Journal of Cellular Biochemistry 57:655–665 (1995)

Long-Term Exposure to Chromium(VI) Oxide Leads to Defects in Sulfate Transport System in Chinese Hamster Ovary Cells

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Abstract Chromium(VI) resistant Chinese hamster ovary (CHO) cell lines were established in this study by exposing parental CHO-K1 cells to sequential increases in CrO_3 concentration. The final concentration of CrO_3 used for selection was 7 μM for Cr7 and 16 μM for Cr16 cells. Cr16-1 was a subclone derived from Cr16 cells. Next, these resistant cells were cultured in media without CrO_3 for more than 6 months. The resistance of these cells to CrO_3 was determined by colony-forming ability following a 24-h treatment. The LD_{50} of CrO_3 for chromium(VI) resistant cells was at least 25-fold higher than that of the parental cells. The cellular growth rate, chromosome number, and the *hprt* mutation frequency of these chromium(VI) resistant cells were quite similar to their parental cells. The glutathione level, glutathione S-transferase, catalase activity, and metallothionine mRNA level in Cr7 and Cr16-1 cells were not significantly different from their parental cells. Furthermore, Cr16-1 cells were as sensitive as CHO-K1 cells to free-radical generating agents, including hydrogen peroxide, nickel chloride, and methanesulfonate methyl ester, and emetine, i.e., a protein synthesis inhibitor. The uptake of chromium(VI) and the remaining amount of this metal in these resistant and the parental cell lines were assayed by atomic absorption spectrophotometry. Experimental results indicated that a vastly smaller amount of CrO_3 entered the resistant cell lines than their parental cells did. A comparison was made of the sulfate uptake abilities of CHO-K1 and chromium(VI) resistant cell lines. These results revealed that the uptake of sulfate anion was substantially reduced in Cr7 and Cr16-1 cells. Extracellular chloride reduced sulfate uptake in CHO-K1 but not in Cr16-1 cells. Therefore, the major causative for chromium(VI) resistance in these resistant cells could possibly be due to the defects in SO_4^{2-}/Cl^- transport system for uptake chromium(VI). © 1995 Wiley-Liss, Inc.

Key words: sulfate uptake, chromium(VI) uptake, GSH, GST, catalase, cytotoxicity

Chromium is an essential trace metal which is necessary for certain physiological functions, e.g., glucose metabolism [Morris et al., 1992]. However, epidemiological evidence has clearly confirmed that over-exposure to chromium prevalently produces allergic dermatitis; ulceration in the skin, mucous membranes, and nasal septum; renal tubular necrosis; as well as increases risks of cancer in the respiratory tract [Langård and Norseth, 1986; IARC, 1990]. The cytotoxic and genotoxic effects of chromium have been widely demonstrated, i.e., cell death, chromosome aberrations, sister chromatid exchanges, cell transformation, and gene mutations [Sen and Costa, 1986; Sen et al., 1987; Briggs and Briggs, 1988; Biedermann and Landolph, 1990; De Flora et al., 1990; Yang et al., 1992]. Chromium compounds enhance the transcription activities of certain genes, decrease the fidelity, and increase the processivity of polymerases during DNA replication [Tkeshelashvili et al., 1980; Hamilton and Wetterhahn, 1989; Snow and Xu, 1991; Manning et al., 1992]. Animals exposed to chromium also exhibit an increased incidence rate of tumors at the site of exposure [Langård and Norseth, 1986].

Both trivalent and hexavalent chromium are stable compounds in nature. However, chromium(VI) compounds are much more effective than chromium(III) compounds in the induction of cytotoxicity and genotoxicity as described above [Langård and Norseth, 1986; De Flora et al., 1990]. Chromium(VI) forms as oxyanion (e.g., CrO_4^{2-}) that mimics physiologically important oxyanions (e.g., SO_4^{2-}) in rapidly entering cells through the anion transport systems, whereas chromium(III) is incapable of entering into cells

Received August 12, 1994; accepted September 14, 1994.

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through membrane transporters [Campbell et al., 1981; Wetterhahn et al., 1989]. Nevertheless, chromium(VI) does not directly interact with DNA in vitro [Wolf et al., 1989]. An important model which accounts for the toxic effects of chromium(VI) is the intracellular reduction of chromium. Once in the cytoplasm of the cells, chromium(VI) is reduced through reactive short-lived chromium intermediates to the ultimate kinetically stable trivalent species [Arslan et al., 1987; Wetterhahn et al., 1989; Aiyar et al., 1991]. The cellular components involved in the reduction of chromium(VI) include ascorbate, glutathione (GSH), cysteine, hydrogen peroxide (H_2O_2), *DT diaphorase*, cytochrome P450 reductases, and the mitochrondial electron transport chain [Wiegand et al., 1984; De Flora et al., 1985; Kortenkamp et al., 1990; Ryberg and Alexander, 1990; Aiyar et al., 1991; Mikalsen et al., 1991; Standeven and Wetterhahn, 1991; Sugiyama et al., 1991]. Upon metabolic reduction, oxidative DNA damage, e.g., single-strand breaks, radical-DNA adducts, chromium-DNA adducts, and chromium-mediated DNA-DNA or DNA-protein crosslinks, are produced [Sugiyama et al., 1986; Shi and Dalal, 1990; Aiyar et al., 1991; Borges et al., 1991; Costa, 1991; Manning et al., 1992]. Although the uptake-reduction model is accepted as a reasonable account for the toxic effects of chromium(VI), which intracellular forms of chromium or byproducts of chromium reduction are the ultimate cytotoxic and/or genotoxic species remain unclear.

