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ACUTE EFFECTS OF MERCURIC COMPOUNDS ON CULTURED MAMMALIAN CELLS

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Abstract—The observed acute effects of methyl mercuric chloride on cultured mammalian cells were: (1) retardation of cell multiplication, (2) cell killing, (3) depression of [^{3}H]thymidine and [^{3}H]uridine uptake, and (4) induction of single-strand scissions of DNA. Among the cellular responses studied, [^{3}H]thymidine and [^{3}H]uridine incorporation were the most sensitive indicators of cellular mercuric poisoning. Toxicities of three mercuric compounds—methyl mercuric chloride, phenyl mercuric acetate and mercuric chloride—were compared by using two indicators, [^{3}H]thymidine incorporation and cell multiplication. Methyl mercuric chloride and phenyl mercuric acetate were equally toxic, while mercuric chloride was the least toxic. Addition of glutathione to cells pretreated with methyl mercuric chloride allowed the cells to recover from the toxic effects of methyl mercuric chloride at a faster rate than those left without glutathione.

Mercury poisoning has been reported throughout the world and mercury is now considered to be one of the major potential pollutants of the biosphere [1–4]. The main effector of mercury poisoning is thought to be an alkylated form(s) of mercury [5, 6], to which metallic mercury and/or mercuric ions can be converted [7, 8]. Although mercury poisoning has been studied in animals and men, little is known about the nature of mercury poisoning at the cellular level except that a high incidence of chromosome aberrations was found in lymphocytes cultured from men who had a high tissue level of mercury [9].

The present study was initiated to investigate the cellular responses of cultured mammalian cells to three mercury compounds.

MATERIALS AND METHODS

Cell line. Mouse leukemic L5178Y cells were cultured in Fischer's medium with 10% horse serum [10, 11]. Cells used were always in the exponential growth phase with a population doubling time of 8·5 hr.

Chemicals. Methyl mercuric chloride, phenyl mercuric acetate and mercuric chloride were donated by Dr. T. Suzuki of Tohoku University, Sendai, Japan. Methyl mercuric chloride and mercuric chloride were dissolved in a small volume of ethanol and diluted to 1×10^{-3}M with Puck's saline G [12]. Phenyl mercuric acetate was dissolved in ethanol and diluted to 2×10^{-4}M with Puck's saline G.

Glutathione (Yamano-uchi Pharmaceutical Co., Tokyo, Japan) was dissolved in Fischer's medium without horse serum and its pH was adjusted to 7 to 7·5 by the addition of 2 N NaOH.

Estimation of survival. The cells were treated with one of the mercuric compounds for 60 min, rinsed once with cold Fischer's medium and plated in culture tubes containing 5 ml of Fischer's medium with 15% horse serum and 0·12% agar (purified agar, Difco Laboratories, Detroit, Mich., U.S.A.) [13]. After 8–9 days of growth, the number of colonies was counted. The plating efficiency of the untreated controls was 70–90 per cent. The survival of the treated cells is the ratio of the plating efficiency of the treated cells over that of the untreated cells.

Incorporation of [^{3}H]thymidine and [^{3}H]uridine into the acid-insoluble fraction. The cells were exposed to one of the mercuric compounds for 60 min in the presence of [^{3}H]thymidine (1 μCi/ml, 5 Ci/m-mole) or [^{3}H]uridine (2 μCi/ml, 5 Ci/m-mole) (Dai-ichi Pure Chemicals Co.). After this, the cells were centrifuged, lysed with 0·2 ml of 2% sodium dodecyl sulfate, and placed on a filter paper disc. The filter discs were washed three times with 5% trichloroacetic acid, rinsed three times with ethanol, and dried. The dried discs were placed in vials containing toluene-PPO–POPOP† scintillator [14] and counted by a Beckman DPM-100 liquid scintillation counter.

Alkaline sucrose gradient centrifugation. The cells (5×10^3 cells in 0·025 ml) were lysed for 150–180 min at room temperature in 0·2 ml of lysing solution (0·05 N NaOH, 0·95 N NaCl, 10 mM EDTA), which was then placed on the top of 4·8 ml of a 5–20% sucrose gradient (0·1 N NaOH, 0·95 N NaCl, 3 mM EDTA) [15].

The gradient was centrifuged at 4000 rev/min for 15

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† PPO = 2,5-diphenyloxazole; POPOP = 1,4-bis-2-(4-methyl 5-phenyloxazolyl) benzene.

