmium and zinc among cell fractions was then measured to see how the zinc

Fig. 1. The effect of zinc status and cadmium on the growth of Ehrlich ascites tumours in mice maintained on $\text{Zn}(-)$ chow

●, Control animals given 80 g of zinc/ml of water; ○, cadmium-injected animals given $80 \mu\text{g}$ of zinc/ml of water; ▲, control animals given zero zinc in water; △, cadmium-injected animals given zero zinc in water. Cadmium (50 ng-atom doses, as CdCl_2) was injected intraperitoneally on days 7, 9, 11, and 13 (arrows).

Table 1. Effect of dietary zinc on cadmium and zinc uptake

Cadmium was given in injections of 50 ng-atom ; for treatment with a total of 200 ng-atoms , injections were given on days 7, 9, 11 and 13; for 150 ng-atom , on days 9, 11 and 13; for 100 ng-atom , on days 11 and 13. Zinc was given in injections of 1000 ng-atom on days 7, 9, 11 and 13. Data are presented as means $\pm$ S.E.M., with the number of samples in parentheses.

Zinc in		Metal injected (ng-atom)	Metal in			
Chow	Water ($\mu\text{g/ml}$)		Cells (ng/atoms/mg of protein)		Fluid (μM)	
			Cd	Zn	Cd	Zn
Zn(—)	0	—	—	2.16 ± 0.31 (6)	—	3.21 ± 0.95 (4)
Zn(—)	80	—	—	2.90 ± 0.51 (3)	—	10.44 ± 3.22 (2)
Zn(+)	Tap water	—	—	3.17 ± 0.21 (13)	—	11.31 ± 2.51 (14)
Zn(—)	0	Zn (4000)	—	3.17 ± 0.35 (7)	—	24.52 ± 6.40 (13)
Zn(—)	0	Cd (100)	0.29 ± 0.07 (4)	2.38 ± 0.1 (4)	—	—
		Cd (150)	0.42 ± 0.13 (5)	2.24 ± 0.10 (5)	0.70 ± 0.15 (7)	2.76 ± 0.43 (7)
		Cd (200)	0.62 ± 0.05 (13)*	2.04 ± 0.09 (13)	0.79 ± 0.18 (8)†	3.29 ± 0.99 (7)
Zn(—)	80	Cd (200)	0.35 ± 0.03 (8)*	3.17 ± 0.05 (6)	0.46 ± 0.22 (8)†	10.54 ± 3.35 (8)

* Total accumulation of cadmium was 24% for $\text{Zn}(-)$ animals receiving no zinc in water and 34% for $\text{Zn}(-)$ animals receiving $80 \mu\text{g}$ of zinc/ml of water.

† In each case the total cadmium in fluid is about 1% of the total injected dose.

Table 2. *Distribution of cadmium and zinc in cell fractions*

Zinc-sufficient animals received Zn(+) chow and distilled water; Zn-deficient animals received Zn(−) chow and distilled water. The dose of cadmium was 200 ng-atom.

		Percentage of total metal in fraction				
		Control zinc-sufficient animals	Zinc-sufficient animals + cadmium		Zinc-deficient animals + cadmium	
Fraction	Metal . . .		Zn	Cd	Zn	Cd
Nuclei and cell debris		23	29	15	28	12
Nuclear wash		25	19	26	20	20
Mitochondria		8	3	3	2	2
Mitochondrial wash		7	3	5	4	5
Microsomal fraction		8	11	4	9	6
Supernatant		29	35	48	38	54
Total metal (nmol/10 ⁹ cells)		630	750	86	400	120

status of the tumour affects the localization of cadmium. Typical results are shown in Table 2. Variation of the zinc available to the Ehrlich cells had no dramatic effect on either the zinc or cadmium distributions. About one-half of the cadmium was found in the particulate components of the cell and the other half in the supernatant. This observation also holds for zinc-deficient tumour cells subjected to 150 ng-atoms of cadmium on days 7, 9, and 11 and examined on day 14 (results not shown). Cadmium was found in all of the cell fractions. When pellets of both nuclei and mitochondria were washed by resuspension and centrifugation, some cadmium remained bound to these fractions, whereas a portion of it was less firmly bound and was washed free of the pellets. The Sephadex G-75 profiles of zinc and cadmium in the 149 000 × *g* supernatant were then obtained.

