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USE OF MAMMALIAN DNA REPAIR-DEFICIENT MUTANTS TO ASSESS THE EFFECTS OF TOXIC METAL COMPOUNDS ON DNA

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Abstract—Wild-type and repair-deficient cell lines (EM9) of Chinese Hamster Ovary cells were utilized to assess cytotoxic responses towards metals that produce lesions in DNA. Alkaline elution studies indicated that both $CaCrO_4$ and $HgCl_2$ induced single-strand breaks in the DNA. $CaCrO_4$ and $HgCl_2$ treatments of intact Chinese hamster ovary cells also caused the induction of DNA cross links. The mutant cells, which are thought to have a defect in the repair polymerase enzyme and therefore exhibit greater sensitivity towards a variety of agents that produce lesions in the DNA such as X-rays and ultraviolet-light, also displayed a greater sensitivity, compared to wild-type cells, towards the cytotoxic response of $HgCl_2$ and $CaCrO_4$. For example, the IC_{50} (concentration producing a 50% growth inhibition) following exposure for 6-hr to $CaCrO_4$ or 1 hr to $HgCl_2$ was 3.4-fold or 1.8- to 3.9-fold greater in wild-type cells compared to repair-deficient cells respectively. Mutant cells compared to wild-type cells were not more sensitive to growth inhibition by agents whose primary site of action was not at the DNA level (i.e. amphotericin B, trifluoroperazine and cycloheximide). The DNA crosslinks induced by exposure to 10 μM $CaCrO_4$ for 6 hr were almost completely repaired in wild-type cells within 24 hr, whereas in similarly exposed mutant cells this lesion was initially more pronounced and was only partially repaired following a 24-hr recovery period in the absence of $CaCrO_4$. The repair of single-strand breaks induced by $CaCrO_4$ was more rapid and similar in both wild-type and mutant cells. Since Hg(II) inhibits repair of single-strand breaks, we could not study repair of this lesion induced by this agent; however, at very low concentrations (1 μM) binding of ^{203}Hg(II) to DNA was greater in the mutant cells compared to the wild-type cells. Following removal of ^{203}Hg(II) from the media, mutant cells generally retained more ^{203}Hg bound to DNA relative to the total ^{203}Hg(II) present in the cell. These results demonstrate that an important toxic action of $CaCrO_4$ and $HgCl_2$ involves injury to DNA since the concentrations of these metals causing measurable DNA damage were consistent with their respective cytotoxic concentrations and DNA repair-deficient mutants displayed both enhanced cytotoxicity and decreased repair of metal-induced lesions.

Metal compounds of nickel, chromium and arsenic have been implicated in the etiology of human cancer, while a number of other compounds of metals such as Cd(II) and Pb(II) have been demonstrated to induce tumors in experimental animals [1–4]. With the exception of oxidation–reduction reactions, metal compounds undergo little metabolic alteration *in vivo* [5, 6], which makes this group of chemicals an excellent class of carcinogens to study in a cell culture system [4]. While the absence of the complex metabolites observed for organic carcinogens might initially appear to simplify analysis of inorganic compounds, their study is complicated because most metal ions are detached from biological ligands by standard isolation methods. An additional variable is that metal ions of the first transition series may produce reactive oxygen species that have been associated with a number of courses of cellular injury including damage to the DNA or the cell membrane [7, 8].

Several lines of evidence point to the interaction of metals with DNA and the enzymes responsible for its replication and repair. Compounds of nickel and chromium have been shown to induce lesions in DNA, including DNA-protein crosslinks and single-strand breaks [9–12]. $HgCl_2$ has also been demonstrated to cause single-strand breaks in DNA by an X-ray-like mechanism, yet this metal in a number of forms has not been implicated as a carcinogen and there is little evidence to support its mutagenic role [13, 14]. In addition to the production of specific DNA lesions, metal compounds with carcinogenic or mutagenic activity also decrease the fidelity of DNA replication [15]; however, very few metal compounds that are potent carcinogens display any mutagenic activity in either bacterial or mammalian systems [14]. Metal compounds are able to induce

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repair of DNA [16] an effect that has been closely linked to their potential oncogenicity [17].

The identification of critical DNA lesions by direct isolation of metal complexes presents considerable problems in interpretation since many metals may shift their binding sites during DNA isolation procedures. It therefore is essential to utilize methods that monitor lesions in the DNA as a marker of metal interaction with the genetic material. Detection of a DNA lesion in an intact cell requires that the lesion not be recognized or reversed by DNA repair enzymes and occurs at some level or critical time period that exceeds the capacity of the DNA repair system to reverse the lesion. Since the DNA repair system in mammalian cells is complex and not well understood but is central to understanding carcinogenesis of metals, in the present investigation an alternative approach was taken by studying the effects of metal compounds on the growth of cells defective in DNA repair. The assumption was made that, if an agent has an effect on the DNA, then cells with repair defects should be more sensitive compared to wild-type cells possessing normal repair capacity. Our results demonstrate clearly that EM9 cells, which have been characterized as more sensitive to X-ray-induced DNA damage presumably by virtue of a deficient DNA repair system [18], also exhibit at least a 2-fold greater sensitivity of growth inhibition toward $HgCl_2$ and $CaCrO_4$ but do not exhibit greater sensitivity toward agents such as trifluoroperazine, cycloheximide and amphotericin B that do not disrupt cell reproduction primarily by interacting with DNA. Additionally, both the effects of $HgCl_2$-induced injury to the cell membrane and its depletion of reduced glutathione were equivalent in wild-type and mutant cells, further supporting the hypothesis that repair mutants are more sensitive to $HgCl_2$ by virtue of an effect on DNA homeostasis. Finally, examinations of DNA lesions produced by $HgCl_2$ and $CaCrO_4$ and their repair indicated that mutant cells appear to differ from wild-type cells in their ability to contend with repair of DNA crosslinks produced by $CaCrO_4$ and to remove ^{203}Hg that became bound to DNA when cells were treated with noncytotoxic concentrations of $HgCl_2$. Although these differences are not striking, they are consistent with the differences in cytotoxicity and thus formulate support for the hypothesis that metals lead to cell death by producing lesions in the DNA.

