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TEMPERATURE AND SPECIES DIFFERENCES IN SUSCEPTIBILITY OF KIDNEY CELL CULTURES TO MERCURY TOXICITY

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SUMMARY

The effect of temperature on inorganic mercury toxicity was investigated using kidney tissue culture systems. The relative susceptibility of rabbit (homeothermic) kidney to mercury intoxication was compared to that of Coho salmon (poikilothermic) kidney over temperature ranges consistent with the habitat of each of the two species. It was demonstrated that susceptibility to mercury toxicity is species dependent; that is, the rabbit kidney cells tolerated higher mercury concentrations in the medium than did the fish-derived cells. Within a given species, susceptibility to mercury toxicity was temperature dependent. Decreasing the temperature increased the toxicity of mercury to cultures of rabbit kidney cells, whereas decreasing temperatures decreased the effect of mercury toxicity on the salmon kidney cells. As a consequence, fish taken from arctic waters are liable to be more toxic when introduced into mammalian food chains. Albumin was shown to act as a protective agent in vitro against inorganic mercury toxicity.

Key words: mercury toxicity; salmon kidney cultures; species variation; albumin protection; temperature effects.

INTRODUCTION

Mercury and its compounds, both inorganic (1) and organically bound (2), occupy a position of major concern among current health-associated environmental problems. The kidney and the gastrointestinal tract are the major routes of mercury excretion with the kidney reportedly retaining more mercury than any other organ (3). Mercuric chloride ($HgCl_2$) is a well known nephrotoxic agent; hence it appeared relevant to study the toxicity of varying mercury levels on kidney cells grown in vitro. Inorganic mercury titrations on kidney cells in tissue culture systems were used to develop information on the comparative tolerances of cells derived from poikilothermic versus homeothermic animals to mercurial intoxication. It was noted in the course of study that increasing serum concentrations in the medium diminished apparent mercury toxicity, suggesting protein protection as measured by tissue culture toxic doses to 50% of cell populations ($TCTD_{50}$). Albumin proved the active component, for mere increases in protein concentration afforded no protection against mercurial cytotoxicity.

MATERIALS AND METHODS

Glassware and reagents. All tissue culture glassware was processed by a 15- to 20-min boiling period in 1 to 2% aqueous sodium carbonate, followed by a rinse sequence of copious tap water, moderate-distilled water, and, lastly, limited triple-distilled water. Pipettes and culture tubes (16- by 125-mm) were disposable tissue culture plastic ware.

The inorganic mercury used for toxicity titrations was the chloride salt (Fisher Scientific Co.). The $HgCl_2$ solution was prepared as a 1% aqueous stock solution (3.68×10^{-2} M). Whether sterilized by autoclaving or by filtration, toxicity titration endpoints were the same. The pH of the stock solution was 4.0. Dilutions for inoculation onto monolayers were made in Hanks' balanced salt solution (BSS) adjusted to pH 7.2 to 7.4 and delivered to cultures in volumes of 0.1 ml per tube. Values reported represent final concentrations in 1.0 ml.

Cell culture and media. Kidney cells from Coho salmon (*Oncorhynchus kisutch*) were grown as primary cultures by trypsinizing [0.25% trypsin

(Nutritional Biochemicals Corp.) in Hanks' BSS] the minced kidney at 4° C for repeated 30-min intervals until most cells were freed from the tissue mass. Washed counted cells were suspended in Medium 199 supplemented with 20% newborn bovine serum (NBBS) (both from Grand Island Biological Company), and 0.1% yeast extract (Nutritional Biochemicals Corp.) so as to achieve a cell density of 7×10^5 cells per ml. The cells were delivered in 1-ml volumes into culture tubes and slanted for incubation at 18° to 19° C under atmospheric conditions.