Several chromium(VI) resistant bacterial and mammalian cells have already been established. Bacterial resistance to chromium(VI) appears to be due to two different determinants located on chromosome and plasmid through different mechanisms [Cervantes and Sliver, 1992]. In all of these cases, chromium(VI) resistance has resulted from a decreased chromium accumulation in the resistant bacteria. The plasmid chromium(VI) resistance determinants to be a membrane protein and its role in transport remain unknown [Cervantes and Sliver, 1992]. Chromium(VI) resistant Chinese hamster mutants have been selected from high dosages and a single treatment of chromium(VI) [Campbell et al., 1981]. All of these mutants fall into a single complementation group that contains an impaired ability to transport sulfate. In addition, Chinese hamster mutants that are defective in sulfate transport systems have been demonstrated to be resistant to chromium(VI) [Esko et al., 1986]. However, chromium uptake and excretion abilities of the mutants derived from these studies have not yet been examined. Furthermore, the correlation between chromium(VI) resistance and its reduction mechanisms in these chromium resistant mutants have not been explored. We have established chromium(VI) resistant cells derived from Chinese hamster ovary-K1 cells by progressively increasing chromium(VI) oxide (CrO_3) concentrations. The chromium uptake and excretion, and sulfate transport abilities are determined in this study. Several parameters related to the chromium reduction pathways are also examined in our chromium(VI) resistant cells, along with a comparison made with the parental sensitive cells. These parameters include the level of GSH, the activities of glutathione S-transferase (GST) and catalase, and the expression of metallothionine (MT). In addition, we test coresistance to free-radical generating chemicals, i.e., H_2O_2, methanesulfonate methyl ester (MMS), and nickel chloride ($NiCl_2$). Those results suggest that the inability to uptake chromium(VI) through sulfate transport is the major causative for chromium(VI) resistance in our resistant cells. This study also suggests that chronic exposure to chromium(VI) may result in defective membrane anion transport systems.

MATERIALS AND METHODS

Cell Culture

CHO-K1 cells obtained from American Type Culture Collection (Rockville, MD) were grown in a complete medium that consisted of F12/Dulbecco's modified Eagle's medium (DMEM) (1:1; Gibco, Life Technologies Co., Grand Island, NY) supplemented with sodium bicarbonate (0.02% w/v), L-glutamine (0.03% w/v), penicillin (100 U/ml), streptomycin (100 μg/ml), bovine calf serum (5% w/v; Hyclone, Logan, UT) and fetal calf serum (5% w/v; Hyclone). Cell cultures were maintained in exponential growth and incubated at 37°C in a humidified incubator containing 5% CO_2 in air.

Establishment of Chromium(VI)-Resistant Cells

Chromium(VI) resistant CHO-K1 cells were established by progressively increasing the concentrations of CrO_3 (Cat. no. 227; Merck, Darmstadt, Germany) in the culture medium as follows: 1.5, 3, 7, 9, 12, and 16 μM. At each concentration, the cells were subcultured 3

times. Chromium(VI) resistant cell lines Cr7 and Cr16 were established from the survival cells in media containing 7 μM and 16 μM of CrO_3, respectively. These resistant cell lines were then cultured without CrO_3 for more than 6 months. Cr16-1, Cr16-4, and Cr16-9 cell lines were subclones isolated from Cr16 cells.

Cytotoxicity Assay

CrO_3 and chromium(III) chloride hexahydrate ($CrCl_3 \cdot 6H_2O$; Cat. no. 22957-1, Aldrich, Milwaukee, WI) were prepared freshly in MilliQ water to give a concentration of 100 mM and 2.5 M, respectively. Nickel chloride ($NiCl_2$; Cat. no. 6717, Merck) was dissolved in MilliQ water to yield a stock solution of 100 mM. Cells were treated with CrO_3, $CrCl_3 \cdot 6H_2O$, $NiCl_2$ for 24 h in complete media. Methanesulfonate methyl ester (MMS; Cat. no. M-4016, Sigma, St. Louis, MO) was diluted with MilliQ water to produce 100 mM solutions. Cells were exposed to MMS for 1 h in serum-free media. Perhydrol (H_2O_2, 30%; Cat. no. 7210, Merck) was prepared freshly at 20 mM in MilliQ water. Cells were treated with H_2O_2 for 1 h in salts-glucose medium (SGM) that consisted of 50 mM 2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes, pH 7.2), 10 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 5 mM glucose.