MATERIALS AND METHODS

Cell culture techniques. Several lines of Chinese Hamster Ovary (CHO) cells were used for this study. The AA8 parental cell line was used to derive the repair-deficient EM9 cell line [18] from suspension cultures of AA8 cells that were mutagenized with ethylmethane sulfonate. These two lines were obtained from Dr. L. H. Thompson of the Lawrence Livermore Laboratories. Cell maintenance and growth experiments were performed using α-Minima Essential Medium (α-MEM) or McCoy's 5a medium containing 1% antibiotic–antimycotic mixture (Gibco, Inc., Grand Island, NY) and 10% fetal bovine serum (Gibco) that had been heat-inactivated at 56° for 30 min. Cultures were grown as monolayers in an atmosphere of 95% air and 5% CO_2.

Assay methods. Estimates of the inhibition of cell growth by metal compounds were made by plating 10^5 cells in each of six 35 mm wells of a plastic culture dish (Costar). The compounds were applied to the cell monolayers in media containing serum between 4 and 8 hr after the initial plating. Each concentration of metal compound was analyzed in triplicate, and the length of treatment was varied from 1 hr to 3 days. Determinations of cell numbers were performed each day during or after treatment using a Coulter particle counter. Cell growth was also monitored by the incorporation of [^{3}H]thymidine into DNA. After exposure of cells to radiolabel, cells were trypsinized, washed with saline A (0.14 M NaCl, 5 mM KCl, 5 mM dextrose, and 4 mM $NaHCO_3$, pH 7.2), and treated with 10% trichloroacetic acid to precipitate the DNA. The precipitates were collected on 0.45 μm filters (Millipore), and the radioactivity present on each filter was determined. Assessment of the exclusion of Trypan Blue was accomplished with 0.2% dye solution and a standard hemacytometer chamber. Glutathione levels were determined fluorometrically using the dye *o*-phthalaldehyde [19].

Alkaline elution assay. Alkaline elution was carried out by a procedure virtually identical to that previously described [20] to assess the DNA lesions produced by $HgCl_2$ and $CaCrO_4$ and also to monitor the repair of these lesions. Briefly, 10^6 cells were plated in 100 mm dishes and incubated for 24 hr in the presence of 0.02 μCi/ml of [^{14}C]deoxythymidine. The medium was then removed, replaced with 10 ml of medium without isotope, and the cells were incubated at 37° overnight. Following treatment with the metal compounds, cells were removed by trypsinization, and an aliquot containing 8.5×10^5 cells was diluted to 20 ml with ice-cold saline A and deposited onto 25 mm polycarbonate filters (when DNA was assayed for strand breaks) or polyvinylchloride filters (when DNA was assayed for crosslinks). Filters were rinsed with 10 ml of saline A and cells were lysed directly on the filters by passage of 5 ml of 2% sodium dodecyl sulfate (SDS), 0.025 M EDTA, pH 10.1. Cell lysates on the filters were rinsed with 5 ml of 0.02 M EDTA. DNA was eluted with 25 ml of a solution containing 0.025 M EDTA (free acid) plus 2% (final concentration) tetrapropylammonium hydroxide, pH 12.15, at a flow rate of 0.035 ml/min. Fractions of approximately 3 ml were collected and counted in 7 ml of Liquiscint containing 0.7 ml of glacial acetic acid. The filters were digested for 1 hr at 60° in 0.4 ml of 1 N HCl and counted in 7 ml Liquiscint containing 0.4 ml of glacial acetic acid and 2.5 ml of 0.4 N NaOH. The degree and nature of crosslinking in the DNA were assessed by utilizing a test dose of X-rays and/or proteinase K as described [20].

^{203}Hg(II) binding to DNA. CHO cells were grown to a density of $5–8 \times 10^6$ cells in 10 cm dishes and incubated with $^{203}HgCl_2$ for 15–30 min in a salts/glucose maintenance medium [50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), 0.15 M NaCl, 2 mM $CaCl_2$, 5 mM dextrose and 5 mM KCl]. After removal of the cells from the dishes by trypsinization or by scraping, cell number was determined by a Coulter particle counter or by

a hemacytometer, and cellular uptake of $^{203}HgCl_2$ was determined by a Beckman gamma counter in which the counting efficiency was typically 95%. DNA was isolated from the cells by a modification previously described [21]. The cells were lysed in 3 ml of 10 mM Tris–HCl, pH 8.0; 10 mM EDTA; 10 mM NaCl; and 0.5% SDS containing 50 μg/ml proteinase K. The lysate was placed immediately into dialysis against the above buffer but without proteinase K and dialyzed for 20 hr at 20°. The sample was then extracted with chloroform–isoamyl alcohol (24:1), precipitated with ethanol and digested with RNAse A (100 μg/ml) in 40 mM Tris–HCl, pH 7.2; 5 mM EDTA; and 10 mM NaCl. The DNA was collected by ethanol precipitation; typical yields ranged between 4.5 and 6 μg for one million AA8 or EM9 cells. The quantitation of DNA was determined by the absorbance at 260 nm and was also analyzed by reaction with diphenylamine [22]. Occasional contamination of the DNA fraction with RNA occurred as determined indirectly by overestimates of the DNA by A_{260} values compared to values obtained by the diphenylamine reaction; however, complete removal of the RNA fraction by alkaline hydrolysis at this stage of the isolation never resulted in the loss of more than 5% of the $^{205}Hg(II)$ from the DNA fraction. No protein could be detected in the DNA fraction by the micro-Biorad Assay.