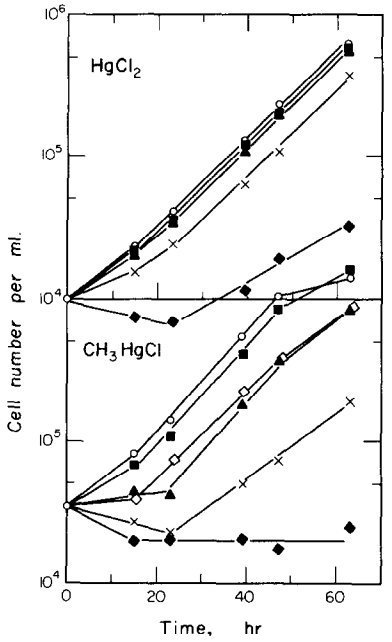

Fig. 1. Growth curves of cells treated with mercuric chloride or methyl mercuric chloride for 60 min. The mercury compounds were then removed at 0 time and cell growth was observed. Upper panel, cells treated with mercuric chloride: (○) control, (■) 5×10^{-6}M, (▲) 1×10^{-5}M, (×) $1{\cdot}5 \times 10^{-5}$M and (◆) 2×10^{-5}M. Lower panel, cells treated with methyl mercuric chloride: (○) control, (■) 1×10^{-6}M, (▲) 2×10^{-6}M, (×) 3×10^{-6}M, and (◆) 4×10^{-6}M. (◇) treated with 4×10^{-6}M same as (◆), but grown in medium containing 0·5 mM glutathione from zero time.

min and then at 38,000 rev/min (180,000 gravity units at the tube bottom) for 60 min at 12° with an SW50-1 rotor in a Beckman L3-50 ultracentrifuge. The sucrose gradient was fractionated into 0·2-ml fractions and each fraction was placed on a filter paper disc. After being dried, the discs were placed in vials and counted as described previously.

Estimation of single-strand scissions of DNA. The cells were labeled for 20 hr (about two generations) with [^{3}H]thymidine (0·5 μCi/ml, 5 Ci/m-mole), washed and resuspended in Fischer's medium with 10% horse serum. The cells were then treated with one of the mercuric compounds for 60 min, and afterwards resuspended in medium without horse serum. They were then subjected to alkaline sucrose gradient centrifugation. The extent of the single-strand scissions was qualitatively estimated from the shift of the DNA peak toward the top of the centrifuge tube.

Elongation of replicating strands of DNA. The cells were pulse-labeled with [^{3}H]thymidine (20 μCi/ml, 5 Ci/m-mole) for 20 min. They were then "chased" with Fischer's medium containing 10^{-4}M thymidine and 10^{-5}M deoxycytidine for various lengths of time in the presence or absence of mercuric compounds. Next, they were subjected to alkaline sucrose gradient centrifugation and the rate of elongation of the replicating DNA strands was estimated from the manner in which the labeled DNA increased its size with increasing incubation time.

RESULTS

Cell multiplication. Cells were treated with various concentrations of methyl mercuric chloride for 60 min, then placed in fresh medium and their growth was followed for 60 hr. These growth curves are shown in Fig. 1. Cell growth was not affected by methyl mercuric chloride at 1×10^{-6}M or less, or by mercuric chloride at 1×10^{-5}M or less. When concentrations of the mercuric compounds were increased above these levels, the growth curves of the treated cells showed a time lag before resumption of cell multiplication and this resumed cell growth also showed a longer population doubling time than the controls. At concentrations of methyl mercuric chloride of 4×10^{-6}M and higher or inorganic mercuric chloride of $2{\cdot}5 \times 10^{-5}$M and higher, cell growth ceased completely during the observation period of 3 days.

Survival. Figure 2 shows the survival curve of cells treated with methyl mercuric chloride for 60 min. D_0, D_q and n (cf. S. Okada [16]) were $0{\cdot}5 \times 10^{-6}$M, $2{\cdot}2 \times 10^{-6}$M and 55 respectively.

Induction of single-strand scissions of DNA. Cells were labeled with [^{3}H]thymidine for two generations, treated with methyl mercury at various concentrations for 1 hr, and then subjected to alkaline sucrose gradient centrifugation. Figure 3A is an example of the sedimentation profile of untreated cells with an average molecular weight of $2{\cdot}3 \times 10^8$ daltons. There is a

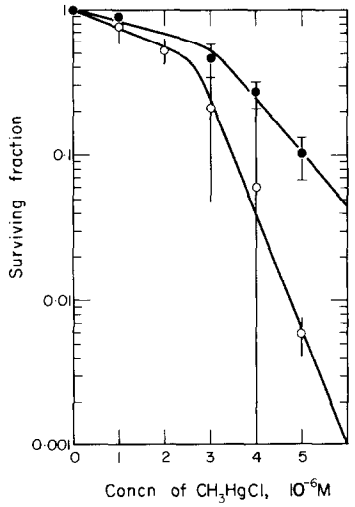

Fig. 2. Survival curves of cells treated with methyl mercuric chloride and cultured in medium with (solid circles) or without (open circles) 0·5 mM glutathione. Bars indicate standard deviation.