Cadmium and zinc-binding protein fraction of supernatant

Fig. 2 shows the typical pattern of zinc and cadmium in the effluent fractions from gel exclusion chromatography. In Ehrlich cells from either Zn(+) or Zn(−) tumours, cadmium was localized almost entirely in a protein fraction that behaved like a molecule of mol.wt. 10 000 in this chromatographic system. There was little absorbance at 280 nm associated with the fraction; however, more absorbance at 250 nm was detected. In the zinc-sufficient animals, these fractions also contained large amounts of zinc. The average Zn/Cd atomic ratio was 2.8. These properties are consistent with the protein being a metallothionein. Nevertheless, firm identification will await further study. At present, it will be called a binding protein.

The cadmium-binding protein was also present under zinc-deficient conditions, as illustrated in Fig. 2(b). Furthermore, there was a marked alteration in

the metal composition of the fraction; the Zn/Cd atomic ratio was drastically reduced to 0.65. Although this shift is partly due to an increase of cadmium in the fraction, it is principally due to a major decrease in the amount of zinc. Again, virtually all of the cadmium was localized to the binding protein.

Exposure of the tumour to extra zinc, as described in Table 1, increases the amount of zinc in the 10 000-mol.wt. area of the chromatographic effluent profile. An approx. 4-fold enhancement in the zinc content of the binding-protein fractions was observed. Increased amounts of zinc also appeared in higher and lower molecular weight fractions.

The finding that cadmium binds to a single cytosol fraction is in accord with many other studies demonstrating the presence of metallothionein in cells after exposure to cadmium. However, when the metal profile of the cytosol of Ehrlich cells untreated with cadmium was examined, the 10 000-mol.wt. region of the chromatogram was occupied by a zinc-containing protein (Table 1). The overall profiles of Zn(+) and Zn(−) cell supernatants and the amount of zinc associated with the binding protein in zinc-normal and zinc-deficient cells are similar and are comparable with the level in the cadmium and zinc-binding protein in Fig. 2(a).

Characterization of the cadmium- and zinc-binding protein

In some experiments, 5 mM-2-mercaptoethanol was added to the cell homogenate before Sephadex G-75 chromatography. This modification was based on studies with a rat-liver model which showed that the metal content of metallothionein markedly changed under oxidizing conditions, leading to shifts of zinc from metallothionein into higher and lower molecular weight fractions (D. T. Minkel, K. Paulsen, S. Wielgus, C. F. Shaw & D. H. Petering,

Fig. 2. Sephadex G-75 profiles of cadmium (O), zinc (●) and A_{250} (---) in the supernatants of Ehrlich cells grown in mice injected with 200 ng-atom of cadmium

The mice were maintained on Zn(—) chow and given (a) 80 μg of zinc/ml or (b) zero zinc in their water. The zinc/cadmium ratios in the zinc, cadmium-binding protein fraction are (a) 2.8 and (b) 0.66. The bars show molecular weight calibrations.

unpublished work). Fig. 3 shows the Sephadex chromatography elution profile for the supernatant of Ehrlich cells not exposed to cadmium. Strikingly, there was very little zinc in the low-molecular-weight region between fractions 50 and 60. Furthermore, 60% of the supernatant copper was found in the fractions containing the zinc-binding protein. In comparison with liver, there was little Cu,Zn-superoxide dismutase in the region between fractions 25 and 30, which corresponds to a mol.wt. of 30 000.

The zinc, copper-binding protein reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) in a complex way. Fig. 4 shows that about 50% of the total reactive thiol groups react within the mixing time of the assay mixture. This is followed by a much slower reaction. The initial reaction is characteristic of free thiol reacting with 5,5'-dithiobis-(2-nitrobenzoic acid), according to studies with horse-kidney metallothionein (Li *et al.*, 1979). The second phase is similar to the reaction observed between 5,5'-dithiobis-(2-nitrobenzoic acid) and thiol groups bound to cadmium, zinc, or copper in the horse kidney protein (Li *et al.*, 1979). The molar ratio of slowly reacting thiol to metal is 3.03; assuming a stoichiometry of 3 thiol/Zn and 1 thiol/Cu the calculated ratio is 2.21, in reasonable agreement with the experimental value.