Experiments were performed to determine the amount of exchange between ligand $^{203}Hg(II)$ complexed with cellular ligands and DNA that might occur after lysis of the cells. Complexes of $^{203}Hg(II)$ with cellular ligands were isolated by sonication of cells that had been treated with 2.5 or 5 μM $^{203}HgCl_2$ (*vide supra*) and sedimentation at 3000 *g* to remove cell membranes, organelle structures and the DNA (as chromatin). These complexes were added to lysates of cells that had not been treated with $^{203}HgCl_2$. The amount of $^{203}Hg(II)$ bound to DNA was determined as described above and found to be approximately one-half the amount bound to DNA when measurements were performed on cells that were treated with $^{203}HgCl_2$ as monolayers. These results were used to correct the amount of $^{203}Hg(II)$ bound to DNA attributed to post-lysis exchange.

RESULTS

Alkaline elution analysis of DNA lesions produced by $CaCrO_4$ *and* $HgCl_2$. Figure 1 demonstrates that $CaCrO_4$ was capable of producing both single-strand breaks and DNA crosslinks in intact CHO cells. The extent of crosslinks was greater at 6 hr than at 3 hr as indicated by an enhanced retention of DNA on the filters in $CaCrO_4$-treated cells that had received a test dose of X-rays (Fig. 1, line f) compared with untreated cells exposed to a similar test dose of X-rays alone (Fig. 1, line d). Some strand breaks were observed by the faster elution rate of DNA after the proteins were digested by proteinase K (line e). A relatively high concentration of $CaCrO_4$ (50–100 μM) was required to observe single-strand breaks following 3–6 hr of exposure; however, DNA crosslinks could be detected at considerably lower concentrations of $CaCrO_4$ (10–20 μM) during this same exposure period (not shown). While the types of DNA

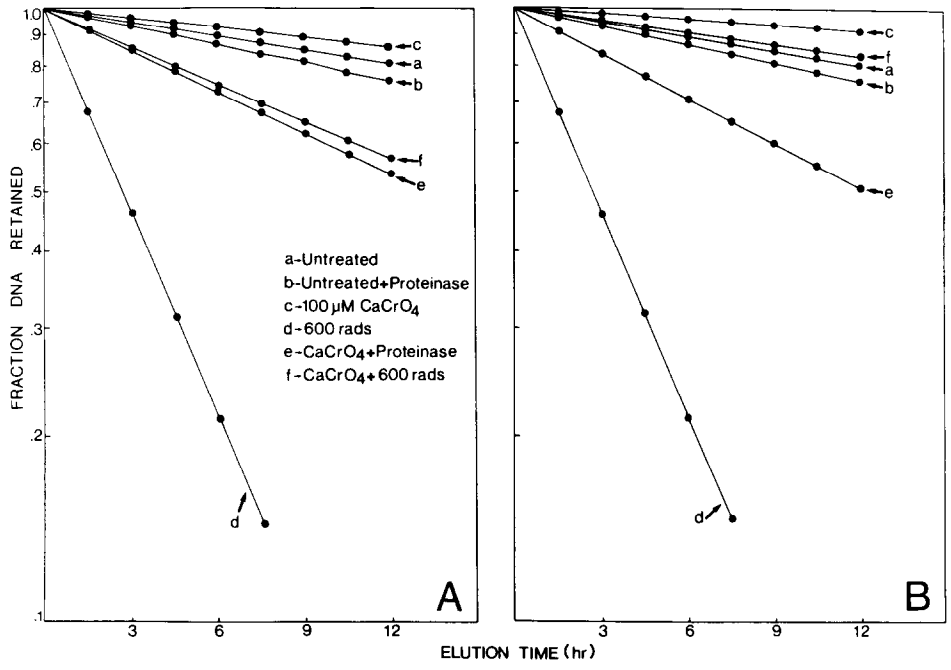

Fig. 1. Induction of DNA strand breaks and DNA crosslinks by hexavalent chromium. AA8 cells were treated for 3 hr (A) or 6 hr (B) with 100 μM $CaCrO_4$ in McCoy's medium and then exposed to X-rays (600 rads) or digested with proteinase K, 0.5 mg/ml, for 60 min or applied and lysed onto polyvinylchloride filters without additional post-incubation treatment, as shown in the figure. DNA was analyzed by alkaline elution as described in Materials and Methods.

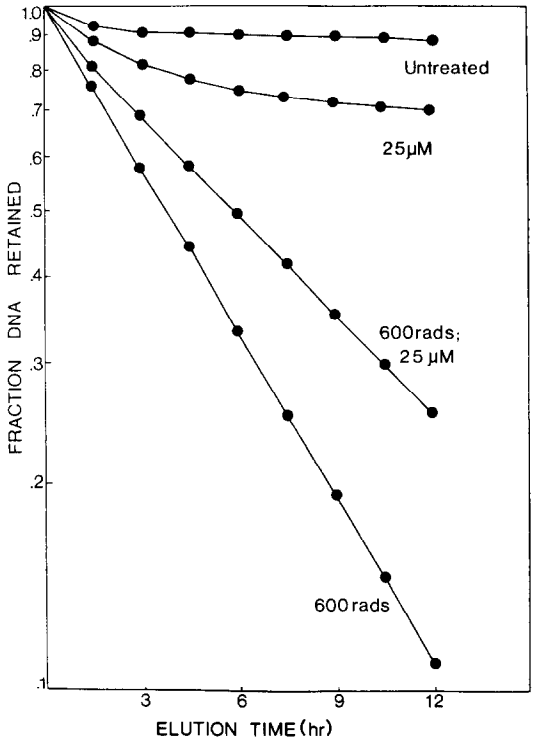

Fig. 2. Analysis of DNA lesions produced by $HgCl_2$. Cells were exposed to 25 μM $HgCl_2$ in McCoy's medium for 6 hr. Selected cultures on ice were exposed to 600 rads prior to elution as shown in the figure. For each of the treatments shown, an equivalent fraction of cells was treated with proteinase K; there was essentially no change in the elution profile. Alkaline elution assay was performed as described in Materials and Methods.