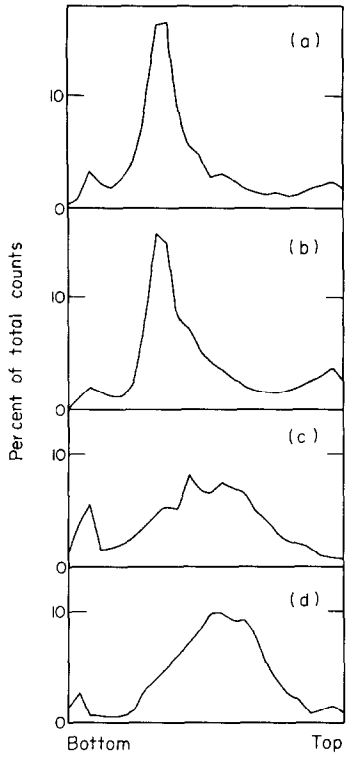

Fig. 3. Alkaline sucrose gradient sedimentation pattern of cellular DNA treated with (B, C) methyl mercuric chloride or (D) mercuric chloride at concentrations of: (A) none, (B) 1×10^{-6}M, (C) 1×10^{-5}M and (D) 1×10^{-4}M.

small peak at the bottom of the tube which may be due to incompletely lysed or tangled DNA. The small peak at the top is probably due to a small amount of DNA trapped with a protein-and-lipid layer in the lysing solution. These two small peaks always constituted less than 10 per cent of the total labeled DNA in the gradient.

When the cells were treated with mercuric compounds, the sedimentation profiles became broader and shifted toward the top of the centrifuge tube with increasing concentration of these compounds (Fig. 3, B, C and D). The shift of the sedimentation pattern toward the top results from the introduction of strand scissions in the cellular DNA molecules. A significant alteration of the sedimentation profiles at a concentration of 1×10^{-5}M of methyl mercury was comparable to that of 1×10^{-4}M $HgCl_2$.

DNA synthesis. Incorporation of exogenous [^{3}H]thymidine into the acid-insoluble fraction of the cells was depressed when concentrations of methyl mercuric chloride in the medium exceeded 2×10^{-7}M, when phenyl mercuric acetate exceeded 2×10^{-7}M, or mercuric chloride exceeded 2×10^{-6}M (Fig. 4).

In order to find out whether the mercuric compounds affected the DNA synthetic machinery *in situ*, the cells were labeled with [^{3}H]thymidine for 20 min in the absence of mercuric compounds (Fig. 5A). When the cells were chased with cold thymidine, the labeled fraction moved toward the bottom with increasing chase time (Fig. 5B) and reached the distribution of the bulk DNA after a 2-hr chase period (Fig. 5C). This indicates that it takes about 2 hr for the 20-min labeled fraction to reach the bulk DNA distribution of the untreated cells. Subsequently, the 20-min labeled cells were chased with cold thymidine in the presence of various concentrations of methyl mercury and the elongation of the 20-min labeled DNA fraction was followed: Fig. 5, D and E, shows that methyl mercury

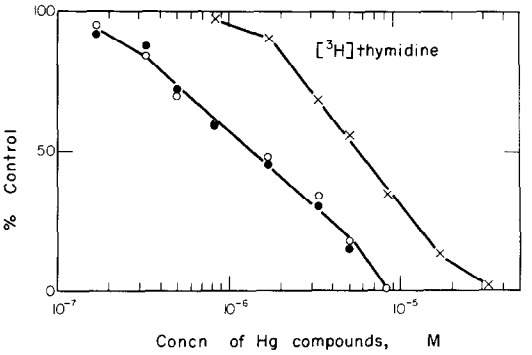

Fig. 4. Cells were exposed to [^{3}H]thymidine in the presence of various concentrations of mercury compounds for 1 hr and the incorporation of [^{3}H]TdR into the acid-insoluble fraction of the cells was determined. (●) Methyl mercuric chloride, (○) phenyl mercuric acetate or (×) mercuric chloride.