Having found a zinc-binding protein normally present in the Ehrlich cells that appears to contain

Fig. 3. Sephadex G-75 elution profile of zinc (●) and copper (O) in the supernatant of Ehrlich cells from animals not exposed to cadmium and given Zn(—) chow + 80 μg of zinc/ml of water

Fig. 4. Kinetics of reaction (semi-logarithmic plot) of the zinc- and copper-binding protein with 5,5'-dithiobis-(2-nitrobenzoic acid), measured by the formation of thionitrobenzoate at 412 nm

Lyophilized and redissolved binding protein was reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) (10mM) in a sealed 3ml cuvette under a N_2 atmosphere at pH 7.8 in 0.2M-phosphate buffer. The reagent/metal ratio was 100:1. The last fast step, which corresponds to less than 10% of the total reaction, was reproducible for several independent preparations of the protein. A_{∞} , absorbance at end of reaction.

unsaturated binding sites for metal, the question arose whether cadmium *in vivo* was simply binding to pre-existent zinc-binding protein. A short incubation of Ehrlich cells with cadmium *in vitro* was carried out. Typical results are given in Fig. 5. Cadmium bound in all regions of the profile, including the fractions corresponding to the zinc-binding protein. Significantly, the binding of the cadmium occurs without displacement of zinc or copper. Thus, the enhanced amount of cadmium in the 10000-mol.wt. fraction appears to be caused at least in part by the presence of unsaturated binding sites in pre-existent zinc-binding protein.

Functional effects of exposure to cadmium

Because cadmium inhibits the growth of Ehrlich cells *in vivo*, as shown in Fig. 1. DNA and RNA synthesis were measured in the Ehrlich cell model. Fig. 6 shows the typical time courses of thymidine uptake into cells and its incorporation into the DNA of the tumours under several different conditions during their growth. Control tumour populations from animals maintained on Zn(+) and Zn(-) diets showed kinetics of radioactive label uptake and incorporation into DNA similar to those observed in

Fig. 5. Comparison of the Sephadex G-75 elution profile of cadmium in the Ehrlich cell supernatant following exposure *in vivo* and exposure *in vitro* for 20 min
●, Cadmium for 20min; ○, cadmium *in vivo*;
----, A_{280} ; ▽, A_{250} .

previous studies (Minkel *et al.*, 1979; Petering & Saryan, 1979). In tumours from zinc-deficient animals, not only was the incorporation of thymidine into DNA greatly depressed, but its uptake into the cells was also greatly inhibited. Patterns of movement of the label into cells and into DNA qualitatively similar to those observed in the Zn(-) tumour were seen in cells previously exposed to cadmium in the presence and absence of dietary zinc. In fact, both uptake and incorporation into DNA were suppressed to even a greater extent than in the control Zn(-) tumours.

Although there was a significant effect upon DNA synthesis that parallels the macroscopic inhibition of growth of these tumours by cadmium, there was no clear perturbation of RNA synthesis, as measured by [^3H]uridine incorporation into RNA (Fig. 7). These results agree with earlier studies that zinc deficiency alone does not alter either uptake or incorporation of radioactive label into RNA (Minkel *et al.*, 1979; Petering & Saryan, 1979). The slight differences in the uptake of uridine over time between cadmium-treated tumours with differing dietary concentrations of zinc were not reflected in the amount of radioactive label fixed in RNA. The viability of the cells in the cell populations used in the

Fig. 6. Utilization of [^3H]thymidine by ascites tumour cells

The upper portion of the figure (dashed lines) illustrates the loss of ^3H from the incubation medium (a measure of cellular uptake); the lower portion (solid lines) represents incorporation of [^3H]thymidine into DNA. Open symbols represent cadmium-treated animals; closed symbols represent untreated animals. Circles represent animals given zinc-free water; triangles represent animals given $80\mu\text{g}$ of zinc/ml of water.

experiments of Figs. 6 and 7 was assessed using a dye exclusion test with Trypan Blue. In all cases, viability was greater than 95%. Therefore, there appeared to be a selective effect of cadmium upon the mechanism of DNA synthesis in the liver Ehrlich cells.

Discussion

The studies described here were begun with the hypothesis that Ehrlich ascites tumour could be used as a model for the examination of the interactions of cadmium with cells. This was substantiated by the finding that Ehrlich cells exposed to CdCl_2 *in vivo* bind virtually all their cytoplasmic cadmium in a single protein that has properties similar to mammalian metallothioneins, which are known to bind cytosolic cadmium, zinc, and traces of copper.

Fig. 7. Utilization of [^3H]uridine by ascites tumour cells
The legend follows that for Fig. 6, except that the lower portion (solid lines) represents [^3H]uridine incorporation into RNA.