The survival of cells was determined by colony-forming ability. Briefly, 1×10^6 cells were plated on a 100-mm Petri dish and incubated for 18 h. Next, the cells were exposed to various concentrations of chemicals in conditions as described above. At the end of the treatment, the cells were washed twice with PBS, trypsinized and replated at 200 cells per 60-mm Petri dish in triplicate, and incubated in complete media for 7 days. The dishes were then fixed with 95% ethanol, stained with a 10% Giemsa solution, and colony numbers were counted for determination of cytotoxicity.

The genetic locus of emetine resistance is closely linked to chromium resistant locus in CHO cells [Campbell and Worton, 1980]. Emetine is a protein synthesis inhibitor. In this study, whether our chromium(VI) resistant cells are coresistant to emetine was determined by exposing 200-10^5 cells to emetine (dissolved in H_2O; Cat. no. E-2375, Sigma) for 7 days in complete media. The cells were fixed and stained as described above to determine colony-forming ability in situ.

Mutagenicity Assay

Cells were examined for 6-thioguanine resistance to detect mutation frequencies in the hypoxanthine (guanine) phosphoribosyltransferase (*hprt*) gene of Cr16-1 and CHO-K1 cells. 6-Thioguanine assay was performed as previously described by Yang et al. [1992].

Determination of Intracellular Glutathione (GSH)

The procedure for GSH determination was described by Cohn and Lyle [1966]. Cells in exponential growth were harvested using a rubber policeman and washed twice with phosphate-buffered saline (PBS). The cell suspension was centrifuged at 1,000 rpm for 10 min at 4°C. The cell pellets were then suspended in 200 μl ice-cold distilled water. One-half of the cell suspension was transferred to a 1.5 ml eppendorf and sonicated 4 times with 15 s per sonication; in addition, 25 μl of 25% metaphosphoric acid (Cat. no. 546, Merck) was added. Following centrifugation at 8,000 rpm for 15 min at 4°C, 40 μl of the clear supernatant was transferred to a tube containing 2 ml distilled water. Next, 0.5 ml of 0.1 M potassium phosphate buffer, pH 8.0, and 0.1 ml of 0.1% O-phthalaldehyde (dissolved in methanol) were added and mixed well. The mixture was maintained at room temperature for 15 to 20 min. The fluorescence (excitation wavelength 350 nm, and emission wavelength 420 nm) was measured with a fluorescent spectrophotometer (Model F-4000, Hitachi Ltd., Tokyo, Japan). The protein concentrations were determined by the bicinchoninic protein assay kit purchased from Pierce (Rockford, IL) using bovine serum albumin as a standard. The absorbance at 562 nm was measured with a spectrophotometer (Model U-2000, Hitachi).

Determination of Glutathione S-transferase (GST) Activity

GST activity was measured by the previous method of Habig et al. [1974] using 1-chloro-2,4-dinitrobenzene (CDNB; Sigma C-6396) and GSH as substrates. The cell pellets, obtained as described in GSH determination, were resuspended in 500 μl of 100 mM potassium phosphate (pH 6.8), and sonicated as described above. The debris was removed by centrifugation at 12,000 rpm for 30 min at 4°C. Assays were performed at room temperature in a 1 ml mixture containing clear cell lysate, 100 mM potas-

sium phosphate-1 mM EDTA (pH 6.5), 20 mM GSH, and 20 mM CDNB. The absorbance at 340 nm was continuously recorded for 2 min with a spectrophotometer (Model U-2000, Hitachi). The protein concentration was determined as described above. The enzyme activity was expressed as nmole/min/mg protein.

Determination of Catalase Activity

Catalase activity was measured as described by Aebi [1984] with minor modifications. Briefly, cells in exponential growth were harvested using a rubber policeman and washed with PBS. The cell suspension was centrifuged at 1,000 rpm for 5 min at 4°C. The cell pellets were then resuspended in 50 mM potassium phosphate buffer, pH 7.0, sonicated and centrifuged as described above. Assay were performed at room temperature in a 1 ml mixture containing clear cell lysate, 200 mM potassium phosphate buffer, pH 7.0, and 10 mM H_2O_2. The decomposition of H_2O_2 was followed directly by a decrease in absorbance at 240 nm. The enzyme activity was expressed as nmole of H_2O_2 decreased/min/mg protein.