crosslinks were not determined for the conditions used for Fig. 1, preliminary experiments using $CaCrO_4$ treatments in a salts/glucose medium have indicated that both DNA-protein and DNA-DNA crosslinks are produced (unpublished observations). Other investigators have reported previously that the crosslinks produced by $CaCrO_4$ are a mixture of both DNA-DNA and DNA-protein based upon alkaline elution analysis [10, 11]. Figure 2 demonstrates that $HgCl_2$ induced both single-strand breaks and some DNA-DNA crosslinks but no DNA-protein crosslinks, since proteinase K treatment of cell lysates did not alter the elution patterns (see legend to Fig. 2). During early treatment times (1 hr) the predominate lesion observed was single-strand breaks; however, following 5 hr of exposure substantial DNA-DNA crosslinks were also detected (Fig. 2). The strand breaks caused by 25 μM treatment with $HgCl_2$ reduced the DNA retained on the filter to 75% compared to 87% in the untreated cells. The DNA-DNA crosslinks were observed as a slower elution rate following treatment with 25 μM $HgCl_2$ for 5 hr of cells given a test dose of X-rays compared to the test dose given to untreated cells. DNA-protein crosslinks were not present following $HgCl_2$ treatment since the elution rate of DNA was unaffected by the addition of proteinase K to the filters.

Assessment of the effect of $HgCl_2$ *on cell membrane and on glutathione levels in wild-type and EM9 repair-deficient mutants.* During the initial testing of the toxicity of $HgCl_2$, several observations prompted an investigation of the effect of this compound on the cell membrane. At concentrations of 50 μM $HgCl_2$ or greater in α-MEM medium with 15% fetal bovine serum the cells developed a rounded appearance coincident with reduced attachment to the substrate. Additionally, cells treated with this concentration of $HgCl_2$ were hypersensitive to lysis by trypsin as compared to untreated cells or those cells treated with $CaCrO_4$ or $NiCl_2$. These observations, coupled with prior results that Hg(II) enters the cell more rapidly than Cr(VI) or Ni(II) [8, 23], suggested that Hg(II) was having an effect on the cell membrane. The effect of Hg(II) on the cell membrane was therefore investigated, using the Trypan Blue exclusion test. Since we wanted to evaluate the sensitivity of the mutant EM9 cells to $HgCl_2$ with respect to their defective repair system, it was important to differentiate between a toxic response due to membrane damage and a toxic response caused by DNA damage. If membrane damage were a major component of Hg(II) toxicity, a cell having a defective repair system would not necessarily be expected to have enhanced sensitivity to Hg(II). Experiments to estimate membrane integrity by Trypan Blue exclusion after 1 hr of treatment with $HgCl_2$ indicated that significant membrane damage did not occur until a concentration of 200–300 μM was reached, at which point the Trypan Blue viability was reduced from 90 to 4%. These concentrations were in great excess of what was required to affect growth in AA8 cells; a concentration of 75 μM caused a reduction of growth to 60% of that of an untreated culture during a similar time period. The possibility of a delay in the appearance of membrane damage at lower concentrations was examined at 4 and 18 hr after removal of the $HgCl_2$, but no significant membrane damage was exhibited at these later times at concentrations severely affecting growth (i.e. 100–500 μM), indicating that the growth inhibition at these concentrations was most likely not due to a major effect on the membrane, although minor effects could not be eliminated with the Trypan Blue techniques. Membrane damage by $HgCl_2$ leading to significant amounts of cell death appears to occur immediately and precipitously at very high concentrations of $HgCl_2$. The concentration at which this occurs varied, and this variation may have been due to the presence of amino acids in the serum or medium since the concentration of these agents can alter significantly the amount of $HgCl_2$ that interacts with cells [24]. While Hg(II) did have an effect on cell membranes, it was not observed either at low concentrations where significant growth inhibition occurred or in experiments that did not require trypsinization of the cells. Additionally, there was no difference in Trypan Blue exclusion in the wild-type or EM9 cells exposed to $HgCl_2$. These observations led to the elimination of plating efficiency as a measure of sensitivity to $HgCl_2$ in the mutant and wild-type cell lines since immediate trypsinization after metal treatment would have been required and it was difficult to control for the same level of trypsin action

on cells in replicate experiments because of self-digestion of trypsin solutions with time.

Another factor capable of producing a difference in the sensitivity of EM9 cells to $HgCl_2$ would be a low basal level of reduced glutathione. If effects on DNA are important to Hg(II) toxicity, cellular levels of glutathione could alter the extent of this reaction or other injurious reactions since glutathione can react with Hg(II) [23]. The basal levels of glutathione ranged from 8.14 to 7.93 nmoles/mg protein in the wild-type and EM9 cells. When treated with a range of $HgCl_2$ concentrations, the depletion of reduced glutathione was similar in both cell lines, e.g. 150 μM $HgCl_2$ exposure for 1 hr reduced glutathione to 6.83 nmoles/mg protein in AA8 cells and to 6.70 nmoles/mg protein in EM9 cells. Thus, enhanced sensitivity of EM9 cells to $HgCl_2$ cannot be explained on this basis. In the course of these experiments, however, it was observed that reduced glutathione depletion by $HgCl_2$ depended upon the medium used. Medium containing high levels of amino acids that bind Hg(II), such as cysteine, caused cells to be more resistant than those containing lower levels of metal-binding amino acids. These findings are attributed to the inhibition of uptake of $HgCl_2$ into cells, which has been described previously [24], and possibly also to an increased synthesis of glutathione in media rich in cysteine. It was established that $HgCl_2$ was approximately ten times more cytotoxic in a salts/glucose medium than in McCoy's medium and three times more cytotoxic in the latter medium compared to α-MEM medium.