These findings are similar to those reported by Rudd & Herschman (1978) and by Hidalgo *et al.* (1978b). HeLa cells in culture, and a mouse hepatoma, both incorporate cadmium into a metallothionein-like protein. Furthermore, the hepatoma cells normally contain a zinc-binding protein described as a metallothionein, although no copper was found in the material. Ehrlich cells, too, normally contain a zinc- and copper-binding protein basically having the same properties as the cadmium-binding protein described above.

The exposure of these cells to cadmium may simply lead to the binding of metal to pre-existent protein, not to the induction of new binding protein, as occurs when rat liver encounters cadmium ion (Andersen *et al.*, 1978; Richards & Cousins, 1975). Experiments *in vitro*, summarized in Fig. 5, show that cadmium rapidly binds to the zinc- and copper-binding protein fraction without displacing either zinc or copper. The rapidity of the effect is evidence against induction of synthesis *de novo* of binding protein, and also suggests that the native protein has unsaturated metal binding sites that pick up cadmium entering the cells. The rapid initial reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) with

the copper- and zinc-binding protein isolated under anaerobic conditions also supports the idea that there are unsaturated binding sites. In this respect, Ehrlich cells are not a model for the delayed induction of thionein in rat liver after exposure to cadmium. However, they can be used to study the metal-binding properties of pre-existent zinc- and copper-binding protein in a complex cellular environment. Additionally, given the recent demonstration that zinc-thionein is induced in a number of normal physiological responses, the Ehrlich-cell model may be useful for examining some aspects of normal zinc metabolism and its interactions with cadmium.

The relative growth rates of Zn(+) and Zn(-) tumours, their zinc concentrations in fluid and the cellular zinc concentration of Zn(+) Ehrlich cells found here agree with previous studies (Minkel *et al.*, 1979), although the 33% decrease in zinc content of tumour cells in Zn(-) animals was not observed earlier. There is no marked, repeatable difference in the profile of the zinc in cell cytosol or particulate fraction between Zn(+) or Zn(-) tumours (Fig. 5 and Table 2). Thus, the effects of zinc deficiency on Ehrlich cells documented elsewhere are not paralleled by noticeable losses of zinc from the cells or shifts between cellular components.

After the discovery of a naturally occurring zinc-binding protein in Ehrlich cells, it was found that isolation and Sephadex chromatography of cell supernatant in the presence of 2-mercaptoethanol eliminates the presence of the low-molecular-weight band of protein-bound zinc observed in Fig. 2, as can be seen in Fig. 3. The basis for the inclusion of 2-mercaptoethanol came from current studies with rat-liver metallothionein, which show that the protein is readily oxidized during preparation, losing some of its zinc into a lower-molecular-weight fraction (D. T. Minkel, K. Paulsen, S. Wielgus, C. F. Shaw & D. H. Petering, unpublished work). When isolated in the presence of 2-mercaptoethanol, the binding protein contains the majority of the cytoplasmic copper. Interestingly, it is bound in stable form even under aerobic conditions, which for simple systems containing Cu(II) or Cu(I) and thiols would lead to thiol oxidation (Jocelyn, 1972). The finding of large amounts of both zinc and copper in a metallothionein-like protein raises the possibility that copper and zinc metabolism interact in this protein fraction, as has been suggested by other workers (Evans *et al.*, 1970).

Treatment of zinc-deficient tumours with massive amounts of zinc does not raise their cellular zinc concentration measurably above that of tumours from Zn(+) animals. The fact that ascites-fluid zinc concentrations vary over an 8-fold range between Zn(-) cells and cells exposed to excess metal, whereas the cellular zinc concentration only in-

creases from 2.16 to 3.17 ng/atoms/mg of protein confirms that there is no detectable equilibration of cellular and fluid pools of zinc in the tumour (Minkel *et al.*, 1979). Nevertheless, the amount of zinc bound to binding protein does increase above that found in either Zn(-) or Zn(+) tumours (Fig. 5).