Determination of Intracellular Chromium Level

For chromium uptake, 1×10^6 cells were plated on a 100-mm Petri dish and incubated at 37°C overnight. The cells were then exposed to various concentrations of CrO_3 in complete media for 2–6 h. At the end of treatment, the cells were washed six times with 2 ml PBS. In chromium remaining experiments, the cells were exposed to CrO_3 for 4 h, washed as described above, refed with complete media, and incubated for another 5 h. Cells were trypsinized and resuspended in complete media, and the number of cells were determined. The cell suspension was centrifuged at 1,500 rpm for 10 min. The cell pellet was resuspended in 500 μl distilled water, vortexed for 30 s and then treated ultrasonically for 60 s [Wataha et al., 1993]. Total chromium concentration in this solution was analyzed by a polarized Zeeman atomic absorption spectrophotometer (AAS, Hitachi Z-8000) equipped with an autosampler and a graphite furnace. The AAS analytical conditions for chromium were set up as follows: the absorption wavelength at 359.3 nm, the lamp current at 7.5 mA, and the atomizing temperature at 2,900°C.

Sulfate Uptake

A million cells were plated on a 60-mm dish and incubated overnight. The cell number was determined from two replicate dishes immediately before sulfate uptake. Another set of cells was washed twice with warm PBS and maintained at 37°C. One ml of PBS containing 5 μCi $[^{35}S]Na_2SO_4$ (250–1,000 mCi/mmol; NEX-041, New England Nuclear, Boston, MA) and 20 μM or 2.5 mM Na_2SO_4 were added. Uptake of sulfate was stopped by the addition of 3 ml of cold sodium acetate (50 mM, pH 7.2) following exposure for 10, 30, 60 s, 2 min or 20 min. The acetate buffer was quickly aspirated, and the plates were washed six times with 2 ml acetate buffer. Cells were then lysed with a 0.5 ml solution containing 10 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0, and 0.5% SDS. The cell lysate was transferred to Ultrafluor (Packard, Downers Grove, IL) scintillation cocktail. The count per min (cpm) of ^{35}S was determined by liquid scintillation analyzer (Model 1600 TR, Packard). Sulfate uptake rate was expressed as mol/min/10^6 cells.

In experiments in which the effect of extracellular Cl^- on the sulfate uptake was examined, cells were cultured and washed as described, and treated with 1 ml media containing $[^{35}S]Na_2SO_4$ (0.25–12.5 μCi), 0.1–5 mM Na_2SO_4, 1 mM calcium gluconate, 10 mM Tris-Hepes, pH 7.5, and either 150 mM sodium gluconate or 150 mM sodium chloride [Elgavish and Meezan, 1992]. The ratio of SO_4^{2-}:$[^{35}S]SO_4^{2-}$ is 100:1. Sulfate influx was stopped after incubation for 1 min. Cells were washed and lysed, and the amounts of isotope were measured as described above.

RESULTS

Figure 1 shows the growth rates of cells during establishment of chromium(VI) resistance. The population doublings (PDL) of cells that had been exposed to CrO_3 at gradually increasing dosages from 1.5 to 3 μM is nearly the same as that of the untreated parental cells. The growth rate was reduced when the dosages of CrO_3 increased from 3 to 7 μM. Experimental results showed that 7 μM CrO_3 was toxic to Cr3 cells. However, the growth rate of CrO_3 exposed cells achieves the level of untreated cells when the dosages of CrO_3 were stepwisely increases from 9 to 16 μM because the slopes of the growth curves were similar for CrO_3-treated and untreated cells (Fig. 1). Cells that had been

progressively exposed to CrO_3 at dosages up to 7 and 16 μM were then cultured without chromium(VI) for 6 months and defined as Cr7 and Cr16, respectively. Several single colonies, i.e., Cr16-1, Cr16-4, and Cr16-9, derived from Cr16 cell populations were isolated and expanded for further studies. The growth rates of these established cell lines were examined along with a comparison made with CHO-K1 cells. Those results indicated that the growth rates of these chromium(VI) resistant cell lines were the same as the untreated parental cells (data not shown).

The chromosome number of Cr16-1 was similar to that of CHO-K1 cells. More than 75% of the cells contain 20 chromosomes. In addition, the spontaneous mutation frequencies in the *hprt* gene were < 10 per 10^6 viable cells in both Cr16-1 and CHO-K1 cells. Those results indicated that a minor genetic alteration might occur during establishment of chromium(VI)-resistant phenotype. The recessive chromium resistant locus has been demonstrated to be linked to the autosomal emetine resistant locus in chromosome 2q in CHO cells [Campbell and Worton, 1980]. These two genetic markers are cosegregated in a frequency of 42/48 [Campbell and Worton, 1980]. Next, the cytotoxic effect of emetine was analyzed, indicating that Cr16-1 cells are as sensitive to emetine as CHO-K1 cells, i.e., the LD_{50} (dosage that reduces the relative colony-forming ability to 50%) was ~0.015 μM for both cell lines that had been exposed for 1 week. Therefore, the genetic changes of our chromium(VI) resistant cells may differ from that derived from pre-existing chromium resistant cells isolated from lethal dosages of chromium [Campbell et al., 1981].