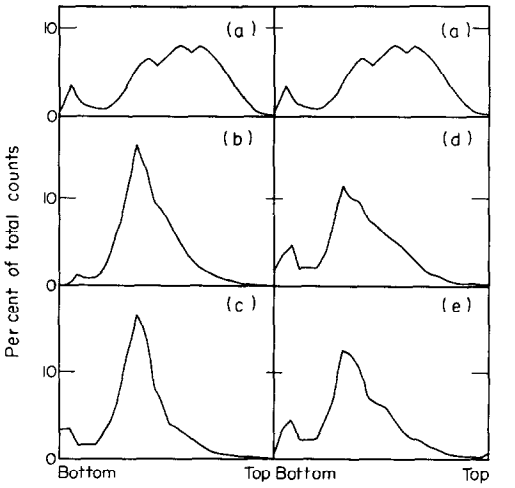

Fig. 5. Effects of methyl mercuric chloride on elongation of DNA strands. Alkaline sucrose gradient sedimentation patterns of DNA from the cells (A) pulse labeled for 20 min, chased for (B) 1 hr and (C) 2 hr in the absence of methyl mercury. Patterns of cellular DNA chased for (D) 1 hr and (E) 2 hr in the presence of 1×10^{-6}M methyl mercury.

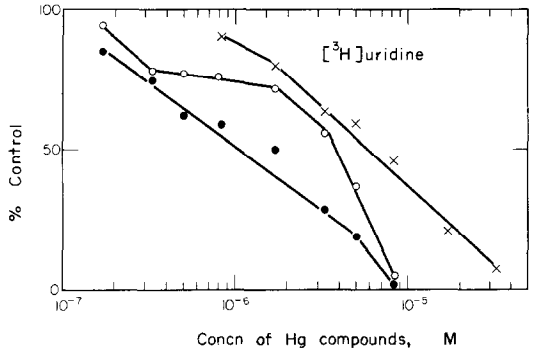

Fig. 6. Cells were exposed to [^{3}H]uridine in the presence of various concentrations of three mercury compounds for 1 hr and the incorporation of [^{3}H]uridine into the acid-insoluble fraction of the cells was determined. Cells were treated with (●) methyl mercuric chloride, (○) phenyl mercuric acetate or (×) mercuric chloride.

at 1×10^{-6}M retarded the elongation of DNA significantly. An almost complete cessation of the DNA elongation process was noted when the concentrations of methyl mercury exceeded 5×10^{-6}M.

RNA synthesis. Incorporation of [^{3}H]uridine into the acid-insoluble fraction of the cells was depressed by mercuric compounds (Fig. 6). The extent of depression of [^{3}H]uridine incorporation was found to be similar to the depression of [^{3}H]thymidine incorporation.

Protection effects by glutathione. Multiplication of cells treated with 4×10^{-6}M methyl mercuric chloride was retarded when they were grown in medium containing no glutathione. When 0·5 mM glutathione was added to the medium, a significant lessening of growth retardation was noted (Fig. 1). When the treated cells were plated in medium supplemented with 0·5 mM glutathione, the survival of the treated cells was found to increase drastically over those without glutathione (Fig. 2). The survival curve characteristics of the glutathione-protected cells were a D_0 of $1{\cdot}3 \times 10^{-6}$M, D_q of $2{\cdot}2 \times 10^{-6}$M, and n of 6·5.

DISCUSSION

The acute effects of methyl mercuric chloride on cultured mammalian cells were: (1) retardation of cell multiplication, (2) cell killing, (3) depression of [^{3}H]thymidine and [^{3}H]uridine incorporation, and (4) induction of single-strand scissions of DNA. Among the cellular responses studied, [^{3}H]thymidine and [^{3}H]uridine incorporation were the most sensitive, followed by cell killing and retardation of cell multiplication. Appearance of single-strand scissions in cellular DNA was the least sensitive to methyl mercuric chloride (Table 1).

The toxicities of the three mercurial compounds were compared with respect to cell multiplication, cell killing, [^{3}H]thymidine incorporation and single-strand scissions of DNA. Table 1 summarizes these results and shows that methyl mercuric chloride and phenyl mercuric acetate were of similar toxicity, while $HgCl_2$ was the least toxic.