Rabbit kidney cells were obtained by primary culture and by serial passage of primary and subsequent cultures. Kidneys from domestic rabbits were minced and the cells released from the tissue pieces by stirring with 0.25% trypsin either at 4° C overnight or at room temperature 3 to 4 hr. Twice-washed cells (Hanks' BSS) were counted and the cell density adjusted to 5 to 7×10^5 cells per ml in medium 199 supplemented with 10% NBBS for tube slants (1 ml per 16- by 125-mm tube) or 7×10^5 cells inoculated into 25-cm^2 tissue culture flasks containing 8 ml of the same medium. Large 75-cm^2 tissue culture flasks containing 30 ml medium received 1 to 2×10^6 cells. Rabbit kidney cultures were incubated at 37° C under atmospheric conditions or under 5% carbon dioxide. The 20% and 10% NBBS supplements give optimal growth conditions for salmon and rabbit kidney cells, respectively.

Salmon as well as initial rabbit kidney cultures employed minced tissue from the entire kidney. In subsequent cultural refinements, the medullary portion of rabbit kidneys was removed and the cortical tissue minced for enzymic dissociation of cells. Rabbit cortex derived kidney cultures displayed the same mercury titration toxicity endpoints as the cultures derived from whole kidney. The cAMP response to highly purified bovine parathyroid hormone (bPTH) of these cultures was accepted as a convenient biochemical marker. For this purpose the assay cells were released from monolayers using calcium-magnesium-free Saline A (4) plus 0.025% EDTA and 0.05% trypsin (STV) (4). The cells were washed, resuspended and counted in Medium 199. The cell densities were adjusted to achieve final cell numbers of 5×10^6 ml, and 200-μl aliquots were dispensed into disposable test tubes. Cells were broken by five freeze-thaw cycles, and 200 μl tris buffer containing 50 mM tris, 8.25 mM Mg^{+2}, and 28 mM theophylline, pH 7.4, were added, followed by 0.6 mg ATP per tube and the bPTH solution. After a 10-min incubation at 37° C, the reaction was stopped by rapid cooling, and the cAMP of the supernate was determined by radioimmunoassay (Schwartz-Mann Kit) based on the procedure of Steiner et al. (5). A linear response was obtained in this system for bPTH concentrations ranging from 0.12 to 2.43 pmol with a precision of ± 0.2 pmol of bPTH.

For passage of monolayers to other bottles or tubes, cell sheets were twice washed with Saline A and cells were dispersed with STV. Volumes of 5 ml for 25-cm^2 flasks and 8 to 9 ml for 75-cm^2 flasks were permitted to act on monolayers for 5 min at room temperature. Flasks were shaken and fluid contents pipetted to effect complete removal and dispersion of cells. Following centrifugation for 10 min at $150 \times g$, cells were resuspended in a small volume of growth medium (10% NBBS in Medium 199), counted by hemacytometer and dispensed into tubes or Falcon flasks. Seed inoculations were the same as for primary cultures.

All media used for cell culture were adjusted to pH 7.2 to 7.4 with 5% aqueous $NaHCO_3$. No antibiotics were added to any of the culture media used in this study.

The mercury toxicity studies were carried out on Coho salmon and rabbit kidney cells grown in tightly capped, slanted 16- by 125-mm culture tubes. Tube monolayers were prepared for toxicity titrations by removal of the growth medium, followed by two 1-ml washes with Hanks' BSS and medium replacement in volumes of 0.9 ml per tube. The replacement medium for salmon kidney cells was 20% NBBS Medium 199 because these cells required high serum concentrations for viability; that of rabbit kidney cells was 3% NBBS Medium 199. The use of 3% NBBS supplement was dictated by a desire to establish comparable growth rates between the two cell systems. Some rabbit kidney cultures received fetal bovine serum rather than NBBS, but this did not change titration endpoints of mercury toxicity. The prepared tube cultures were inoculated in replicates of four with 0.1-ml volumes of each $HgCl_2$ dilution. The $HgCl_2$ concentrations tested ranged from 1:1000 (0.1%) to 1:2,000,000; dilution increments of 100,000 formed the basis of titrations after initial work. Inoculated tubes were returned to respective incubators (salmon kidney at 18° to 19° C and 10° to 12° C; rabbit kidney at 37° C and some at room temperature), and examined for cytopathogenic changes for 3 consecutive days. The toxicity endpoint ($TCTD_{50}$) was calculated