Relative resistance to CrO_3 for Cr7, Cr16, and Cr16-1 compared with CHO-K1 is shown in Figure 2. Cr7, Cr16, and Cr16-1 cells were markedly more resistant to the cytotoxic effect of CrO_3. The values of LD_{50} for these resistant cell lines were more than 25-fold of that for CHO-K1 cells when they were treated with CrO_3 for 24 h. One factor that could possibly affect the cytotoxicity of chromium(VI) is the generation of free radicals or reactive oxygen species (ROS) during chromium reduction in cells [Shi and Dalal, 1990; Aiyar et al., 1991]. Therefore, we examined whether Cr16-1 cells were coresistant to chemicals in which their cytotoxicities were mediated by the formation of ROS. The LD_{50} of Cr16-1 cells that have been exposed to H_2O_2, and MMS for 1 h, and $NiCl_2$ for 24 h were 0.15

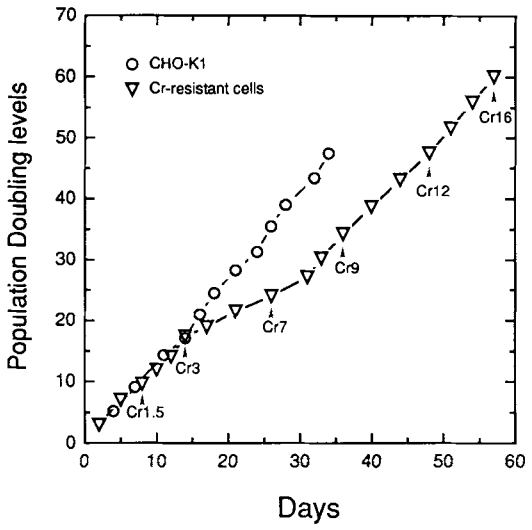

Fig. 1. Cumulative population doublings (PDL) of CrO_3-treated and untreated CHO-K1 cells. Cells were maintained at exponential growth and serially subcultured in complete media, with medium changing every 2–3 days. When the cells reached confluence, they were subcultured at ~1:16 cell dilutions. The untreated CHO-K1 cells (open circles) were subcultured for 34 days. CrO_3-treated cells were continuously subcultured for 57 days with progressive increases in dosage of CrO_3 during establishment of the chromium-resistant cells (open triangles). The total cell number was counted in each subculture to calculate the cumulative PDL. Each symbol represents one subculture, and the PDL between two subcultures N and N + 1 was determined as log (the total cell number of subculture N + 1/the total cell number of subculture N)/log 2.

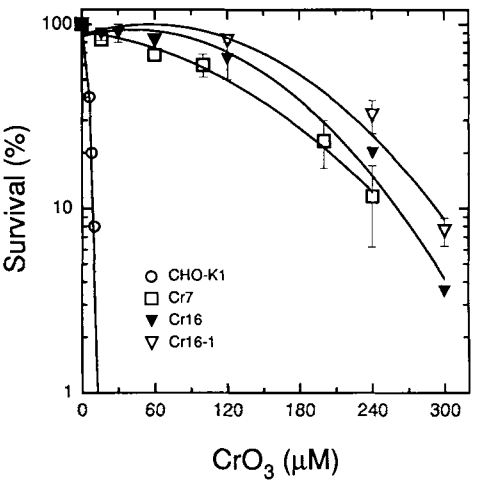

Fig. 2. Cytotoxicity induced by CrO_3 in CHO-K1 and chromium(VI) resistant cell lines. Cells were treated with CrO_3 for 24 h and assayed as described in Materials and Methods. Open circles, squares, triangles, and filled triangles represent the data obtained from CHO-K1, Cr7, Cr16-1, and Cr16, respectively. The results were averaged from at least 4 independent experiments and the bars represent the standard error of the population.

TABLE I. Intracellular Glutathione Level, and the Glutathione S-Transferase and Catalase Activities

Cell lines	Treatment[a]	nmol/mg[b] protein GSH	nmol/min/mg[b] protein GST	Catalase
Cr7	—	14.4 ± 3 (5)	306.2 ± 35 (2)	ND[b]
Cr16	—	14.9 ± 4 (5)	351.3 ± 54 (3)	ND
Cr16-4	—	13.1 ± 3 (5)	299.6 ± 55 (3)	ND
Cr16-9	—	14.5 ± 3 (5)	337.8 ± 38 (3)	ND
Cr16-1	—	16.7 ± 4 (5)	254.3 ± 31 (3)	47.9 ± 6 (2)
	500 μM, 4 h	10.6 ± 1 (3)	254.4 ± 23 (2)	ND
	500 μM, 8 h	10.4 ± 2 (3)	242.0 ± 20 (2)	ND
CHO-K1	—	13.5 ± 3 (5)	339.7 ± 46 (3)	55.7 ± 9 (2)
	50 μM, 4 h	10.9 ± 1 (2)	322.4 ± 35 (2)	ND
	50 μM, 8 h	12.0 ± 3 (3)	344.3 ± 41 (2)	ND

[a]CrO_3 concentration and exposure time.
[b]Each value is the mean ± SD. Parenthesis, number of experiments; ND, not determined.

mM, 1 mM and 0.25 mM, respectively. These results were approximately the same as that of CHO-K1 cells. In addition, the resistance to chromium(III) chloride for CHO-K1 cells was unaltered in Cr16-1 cells; the LD50 of CHO-K1 and Cr16-1 cells that had been treated for 24 h were 13.3 mM and 11.5 mM, respectively.