Methyl mercuric chloride depressed exogenous [^{3}H]thymidine and [^{3}H]uridine incorporation into the cells to an equal extent. This suggests that the compound may have impaired membrane permeability, which led to reduced uptake of exogenous thymidine and uridine into the cells, or alternatively that the mercuric compound damaged the machinery of macromolecular synthesis. To clarify this point, the depression of [^{3}H]thymidine incorporation was investigated in more detail. When the cells were pulse-labeled with [^{3}H]thymidine and chased with cold thymidine, the sucrose gradient centrifugation patterns made it possible to follow the elongation process of the pulse-labeled DNA by observing the shift of label toward the bottom of the tube with increasing chase time. The addition of methyl mercuric chloride to the cells after pulse-labeling with [^{3}H]thymidine made it possible to follow its effect on the DNA elongation rate *in situ* under conditions where the effects of mercury on cell permeability to exogenous [^{3}H]thymidine may be eliminated. The results shown here indicate that the concentrations of mercuric compounds required for depression of DNA replication *in situ* are similar to those required for depression of [^{3}H]thymidine uptake. This indicates that the observed effect of the mercuric compounds was actually due to inhibition of DNA synthesis *in situ* rather than a simple permeability effect.

Single-strand scissions may not be an important contribution to depression of DNA synthesis because

Table 1. Comparison of cytotoxic effects of three mercuric compounds

Cytotoxic effects	Concentration (M)		
	CH_3HgCl	Phenyl-Hg-acetate	$HgCl_2$
90% Inhibition of cell growth	3×10^{-6}		2×10^{-5}
Survival (D_0)	$0{\cdot}5 \times 10^{-6}$		
50% Inhibition of [^{3}H]thymidine incorporation	1×10^{-6}	1×10^{-6}	5×10^{-6}
Induction of single-strand breaks*	10^{-5}		10^{-4}

* The concentrations cited here were not the minimum which induced single-strand scissions, but those which induced comparable degrees of significant single-strand breaks in cellular DNA.

the concentration of the compounds necessary to induce a significant number of single-strand scissions is about 10 times greater than that necessary for depression of DNA synthesis. In the literature, mercuric compounds are reported to bind preferentially with AT-rich regions of DNA [17], to denature DNA of bacterial and mammalian origins *in vitro* [18] and to inhibit membrane-bound DNA polymerase in bacteria [19, 20, 21]. All or some of these mechanisms might be involved in the depression of DNA synthesis shown here.

Since mercuric compounds, such as *p*-chloromercuribenzoic acid, are known to bind specifically to SH groups, and since *N*-ethylmaleimide, a known SH binding agent, was also found to induce single-strand scissions of DNA [21, 22], it seems possible that some of the lesions caused by methyl mercuric chloride occur through its binding to cellular SH groups. If so, then the addition of SH compounds would be expected to reduce the toxic effects of mercuric compounds. The present study shows clearly that the addition of glutathione to cells previously treated with methyl mercuric chloride results in the reduction of two of the toxic effects of methyl mercuric chloride: retardation of cell multiplication and cell killing.

Survival curves can be drawn to display the effect of any toxic agent on a particular biological system. Such a curve is shown here in Fig. 2, which gives the surviving fraction of cells as a function of the mercury concentration. The parameters describing such curves were developed by radiation biologists for target theory, but can be used to describe the effects of agents other than radiation [16]. These parameters are:

D_{37}: The dose which leaves a surviving fraction of 37 per cent is called the D_{37} dose. This can be read directly from the curve in Fig. 2 as the dose corresponding to a 37 per cent survival.

n: Most survival curves are not linear (Fig. 2). Two other parameters help describe this case. If the linear portion of the curve is extended until it intersects the log survival axis, the point of intersection is called n or the extrapolation number.

D_0: The slope of the extended linear curve is called $1/D_0$.

D_q: If a horizontal line is drawn through the 100 per cent survival point until it intersects the extended linear curve, the abscissa value corresponding to the point of intersection is called D_q or the quasithreshold dose. This is an approximation of the dose after which the survival curve becomes linear.

The relationship among the parameters is

$$D_{37} = D_0 + D_q$$

$$(D_q/D_0) = \ln N$$

It was found here that the D_q values for mercury-treated cells with or without glutathione treatment were of the same magnitude. This implies that the cells are killed effectively when the concentration of methyl mercury exceeded the D_q value of $2{\cdot}2 \times 10^{-6}$M and that the protective action by glutathione is mainly due to its alteration of the D_0 value, but not of the D_q value. As to the mechanism of the rescue effect of glutathione, two possibilities could be suggested: one is a stimulation of cellular recovery from mercury-inflicted damage, and the other is a removal of tightly bound cellular mercury. Further studies are needed to clarify the protection mechanisms involved.

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