Ohtake *et al.* (1978) recently found that rat liver cells stimulated to divide by a drug-hormone combination and by partial hepatectomy generate a large amount of new zinc-metalllothionein. They suggested that its induction might be a requirement for carrying out DNA synthesis as the liver moves from a resting state into the S-phase of the cell cycle. The finding of zinc- and copper-binding protein in rapidly-dividing tumour cells may signal a similar requirement. However, the protein is still present in Zn(-) tumours (Fig. 2b) which grow much more slowly and apparently contain a large proportion of resting cells (Minkel *et al.*, 1979; Petering & Saryan, 1979). The concentration of zinc-binding protein in the cytosol of Ehrlich cells was shown not to vary during the first cell cycle after release of Ehrlich cells from a double thymidine block (D. Minkel, C. Krishnamurti, L. A. Saryan & D. H. Petering, unpublished work). Thus, if the binding protein is a requirement for cell-cycle progression, it seems to be a permanent component of Ehrlich tumour, unaffected by the proliferative state of the cell population or the segment of the cell cycle in which the cells are found.

Turning to the interaction of cadmium and zinc in Ehrlich cells, the presence of cadmium in the tumour does not alter the total cellular zinc content relative to control values (Table 2). However, the cadmium content of the cells is a function of the zinc status of the tumour. Almost twice as much cadmium/mg of cell protein is present in the Zn(-) tumour as in the Zn(+) cells. Indeed, although the tumours from zinc-deficient animals contain only about one-third the number of the cells of the zinc-normal tumours, they accumulate two-thirds of the same total dose of cadmium.

Although the total cadmium content varies depending on zinc status, the relative distribution among the cell fractions does not vary. About 50% of the total cadmium is found in the particulate fractions of the cell, 40% of which is not removed by washing the pellet (Table 2). While the nature of the binding sites was not determined, recent work (D. Minkel, S. Wielgus, K. Paulsen, D. H. Petering & C.F. Shaw, unpublished work) has demonstrated that particulate bound cadmium in rat liver is almost all associated with metallothionein.

The amount of cadmium bound to the binding protein is enhanced in zinc deficiency, even though the amounts of zinc in these fractions are similar in Zn(-) and Zn(+) tumour cells unexposed to cadmium. Interestingly, the presence of cadmium

leads not only to cadmium in the binding protein, but also to a much increased content of zinc in the band (Fig. 2).

Finally, some functional effects of cadmium on Ehrlich cells have been observed. Incorporation of thymidine into DNA is inhibited in cadmium-exposed, Zn(+) cells and even more so in the Zn(-) tumours treated with cadmium. Total uptake of thymidine into Ehrlich cells is also decreased in the presence of cadmium. These cell populations are viable according to Trypan Blue staining, and show incorporation of uridine into RNA approx. the same as in control tumours not exposed to cadmium. Hence, there is selectivity to the alterations of metabolism caused by cadmium. This is also suggested by our unpublished studies of the rates of respiration of Ehrlich cells from control and cadmium-exposed tumours. Although oxidative phosphorylation in isolated Ehrlich mitochondria is inhibited by low concentrations of cadmium, only a 25% decrease in the rate of oxygen utilization is seen in the cadmium-treated cell populations either from zinc-normal or zinc-deficient animals. These results are similar to the earlier findings that short-term incubations of Ehrlich cells with cadmium produces only a minor effect on cell respiration (Solaiman *et al.*, 1979).

Comparing the concentration-dependence of the effects of cadmium upon thymidine metabolism in these Zn(+) cells with cells exposed for only 1 h to cadmium, as examined by Solaiman *et al.* (1979), it is seen that inhibition of incorporation of thymidine into DNA occurs at a much lower internal concentration of cadmium (0.32 versus 3.2 ng-atoms/mg of cell protein) and with a corresponding inhibition of uptake of the nucleoside which does not occur after exposure to cadmium *in vitro* (Solaiman *et al.*, 1979). This implies a slow reorganization of heavy metal after initial exposure of the cells, which leads to a different mode of suppression of DNA synthesis.

The effects of cadmium upon macromolecular synthesis in Zn(+) cells are the same as those in zinc-deficient cells (Saryan *et al.*, 1979). In that case, it was suggested that the depression of DNA synthesis in Zn(-) cells is due to a decrease in the population of cells proceeding through the cell cycle. A similar mechanism may be operative here. The intriguing possibility is that in cadmium exposure, there is a direct cadmium-zinc interaction such that cadmium-treated Zn(+) cells behave like Zn(-) cells.

A direct antagonism between cadmium and zinc has been suggested in previous animal experimentation (Petering, 1978). What is new here is that there is defined a specific biochemical process (the incorporation of thymidine into DNA) that may be subject to this competitive relationship.

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