GSH is known to participate in the cellular metabolic reduction pathways of chromium [Wiegand et al., 1984; Aiyar et al., 1991]. Some antioxidant enzymes, e.g., GST and catalase, have been known to play an essential role in the defense systems of ROS-mediated cytotoxicity [Lee et al., 1989; Sugiyama et al., 1993]. We assayed the level of GSH and the activities of GST and catalase in these chromium(VI) resistant cell lines. No significant difference of the levels of these intracellular antioxidants was observed in CHO-K1 and these chromium(VI) resistant cell lines (Table I). In addition, nearly 30% of GSH levels were depleted as compared with their untreated counterparts when Cr16-1 and CHO-K1 cells were treated with CrO_3 at dosages that caused equal cytotoxicity, i.e., 500 μM and 50 μM for 4 h, respectively (Table I).

Metallothionine (MT) is rather crucial for the resistance to metals such as cadmium [Kagi and Schäffer, 1988]. In this study, we examined the induced and basal level of MT mRNA in Cr16-1 and CHO-K1 cells that have been treated with or without very low cytotoxic dosages of cadmium or CrO_3. Those results indicated that an insignificant amount of MT mRNA was detected in both cell lines under the conditions that a positive control cell line, i.e., cadmium resistant cells, had a high level MT mRNA induction (data not shown). Therefore, MT may not be involved in chromium(VI) resistance.

Next, the level of chromium was determined in those cell lines that have been treated with various dosages of CrO_3 for 2–6 h by AAS. As shown in Figure 3, chromium uptake in CHO-K1 exhibited dosage- and time-dependent increases, whereas chromium uptake was substantially reduced in Cr7 and Cr16-1 cells. Whether a correlation occurred between the cytotoxicity and chromium uptake in these cell lines was next determined. Cells were exposed to CrO_3 at various dosages that reduce the relative colony-forming ability to ~90% to ~20%. The uptake of chromium was determined by AAS as described. Those results revealed that uptake of chromium in Cr16-1 and Cr7 cells occurred when these cells were exposed to high cytotoxic dosages of CrO_3 (Table II). Cytotoxicity was directly proportional with the intracellular level of chromium in both chromium(VI) resistant and sensitive cells (Fig. 4). These results indicated that no difference in chromium uptake was observed in chromium(VI) resistant mutants as compared with their parental CHO-K1 cells at the same cytotoxic dosages. The reduced uptake of chromium in these resistant cell lines might have been due to either a faster excretion rate or a slower uptake rate. We measured the excretion rate of chromium in cells that had been treated as described above, and measured the levels remaining after incubation without CrO_3 for 5 h. The results revealed that a slight amount of chromium was excreted from both chromium(VI) sensitive and resistant cells 5 h after treatment (Table II). Therefore, the difference

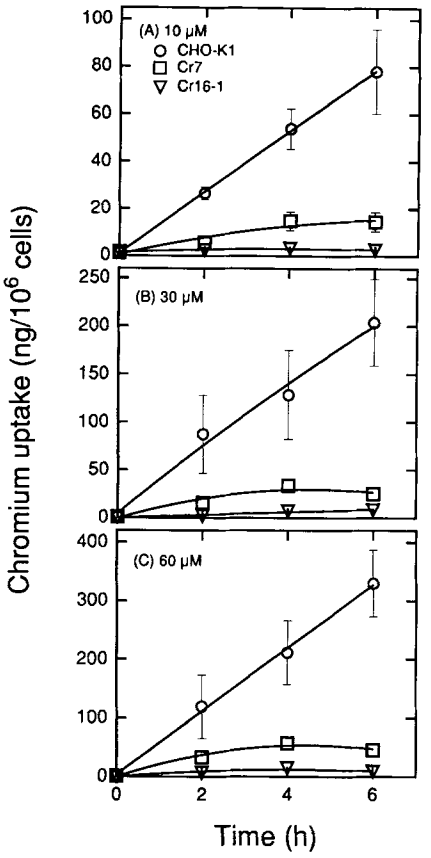

Fig. 3. The relationship of the chromium uptake in CHO-K1 (open circles), Cr7 (open squares), and Cr16-1 (open triangles). Cells were treated with 10 μM (A), 30 μM (B), and 60 μM (C) of CrO_3 for 2–6 h. The amount of chromium per 10^6 cells was measured as described in Materials and Methods. The results were averaged from at least 2 independent experiments.

between chromium(VI) resistant and sensitive cells was that the former had a markedly slower chromium uptake than the latter. These results indicated that plasma membrane barrier was the major reason for chromium(VI) resistance.

