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# Regulatory Role of Extracellular Medium Components in Metal Induced Cyto- and Geno-Toxicity

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Recently, a considerable amount of work in different laboratories has been devoted to establishing and characterizing the mutagenicity and the oncogenic potential of metal compounds in cell culture systems (for reviews see Heck and Costa 1982a,b). Monolayer cultures offer several advantages that make their use attractive for short term studies (Costa 1980). However, the entry of metals into cells can be affected by various types of interactions occurring between metal compounds and medium components (Abbracchio et al. 1982). Different types of sera (newborn vs fetal bovine), different amounts or even different batches of serum significantly affect the toxicity of several metal ions such as $Ni^{2+}$ (Abbracchio et al. 1982), $Cd^{2+}$, $Hg^{2+}$, etc. *(Cantoni, unpublished)*. In this paper we have characterized some of these effects and studied cyto- and geno-toxic actions produced by $HgCl_2$ or $CaCrO_4$ in Chinese Hamster Ovary (CHO) cells grown as monolayer cultures.

## MATERIALS AND METHODS

CHO cells were cultured in MEM supplemented with 10% fetal bovine serum. Cells were grown as monolayer cultures in an atmosphere of 95% air and 5% $CO_2$. $5 \times 10^5$ cells were plated in 60 mm dishes and incubated for 25-30 hr in the presence of [$^{14}$C]-deoxythymidine (0.02 μCi/ml). The medium was then removed and replaced with 10 ml of medium without isotope and cells were allowed to grow at 37°C overnight. $HgCl_2$ or $CaCrO_4$, freshly dissolved in distilled water, were added to the appropriate culture medium and the incubation was continued for another hour. In some experiments, cells were exposed to the metal and various reducing agents or sulphydryl containing compounds at the same time. Cells were then rinsed with an ice-cold phosphate buffered saline (PBS) and, after trypsinization, were analyzed for DNA damage. Alkaline elution of DNA was carried out essentially as described previously (Kohn et al. 1981). Briefly stated, $8 \times 10^5$ cells were loaded onto the surface of 2 μM pore size

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polycarbonate filters (25 mm diameter) and lysed on the filter with 5 ml of 2% sodium dodecyl sulphate, 0.025 M EDTA (pH 10.1) containing 0.5 mg/ml of proteinase K, for 60 min at room temperature. Cell lysates and filters were rinsed with 5 ml of 0.02 M EDTA (pH 10.1). DNA was eluted in the dark with 0.025 M EDTA (free acid) plus 2% tetrapropylammonium hydroxide (pH 12.1) at a flow rate of 0.035 ml/min. Fractions of approximately 3 ml were collected and counted in 10 ml of Atomlight containing 0.07 ml of glacial acetic acid and 2.5 ml of 0.4 N NaOH. Cells used for cyto-toxicity studies were seeded at a density of $1x10^5$ cultured cells in 60 mm Petri dishes containing 5 ml of MEM. After 24 hr cells were incubated for 1 hr in SGM containing varying concentrations of the metal ($HgCl_2$ or $CaCrO_4$) or the metal plus a sulphydryl containing compound or a reducing agent. Following treatment the medium was removed, the cell sheet washed twice with 10 ml of PBS, and cells were incubated for 3 days in 5 ml of MEM. At the end of this period the cell number was determined with a hemocytometer.

## RESULTS AND DISCUSSION

The formation of single strand breaks (SSB) in the DNA of CHO cells was examined following exposure to varying $HgCl_2$ concentrations in three different media whose compositions are given in table 1. The DNA was analyzed by the alkaline elution technique, and data, calculated as fraction of DNA retained on the filter at the 9th hour of elution, was normalized with respect to appropriate controls. The amount of DNA damage for a given $HgCl_2$ concentration was highly dependent upon the composition of the extracellular milieu (Fig. 1A) In fact, whereas 10 μM $HgCl_2$ induced no detectable DNA breakage in Mc Coy's 5a or MEM, a number of DNA SSB comparable to that caused by a 450 rads X-rays dose was observed following treatment with the same metal concentration in the SGM. Addition of either reduced glutathione (1 mM) or cysteine (5 mM) strikingly reduced DNA breakage produced by $HgCl_2$ (Fig. 2A). Consistently, cysteine (5 mM) significantly reduced the growth inhibitory effect of $HgCl_2$ in cells exposed to the metal for 1 hr in the SGM and then allowed to grow in fresh MEM. The formation of DNA SSB was also evaluated in cells treated with $CaCrO_4$ (Fig. 1B). Damage to DNA was higher in cells exposed to chromate in the SGM than in cells treated with the metal in Mc Coy's 5a or MEM, but the difference was not as dramatic as in the case of $HgCl_2$. Addition of ascorbic acid (0.5 mM) to the SGM reduced both DNA breakage (Fig. 2B) and cell killing generated by chromate (table 2). Various in vitro studies have shown that metals exhibit mutagenic activity in bacteria and in mammalian cells (Heck and Costa 1982a), induce DNA damage (Robison et al. 1982; Cantoni et al. 1982, 1984; Cantoni and Costa 1983) and unscheduled DNA synthesis (Robison et al. 1984) in cultured mammalian cells, cause inhibition (Costa 1979) and