TABLE 1

MERCURY TOXICITY ENDPOINTS IN KIDNEY MONOLAYER CULTURES

Monolayer Tissue	N[a]	% Newborn Bovine Serum	$TCTD_{50}$[b] Concentration of Mercuric Chloride
			μM
Coho kidney 12° C	4	20[c]	13±1.3[d]
Coho kidney 18° C	8	20[c]	7.8±0.7
Rabbit kidney 37° C	60	3[c]	6.0±0.6
Rabbit kidney 24° C	8	3[c]	2.2±0.2
Rabbit kidney 37° C	12	20	26±2.5

[a] N = number of titration curves obtained.
[b] $TCTD_{50}$ = tissue culture toxic dose for 50% of monolayer cultures.
[c] These serum concentrations support similar growth rates for the two cell systems.
[d] Mean ± standard deviation.

by the method of Reed and Muench (6). Briefly, the proportionate distance of the 50% mortality endpoint from measured dilutions is calculated by the interpolation from the formula:

$$\frac{\%\text{ accumulated mortality at dilution next above 50\%} - 50\%}{\%\text{ accumulated mortality at dilution next above 50\%} - \%\text{ accumulated mortality at dilution next below 50\%}}$$

The proportionate distance thus calculated is a direct function of the incremental dilution steps used in construction of the titration curve and is added to the dilution next above 50% mortality to yield the $TCTD_{50}$ titer.

Since newborn bovine serum was the medium supplement, albumin protection studies were carried out with Bovine Plasma Albumin Fraction V (Reheis Chemical Co.) prepared as a 20% stock solution in Medium 199. Initial work with albumin protection employed an albumin supplement equivalent to that in 20% serum medium (based on a 4.5% albumin concentration in serum).

Gelatin (J. T. Baker Chemical Co.) prepared as an 8% stock solution in Medium 199 served as the nonspecific protein control. All experiments included quadruplicate cell control tubes given the corresponding medium (0.9 ml) inoculated with 0.1 ml-volumes of Hanks' BSS. When albumin and gelatin titrations were performed, control tubes containing the higher test concentrations were included to rule out nonspecific cytopathogenic effects.

TABLE 2

EFFECTS OF ALBUMIN AND GELATIN ON MERCURY CYTOTOXICITY ON RABBIT KIDNEY MONOLAYERS

Type and Concentration of Medium Protein Supplement	$TCTD_{50}$ Concentration of Mercuric Chloride
	μM
3% NBBS[a]-199	6.0±0.6
20% NBBS-199	26±2.5
3% NBBS-199 fortified with albumin[b]	26±2.5
0.8% Albumin-199	26±2.5
0.8% Gelatin-199	0.37±0.04

[a] NBBS = newborn bovine serum.
[b] Final albumin concentration: 0.9%.

RESULTS

Data for mercury toxicity endpoints are shown in Table 1. Coho kidney at 18° C incubation demonstrated a $TCTD_{50}$ of 2.11 μg $HgCl_2$ per ml (7.8 μM); however, the same Coho salmon kidney cells at 12° C exhibited a $TCTD_{50}$ of 3.55 μg $HgCl_2$ per ml (13 μM). These cells demonstrated decreased susceptibility to mercury toxicity at lower temperatures at probability levels <0.001 (two-tailed t test).

Experiments designed to test the effect of temperature decreases on mercury toxicity to rabbit kidney cells, the mammalian counterpart, revealed increased susceptibility ($p<0.001$). As little as 0.6 μg per ml (2.2 μM) was toxic to 50% of the test population at room temperature compared to 1.64 μg per ml (6.0 μM) at 37° C.

One is tempted to speculate that the increased susceptibility to toxicity at lower than body temperatures in the system derived from the homeothermic animal is the consequence of stress on cells produced by temperature reduction. Certainly, decreased metabolism did not provide increased tolerance to mercury cytotoxicity.