Effect of $HgCl_2$ *and* $CaCrO_4$ *on the growth of wild-type and EM9 repair-deficient mutants.* The hypersensitivity of cell membranes to subsequent trypsin treatment after exposure to $HgCl_2$ prompted the use of a growth assay to study metal effects on cell reproduction. Reproductive capacity of wild-type (AA8) and EM9 cells following treatment with $HgCl_2$ for 1 hr and 3 days was examined at several metal concentrations (Fig. 3). In all experiments, regardless of the treatment time the growth of the EM9

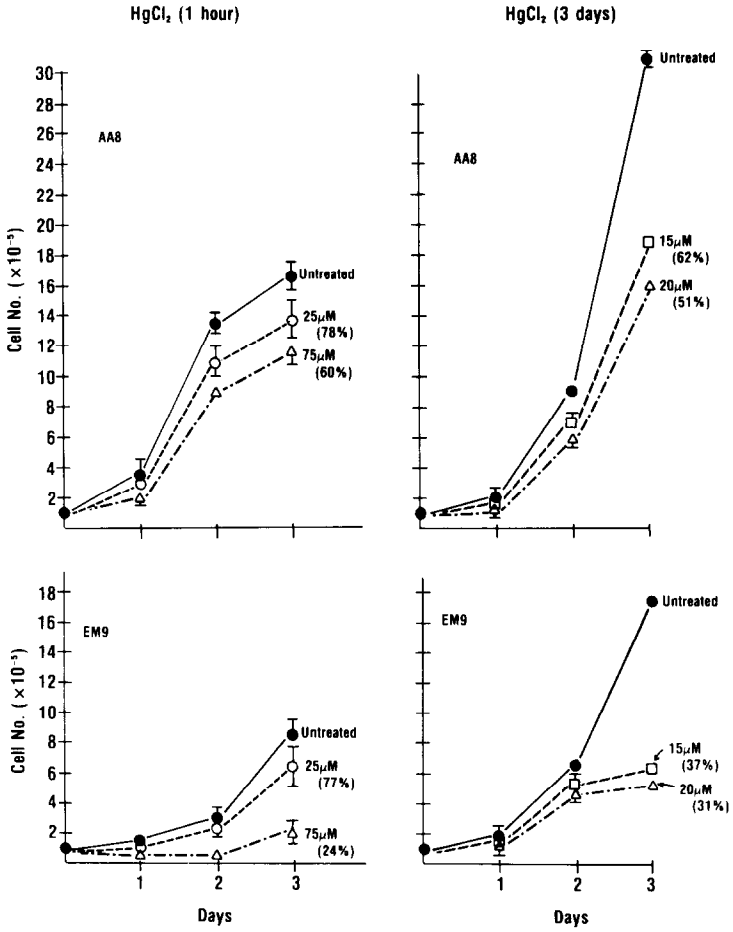

Fig. 3. Effect of $HgCl_2$ on the reproduction of wild-type AA8 and repair-deficient EM9 CHO cells. Cells were plated 4 hr before treatment in α-MEM with $HgCl_2$ for 1 hr or 3 days at the indicated concentration. Cell number was determined by a Coulter particle counter on duplicate samples on days 1, 2 and 3. Each point represents the mean ± S.D. for at least three determinations. Standard errors not shown were smaller than the point plotted in the graph. The numbers in parentheses are the percentages of growth in the treated cultures compared to the untreated cultures on day 3 of the experiment.

Table 1. Effect of $HgCl_2$ and $CaCrO_4$ on growth of wild-type and DNA repair-deficient cells*

Metal salt	Time of treatment	IC_{10}			IC_{20}			IC_{50}		
		AA8†	EM9‡	AA8/EM9§	AA8	EM9	AA8/EM9	AA8	EM9	AA8/EM9
$CaCrO_4$	6 hr	13.0	1.86	(7.0)	15.5	2.70	(5.7)	27.9	8.30	(3.4)
	18 hr	0.81	0.20	(4.1)	1.24	0.36	(3.4)	4.00	2.12	(1.9)
	18 hr	0.85	0.44	(1.9)	1.12	0.63	(1.8)	2.74	1.85	(1.5)
	3 days	1.60	0.62	(2.6)	2.11	0.86	(2.3)	5.10	2.41	(2.1)
	3 days	0.67	0.30	(2.2)	0.93	0.43	(2.2)	2.41	1.24	(1.9)
$HgCl_2$	1 hr	19.8	2.01	(10)	31.2	4.86	(6.4)	154.0	83.7	(1.8)
	1 hr	38.6	12.5	(3.1)	53.7	16.6	(3.2)	148.0	47.0	(3.9)
	3 days	6.70	3.8	(1.8)	8.21	4.2	(2.0)	14.7	7.8	(1.9)
	3 days	4.41	2.76	(1.6)	7.30	3.81	(1.9)	22.6	10.4	(2.2)

* For metal exposures of less than 24 hr, cells were allowed to proliferate in logarithmic phase of growth for 3 days. At the end of this time interval, the number of cells present in each well was determined using a Coulter Counter particle size analyzer. Cells were exposed to the metal compounds continuously for the 3-day exposure condition, and cell number was determined in each well. Each value shown in the table represents the concentration that inhibits cell growth by 10% (IC_{10}), 20% (IC_{20}) and 50% (IC_{50}), determined using three to five different metal concentrations (μM) in each of the experiments shown.