Table 1. Composition of Minimum Essential Medium (with Earle's salts) Mc Coy's 5a (Park and Terasaki modification) and salts/glucose media*

| Components | MEM | Mc Coy's 5a | SGM |
|---|---|---|---|
| L-Alanine | | 13.36 | |
| L-Arginine HCl | 126.40 | 42.14 | |
| L-Asparagine | | 45.03 | |
| L-Aspartic acid | | 19.97 | |
| L-Cysteine HCl | | 31.53 | |
| L-Cystine | 28.42 | | |
| L-Glutamic acid | | 22.07 | |
| L-Glutamine | 292.30 | 219.20 | |
| Glutathione | | 0.50 | |
| L-Glicine | | 5.51 | |
| L-Histidine HCl $H_2O$ | 41.90 | 20.96 | |
| L-Isoleucine | 52.50 | 39.36 | |
| L-Leucine | 52.50 | 39.36 | |
| L-Hydroxyproline | | 19.67 | |
| L-Lysine HCL | 73.06 | 36.54 | |
| L-Methionine | 14.90 | 14.92 | |
| L-Phenylalanine | 33.02 | 16.52 | |
| L-Proline | | 17.27 | |
| L-Serine | | 26.28 | |
| L-Threonine | 47.64 | 17.87 | |
| L-Tryptophan | 10.20 | 3.06 | |
| L-Tyrosine | 45.02 | 22.52 | |
| L-Valine | 46.90 | 17.52 | |
| Ascorbic acid | 0.50 | 0.50 | |
| Biotin | | 0.20 | |
| D-Calcium pantothenate | 1.00 | 0.20 | |
| Choline chloride | 1.00 | 5.00 | |
| Folic acid | 1.00 | 10.00 | |
| i-Inositol | 2.00 | 36.00 | |
| Nicotinic acid | | 0.50 | |
| Nicotinamide | 1.00 | 0.50 | |
| Pyridoxal phosphate | 1.00 | 0.50 | |
| Riboflavin | 0.10 | 0.20 | |
| Thiamine HCl | 1.00 | 0.20 | |
| Vitamin $B_{12}$ | | 2.00 | |
| $CaCl_2$ $2H_2O$ | 264.90 | 132.50 | 292.00 |
| KCL | 400.00 | 400.00 | 370.00 |
| $MgSO_4$ 7 $H_2O$ | 200.00 | 200.00 | |
| NaCl | 6800.00 | 6460.00 | 5800.00 |
| $NaHCO_3$ | 2000.00 | | |
| $NaH_2PO_4$ 2 $H_2O$ | 158.30 | 655.70 | |
| D-Glucose | 1000.00 | 3000.00 | 960.00 |

Table 1 continued

| Components | MEM | Mc Coy's 5a | SGM |
|---|---|---|---|
| Phenol red | 17.00 | 10.00 | |
| Penicillin | 200 U/ml | 200 U/ml | |
| Streptomycin | 200.00 | 200.00 | |
| Fetal bovine serum | 50 ml/l | 50 ml/l | |
| HEPES | 2.86 | | 2.90 |

* Data is expressed as mg/l.

Table 2. Effect of cysteine or ascorbic acid on cell growth inhibition by $HgCl_2$ or $CaCrO_4$.

| Treatment* | GI** |
|---|---|
| 5 μM $HgCl_2$ | 91.7 ± 8 |
| 5 μM $HgCl_2$ + 5 mM cysteine | 12.4 ± 7 |
| 50 μM $CaCrO_4$ | 93.3 ± 4 |
| 50 μM $CaCrO_4$ + 0.5 mM ascorbic acid | 78.2 ± 6 |

* Cells were exposed to the metal with or without cysteine or ascorbate for 1 hr in SGM. Following treatment, the medium was removed, the cell sheet washed twice with PBS and cells were incubated for three days in 5 ml of MEM. Cell number was determined with a hemocytometer.
** Growth inhibition (GI) is expressed as percent of untreated cultures ± SEM.

infidelity (Sirover and Loeb 1976) of DNA synthesis and produce a S-phase-specific blockade (Costa et al. 1983). Furthermore, metal compounds induce transformation in cell culture systems (Heck and Costa 1982b) and are carcinogenic in humans (IARC Monography on the Evaluation of Carcinogenic Risk of Chemicals to Humans 1980) and experimental animals (Costa 1980). The function of metals in these processes is not well understood and several laboratories are investigating in cultured cells the role that metals play in the progression of a normal cell to a transformed cell. However, metals, depending upon their chemical reactivity, bind or interact with several medium components resulting in a decreased availability of the metal for cells. The results of this study indicate that the composition of the extracellular medium can indeed modulate geno- and cyto-toxic actions produced by metal compounds. $Hg^{2+}$ ions show a strong