Experiments with rabbit kidney monolayers to assess the effects on mercury toxicity of serum concentrations in the range used in the salmon kidney cultures revealed that 20% NBBS exerted protective activity for the former cell populations. Under these conditions the rabbit kidney cells exhibited a $TCTD_{50}$ of 7.09 μg per ml or 26 μM $HgCl_2$ and thus appeared less susceptible to mercury intoxication than salmon kidney monolayers (Table 1).

To determine whether the protective activity depended on the albumin concentration of serum or merely on the protein concentration of the medium, mercury titrations were performed using albumin and gelatin as supplements to approximate the protein concentration of the 20% NBBS medium. Table 2 shows that supplementation of 3% NBBS 199 with albumin to a total concentration of 0.9%, i.e. the final protein concentration of 20% NBBS fortified medium, obtained the same endpoint of 26 μM $HgCl_2$. Supplementation of Medium 199 with 0.8% albumin alone gave similar titration endpoints. In contrast, gelatin provided no protection even at high concentrations. Levels greater than 1.2% gelatin were degenerative for these cells. Clearly, the albumin fraction of serum exhibited a protective effect against mercurial cytotoxicity.

Discussion

Coonrad and Paterson (1) studied the nephrotoxic action of mercuric chloride by intramuscular injection of the salt into rats and assay for renal injury via the appearance of β-glucuronidase in urine. Lauwerys and Buchet (7) probed the mechanism of lysosome labilization by isolating rat liver and kidney lysosomes in vitro to study the kinetics of the mercury-membrane interaction. Their results describe the labilization process as a sequence of events triggered by the binding of the mercury to lysosomal membranes, probably to thiol groups. This is followed by an autocatalytic decomposition of remaining lysosomes through the initially-released lysosomal hydrolases.

Though other organs and tissues are injuriously affected, the major target of inorganic mercurial intoxication is the kidney (8), primarily the cortical and subcortical regions (9,10).

Tissue culture studies can be a valuable asset to investigations of comparative toxicology. They are relatively economical when compared to the use of whole animals and permit precise measurements under easily controlled conditions of environment. The technique yields reproducible data.

The present study demonstrates that concentrations of 26 μM $HgCl_2$ are cytotoxic to 50% of rabbit kidney cell populations under appropriate conditions of temperature and albumin concentrations (0.8 to 0.9% albumin). The coefficient of variation over many studies performed by the authors in two different laboratories was of the order of 9.8%. Rabbit spleen cells in 20% serum concentrations (0.9% albumin) reported by Pauly, Caron and Suskind (11) exhibit cytotoxicity at 40 μM $HgCl_2$ concentration, a figure well within the order of magnitude found in our current studies.

Kidney cells from the poikilothermic salmon demonstrated greater resistance to inorganic mercury cytotoxicity at 10° to 12° C than at 18° C. The data suggest that poikilothermic animals residing in arctic waters tolerate greater mercury levels before exhibiting toxicological manifestations than do fish living in more temperate climates. These animals introduced into the human chains then may harbor even greater than usual potential for mercury poisoning to their consumers.

The diametrically opposed effect of temperature reduction on the cells derived from the kidneys of two different subphyla of vertebrates suggests basic differences between them. Cells from the essentially more primitive poikilothermic animals appear to afford greater adaptability to environmental changes. Thus interaction between mercury and membranes of poikilothermic origin may be slowed by reduced temperatures without cellular stress. Cells from the homeotherm, on the other hand, exhibit greater inflexibility to environmental changes. The stress produced by temperature decreases made these cells sensitive to $HgCl_2$ concentrations as low as 2.2 μM.

While not totally unexpected, the protective effect of albumin on mercury cytotoxicity was interesting. Ovalbumin, often employed in mercury poisoning as an emetic, also may serve therapeutically because it adsorbs mercury and thereby prevents cellular penetration.

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