Chromium(VI) resistance mammalian cells have been known to be associated with the failure of sulfate uptake [Campbell et al., 1981]. In addition, CHO mutants deficient in sulfate transport systems have been shown to be resistant to the cytotoxic effect of chromium(VI) [Esko et al., 1986]. As shown in Figure 5, sulfate efficiently entered into CHO-K1 cells in solution containing both low and high concentrations of sulfate. This phenomenon has not been observed in both Cr7 and Cr16-1 cells. The uptake of sulfate was slightly greater when Cr7 cells were incubated in a low sulfate solution for 20 min or in a high sulfate solution for 1 min. Cr16-1 cells were incapable of taking up sulfate in a low sulfate

TABLE II. Cytotoxicity, Chromium Uptake, and Excretion in CHO-K1, Cr7, and Cr16-1 Cells

Cell lines	CrO_3 (μM)	Survival (%)[a]	Chromium[b] Uptake (ng/10^6 cells)	Chromium[b] Remaining (%)
CHO-K1	16	89.1	83 ± 1 (2)	80 ± 9 (2)
	60	65.0	211 ± 77 (2)	121 ± 58 (2)
	100	26.9	465 ± 7 (2)	111 ± 9 (2)
Cr7	60	95.0	71 ± 38 (4)	56 ± 24 (4)
	120	86.8	84 ± 44 (2)	83 ± 18 (2)
	1,000	42.8	385 ± 163 (2)	120 ± 30 (2)
Cr16-1	60	99.0	14 ± 10 (2)	69 ± 64 (2)
	300	95.1	81 ± 19 (2)	75 ± 42 (2)
	1,500	21.7	490 ± 28 (2)	94 ± 0 (2)

[a]Data obtained from the average of 2–4 experiments.
[b]Cells were treated with CrO_3 for 4 h and the amount of chromium in the cells was measured immediately (uptake experiments) and following a 5 h-incubation in chromium-free media (remaining experiments). Chromium remaining percentage was determined as the amount of chromium measured at 5 h after a 4 h-treatment divided by the amount of chromium measured immediately after a 4 h-treatment. Parenthesis, number of experiments.

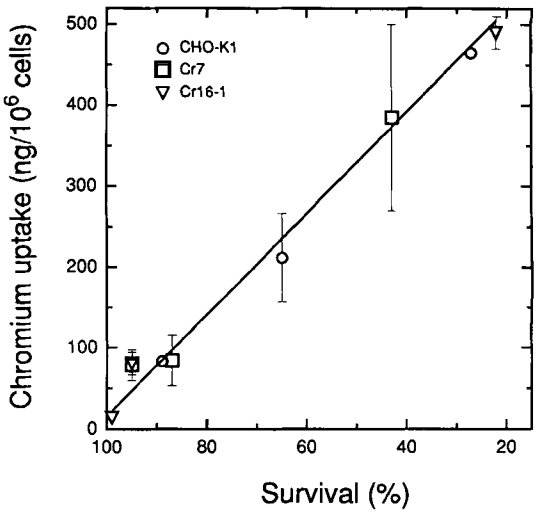

Fig. 4. The correlation between cytotoxicity and chromium uptake. Cells (CHO-K1, open circles; Cr7, open squares; Cr16-1, open triangles) were treated with various dosages of CrO_3 for 4 h and then washed as described. The chromium levels in cells that were assayed immediately after treatment were plotted as a function of percent colony forming ability. Simple linear regression analysis was performed to detect the correlation between cytotoxicity and chromium accumulation.

solution, whereas they took up a low level of sulfate at a constant rate in a high sulfate solution (Fig. 5). In addition, extracellular chloride (150 mM) reduced the efficiency of sulfate uptake in CHO-K1 but not in Cr16-1 cells (Fig. 6).