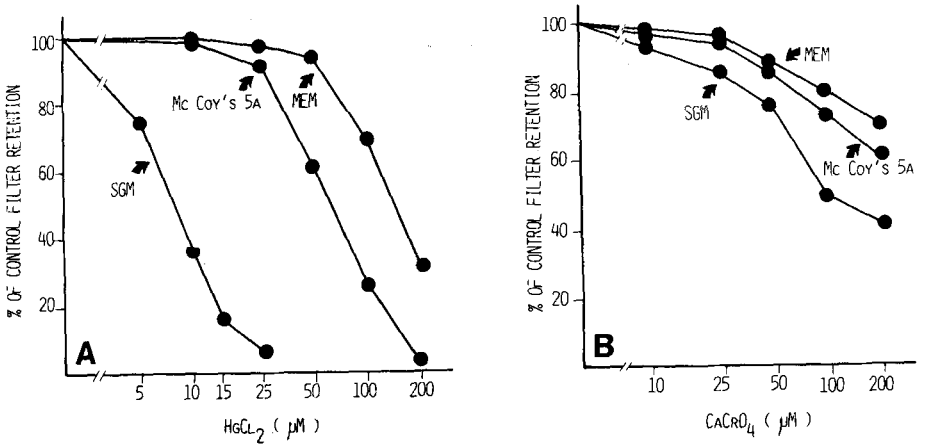


*Figure 1 - Induction of DNA SSB in cells exposed to varying concentrations of $HgCl_2$ (A) or $CaCrO_4$ (B). Cells were treated with the metal compound for 1 hr in each of three different media (MEM, Mc Coy's 5a or SGM) and DNA was analyzed by the alkaline elution technique (see methods section). Data, calculated as fraction of DNA retained in the filter after 9 hr of elution, has been normalized with respect to appropriate controls.*

affinity for -SH and S-S groups of proteins or non protein medium components (Valee and Ulmer 1972) and this probably explains why the metal is more toxic in cells exposed in a simple maintenance solution free of sulphydryl containing compounds than in cells treated in a complete medium. As far as chromate is concerned, it should be emphasized that the oxidation state of chromium is critical for the production of geno- and cyto-toxic effects, since trivalent chromium is considerably less potent than the hexavalent form (Levis et al. 1978; Petrilli and De Flora 1977). The difference in activity between hexavalent and trivalent chromium has been attributed to their uptake since $CrO_4^{2-}$ readily crosses the cell membrane by the $SO_4^{2-}$ transport system, whereas the membrane is relatively impermeable to $Cr^{3+}$ (Jenette 1981). Consistently with other Authors (Petrilli and De Flora 1978) we have found that reducing agents in complete media or added to the SGM, inhibit chromate induced geno- and cyto-toxicity. It is likely that this effect results from the

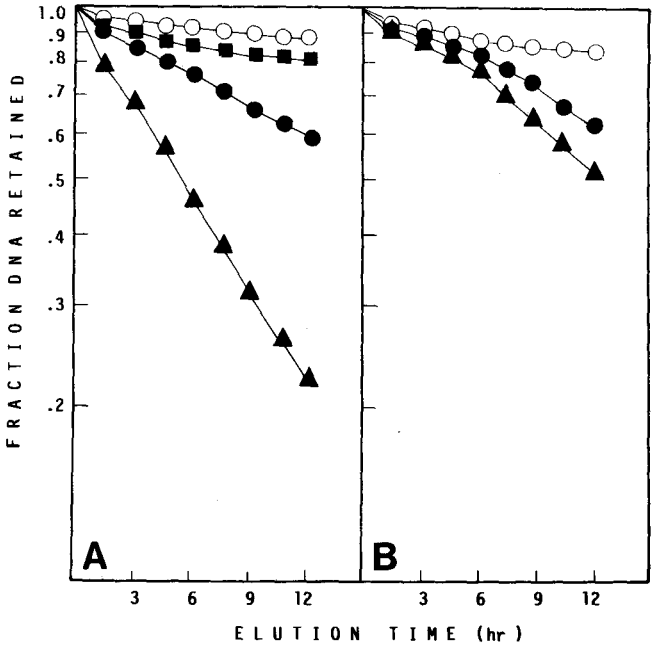


*Figure 2 - Alkaline elution profiles for DNA from cells treated in SGM with $HgCl_2$ (A) or $CaCrO_4$ (B). Treatments for (A) were: O—O untreated control or cells which received 5 mM cysteine or 1 mM GSH; ▲—▲ 10 μM $HgCl_2$, 1 hr; ●—● 10 μM $HgCl_2$ in the presence of 1 mM GSH, 1 hr; □—□ 10 μM $HgCl_2$ in the presence of 5 mM cysteine, 1 hr. Treatments for (B) were: O—O untreated control or cells exposed to 0.5 mM ascorbic acid, 1 hr; ▲—▲ 50 μM $CaCrO_4$, 1 hr; ●—● 50 μM $CaCrO_4$ in the presence of 0.5 mM ascorbic acid, 1 hr.*

formation of the hexavalent chromium reduction product (trivalent chromium) that does not penetrate the cell membrane.
In conclusion, tissue cultures represent a suitable approach for an "in vitro" study of metal carcinogenesis, however, attention must be paid in comparing data from different laboratories or from different experiments in the same laboratory because the type of extracellular medium or even different lots of serum can significantly modify experimental results.

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