† AA8—Wild-type Chinese Hamster Ovary cells.

‡ EM9—DNA repair-deficient cells sensitive to X-rays.

§ Ratio of concentrations that were growth inhibitory at the indicated percentages.

cells was always inhibited to a greater extent than that of AA8 cells by $HgCl_2$ concentrations that slowed the reproduction of AA8 cells. Differences in the inhibition of reproduction were quantitatively assessed by comparing median inhibitory concentrations (IC_{50} values, Table 1). Estimates of inhibitory concentrations were considered separately for individual experiments since the specific concentration capable of producing a 50% inhibition of growth was subject to some variation, possibly due to differences in the content of amino acid or other metal-binding ligands in the serum. The ratio of the IC_{50} for $HgCl_2$ growth inhibition of AA8 cells compared to that for EM9 cells (AA8/EM9) was always greater than 1.8 for 1-hr or 3-day treatments. This increased level of Hg(II) sensitivity for EM9 cells was approximately the same as the sensitivity difference that was observed for X-rays in these two cell types [18].

Growth inhibition by $CaCrO_4$ was also examined for a short (6 hr) and a long treatment period (3 days). Six hours was chosen for the short treatment period rather than 1 hr as for Hg(II) since the cellular uptake of Cr(VI) was slower than Hg(II). The effects of two concentrations of $CaCrO_4$ for each treatment period that inhibited cell growth of AA8 and EM9 cells are shown in Fig. 4, and the results demonstrate a greater effect of $CaCrO_4$ on the EM9 cells relative to the wild-type cells. Quantitatively the response of the EM9 cells to Cr(VI) was similar to that for Hg(II) with the AA8/EM9 ratio of IC_{50} values falling between 1.5 and 2.1 for the 3-day treatment period. For the 6-hr treatment this ratio increased to 3.4.

When IC_{50} or IC_{20} values were compared for the two cell types, the ratios of these values were higher for the short treatment period than for the 3-day exposure, indicating that EM9 cells were even more sensitive to acute metal treatments than to chronic metal exposure (Table 1). In the case of the IC_{50} values, the AA8/EM9 ratio varied from 3 to 10 for the 1-hr treatment time with $HgCl_2$. A greater variation was expected for IC_{10} values than for IC_{50} values since the concentration range was chosen to optimize for the best estimate of the IC_{50}. Nevertheless, the consistently larger AA8/EM9 ratios of IC_{50} values for acute treatments of both Hg(II) and Cr(VI) argue that acute treatments of relatively high doses overload the repair system to a greater extent than the lower concentrations applied to cells for 3 days. The consistent 2-fold difference in IC_{50} values for both Hg(II) and Cr(VI) and the enhanced sensitivity of EM9 for acute treatments suggest that a direct effect of metals on DNA and also possibly on the enzymes of DNA repair was a critical feature of toxicity of these metals.

Effects of trifluoroperazine, cycloheximide and amphotericin B on growth of wild-type and EM9 repair-deficient mutants. Analysis of the sensitivity of the repair-deficient cells compared to the parental cell toward agents whose primary mechanism of toxicity did not involve DNA interaction was sought to verify that the increased metal sensitivity in EM9 cells was related to a defect in the DNA repair system. Agents chosen for examination were trifluoroperazine which is known to produce toxicity by interacting with calmodulin and thus blocking critical cellular functions mediated by calcium; cyclo-

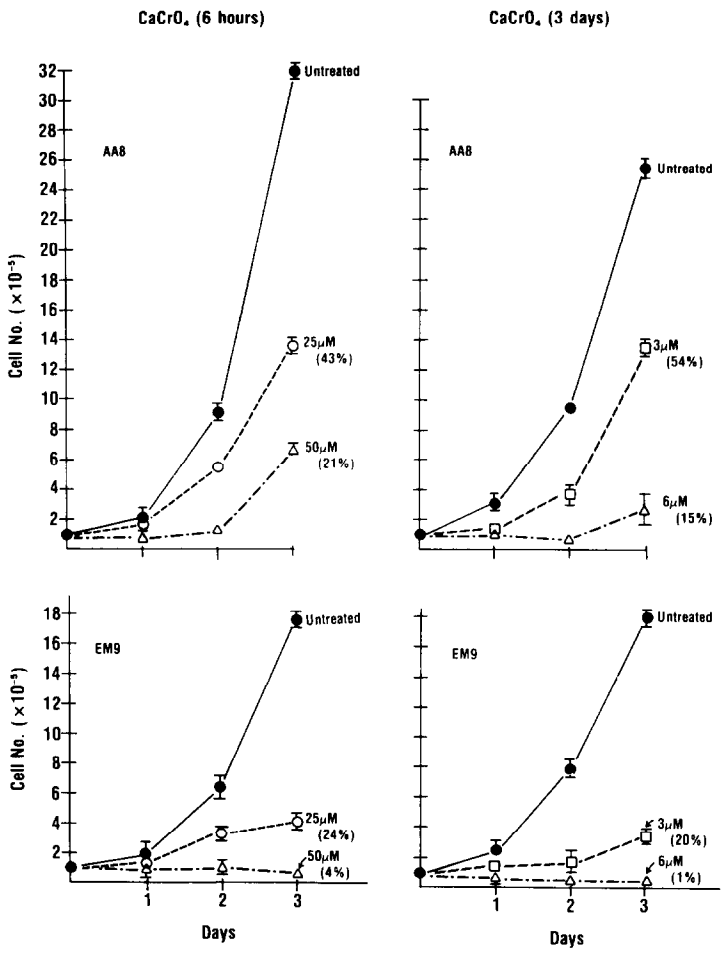

Fig. 4. Effect of $CaCrO_4$ on the reproduction of wild-type and repair-deficient CHO cells. All procedures were performed as described in the legend to Fig. 3 except that acute treatment was for 6 hr instead of 1 hr.

heximide, an inhibitor of protein synthesis; and amphotericin B, a drug that disrupts cell membrane integrity. The effects of these compounds on growth of AA8 and EM9 cells were monitored by the incorporation of [^{3}H]thymidine into DNA. The cells were plated in the same manner as for growth experiments and then treated with the compounds on day 1 or 2 after plating. The treatment time was for 6 hr and the [^{3}H]thymidine was added during the last 2 hr of the treatment. This method of analysis of growth inhibition was comparable to estimates made by determinations of cell numbers. For example, the IC_{50} values of $HgCl_2$ using the [^{3}H]thymidine method were 15.8 and 32.5 μM for EM9 and AA8, respectively, and represent similar differences in sensitivity in the two cell lines as shown by the growth inhibition method. The relative sensitivities of the two strains to trifluoroperazine were examined in three experiments and found to be essentially equal. The average IC_{50} value for the three experiments was 28.0 μM for AA8 and 30.5 μM for EM9. Similar findings were observed with cycloheximide and amphotericin B where in two experiments the ratios of IC_{50} values (AA8/EM9) were 0.92 and 0.87 respectively. Although these results do not prove that EM9 cells were more sensitive to $HgCl_2$ and $CaCrO_4$ because they have a defective DNA repair system, they do indicate that EM9 cells were not generally more sensitive to agents whose primary toxic action does not involve DNA.

Comparison of the effects of $CaCrO_4$ *and* $HgCl_2$ *on the DNA of wild-type and EM9 repair-deficient mutants.* Figure 5 shows the induction of DNA crosslinks by $CaCrO_4$ and examines the repair of these crosslinks 24 hr following wash out of this agent in either wild-type or EM9 repair-deficient mutants. Note that treatment of both EM9 and wild-type cells with 10 μM $CaCrO_4$ for 6 hr caused the development of DNA crosslinks as demonstrated by the slower elution of DNA in these cells given a test dose of X-rays compared with untreated cells also given a test dose of X-rays. While at 100 μM $CaCrO_4$ a significant number of single-strand breaks were produced (Fig. 1), at 10 μM there are no detectable single-strand breaks (data not shown) and thus the damage to DNA is essentially all of the crosslinking type. EM9 cells appeared to have more crosslinking initially than wild-type cells. Twenty-four hours following

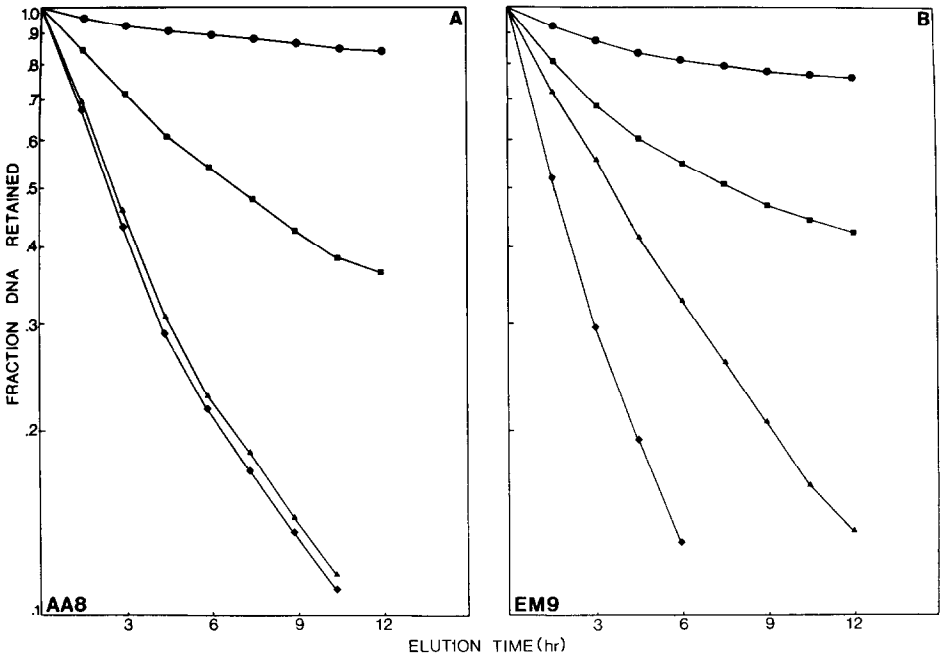

Fig. 5. Alkaline elution analysis of the effect of $CaCrO_4$ in AA8 and EM9 cells. Cells were exposed to 10 μM $CaCrO_4$ for 6 hr only (■—■) or the $CaCrO_4$ was washed out and cells were incubated for 24 hr (▲—▲). The cells treated with $CaCrO_4$ also received a test dose of X-rays and should be compared to the test dose of X-rays performed in untreated cells (◆—◆). Untreated cells were also subjected to alkaline elution analysis without any test dose of X-rays (●—●). Alkaline elution was performed as described in Materials and Methods.

removal of $CaCrO_4$, wild-type cells repaired all the crosslinks whereas in EM9 cells a substantial amount of these crosslinks persisted. Other experiments with alkaline elution have demonstrated that there were no differences in the repair of single-strand breaks by $CaCrO_4$ in either cell type (results not shown).

Since $HgCl_2$ has been shown previously to inhibit repair of its own lesions in the DNA [25], attempts to investigate measurable repair of single-strand breaks induced with $HgCl_2$ were not feasible. However, in Table 2 the binding and removal of Hg(II) from DNA in wild-type and repair-deficient mutants were investigated after treatment with very low concentrations of $^{203}HgCl_2$. The exposure conditions were such that no significant lesions in the DNA could be measured with alkaline elution. EM9 cells accumulated more Hg(II) in the DNA relative to the total Hg(II) concentration present in the cell. While the total cellular loss of $HgCl_2$ from EM9 and wild-type cells was similar following removal of extracellular $HgCl_2$, EM9 cells retained more Hg(II) bound to DNA compared with wild-type cells. The slightly

Table 2. Binding of ^{203}Hg(II) to DNA in EM9 and AA8 cells*

Cell type	Time following exposure (hr)	Ratio of Hg(II) bound to DNA/total cellular Hg(II)
AA8	0	0.020 ± 0.0005
	1	0.010 ± 0.001
	4	0.010 ± 0.001
EM9	0	0.030 ± 0.003†
	1	0.022 ± 0.003†
	4	0.018 ± 0.001†

* Monolayer cultures of each cell line maintained in a minimal salts/glucose medium were treated with various concentrations of $^{203}HgCl_2$ ranging from 0.5 to 2.5 μM for 15 min. The amount of ^{203}Hg(II) taken up into the cells and the amount bound to DNA were determined as described in Materials and Methods. Each number shown in the table represents the ratio of these two values for at least four separate determinations ± S.E.M.

† $P < 0.05$, EM9 cells vs AA8 cells, Student's t-test.

greater accumulation of ^{203}Hg into EM9 cells compared to wild-type cells was dependent upon the exposure concentration. No difference in uptake was seen at 0.5 μM $HgCl_2$, whereas the differences became more striking at higher $HgCl_2$ concentrations (i.e. 2.5 and 5.0 μM). This progressive increase in uptake of ^{203}Hg at higher levels in EM9 cells compared to wild-type cells reflects their enhanced sensitivity to this agent.

DISCUSSION

While metal compounds of both Cr(VI) and Ni(II) have been clearly established as human carcinogens [1], the nature of the critical interactions with DNA leading to the transformed phenotype has not been elucidated. Recent studies have made progress in enumerating the types of DNA lesions produced by different metals and the relative efficiency by which these metals produce the various lesions [9–12, 26]. Both Cr(VI) and Ni(II) are very effective in producing DNA-protein crosslinks based upon alkaline elution analysis [26]. Both of these agents also produce single-strand breaks in the DNA which may be secondary to induction of repair synthesis triggered by DNA crosslinks [25]. Studies with repair-deficient cells were initiated to provide a more sensitive measure of the effects of metals on DNA and to substantiate the role of DNA damage and its repair as an essential feature of the cellular effects of toxic and carcinogenic metals. The 2-fold differences in the sensitivities of EM9 cells to both Cr(VI) and Hg(II), compared with the wild-type, indicate that the action of these metals on DNA is a component of their cellular injury. Additionally, differences of 2-fold or greater were observed for IC_{10} values, and these concentrations were either below levels that produce measurable DNA lesions by the most sensitive technique, i.e. alkaline elution, or near the lower limits of detection for these lesions [8].

The EM9 cell line was chosen for examination of its sensitivites to Cr(VI) and Hg(II) since it is more sensitive to X-rays [18]. The primary lesions to DNA after X-ray treatment were single-strand breaks at random loci; this lesion was also a component of the effect of Hg(II) and Cr(VI) on DNA in intact cells (Fig. 2) [8, 10, 11]. EM9 cells also show increased sensitivity to other mutagens [18]. The yield of thioguanine-resistant cells in the EM9 cell line was approximately 2-fold higher than in the AA8 line when mutagenized with the alkylating agents ethylmethane sulfonate or methylmethane sulfonate [18]. His finding coupled with the 7-fold increase in sister chromatid exchanges is compatible with the EM9 cells having a defect in a repair response that is induced by a number of agents that damage DNA, although the nature of the defect is not known. The levels of single-strand breaks examined in wild-type and EM9 cells in response to Hg(II) and Cr(VI) were equivalent (Figs. 1 and 2) [10, 11] and, if these breaks result from repair of DNA crosslinks, then the endonuclease activities appear comparable in the two cell lines. A defective polymerase in the EM9 cells would explain available information regarding the enhanced sensitivities of these cells. EM9 cells do not appear to differ from wild-type cells in sensitivity to agents whose primary site of action is not at the DNA level (i.e. trifluoroperazine, cycloheximide, amphotericin B) although secondary effects may involve DNA replication. However, the mutant cells were more sensitive to $HgCl_2$ and $CaCrO_4$, both of which have been shown to cause DNA lesions. Differences in the amount of the lesions produced by $HgCl_2$ and $CaCrO_4$ were not striking in the two cell types; however, repair of DNA crosslinks appeared to be delayed in EM9 cells compared to wild-type cells. Additionally, Hg(II) binding to DNA was elevated in EM9 cells relative to the total Hg(II) concentration in the cell and remained elevated following removal of extracellular $HgCl_2$. These findings provide some experimental basis for differences in EM9 cells compared to wild-type cells in terms of the DNA effects of $CaCrO_4$ and $HgCl_2$. Use of the repair mutants provides a fast and efficient way to examine whether an agent produces lesions in the DNA, and this allows the screening of a number of agents for potential DNA damaging activity. Additionally, mechanistic studies can also be conducted with these mutants to understand the importance of the repair process in ameliorating DNA damage.

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