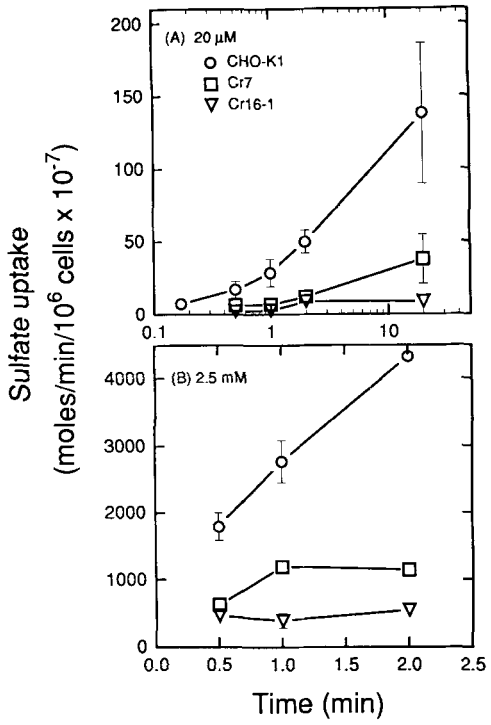

Fig. 5. Sulfate uptake in CHO-K1 (open circles), and Cr(VI) resistant cell lines Cr7 (open squares) and Cr16-1 (open triangles). Cells were treated with 0.02 mM (A) and 2.5 mM (B) of sodium sulfate that containing 5 μCi of $[^{35}S]Na_2SO_4$ for 0.5, 1, 2, or 20 min as described. The amount of sulfate uptake per 10^6 cells was measured by liquid scintillation analysis. The results were averaged from at least 2 independent experiments.

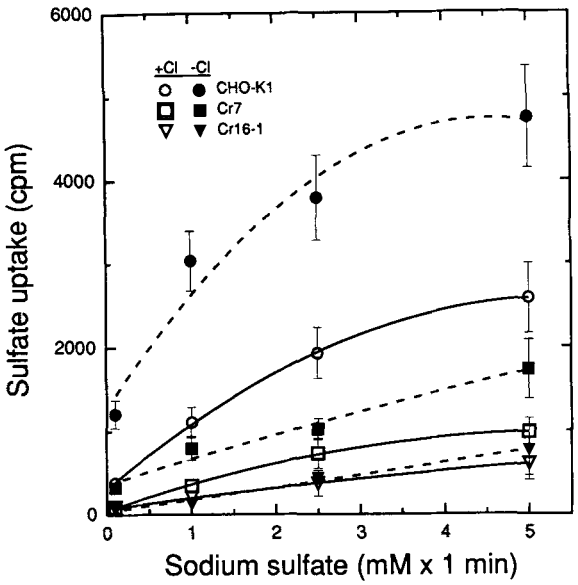

Fig. 6. Effect of extracellular chloride on the efficiency of sulfate uptake. Cells (CHO-K1, circles; Cr7, squares; Cr16-1, triangles) were exposed to media containing various concentrations of SO_4^{2-} (the ratio of $SO_4^{2-}/[^{35}S]SO_4^{2-}$ is 100) with (filled symbols) or without (open symbols) 150 mM sodium chloride for 1 min. The amount of sulfate uptake per 10^6 cells was determined as described. The results were averaged from 4 independent experiments.

DISCUSSION

We have established chromium(VI) resistant CHO cells through progressively increasing dosages of CrO_3. The LD_{50} of chromium(VI) resistant cells are approximately 25-fold higher than that of the parental cells. An extensive characterization of these chromium(VI) resistant cell lines showed that no difference as compared with the parental cells in the growth rates; chromosome number; *hprt* mutation frequency; the intracellular GSH level; GST and catalase activity; the level of MT mRNA; and sensitivity to H_2O_2, MMS, $NiCl_2$, $CrCl_3$, and emetine. The major difference is that the uptake of sulfate and chromium(VI) anions was substantially reduced in the Cr7 and Cr16-1 cell lines. Sulfate uptake of Cr16-1 was independent of the incubation time, i.e., little uptake in a low sulfate buffer (20 μM) and a constant low level of sulfate uptake in a high sulfate concentration (2.5 mM). This finding indicates that a rapid uptake of sulfate through anion transport system was abolished and uptake of sulfate may occur slowly through a different pathway when it was supplied at a high sulfate concentration in Cr16-1 cells. The relatively higher resistance to CrO_3 and uptake of sulfate and CrO_3 in Cr7 cells as compared with Cr16-1 cells might have been due to the fact that Cr7 cells consist of a mixture of functional and nonfunctional cells, whereas Cr16-1 cells are derived from a single clone.

The linear relationship between CrO_3 induced cytotoxicity and chromium uptake in Cr7, Cr16-1, and CHO-K1 cells suggested that once chromium enters the cells, the intracellular levels of chromium would be directly related to cell death (Fig. 4). These results are consistent with the "uptake and reduction" model to account for chromium(VI)-induced cytotoxicity [Arslan et al., 1987; Wetterhahn et al., 1989; Aiyar et al., 1991]. Chromium(VI) can not directly interact with cellular macromolecules. It is metabolited to reactive froms through reduction pathways in cells. Several parameters involved in the reduction pathways include the GSH level and GST and catalase activities which are similar in both chromium(VI) resistant and the parental CHO-K1 cells. In addition, the sensitivities to ROS-generating agents are the same in the chromium(VI) resistant and sensitive cells. Our ob-

servations suggest that the uptake of extracellular chromium(VI) determines cytotoxicity in the parental cells while the cellular ability for intracellular reduction of chromium(VI) to its reactive species may remain intact in our chromium(VI) resistant cells.

Exposure of cells to chromium(VI) have been found to produce a variety of types of DNA damage, i.e., DNA single-strand breaks, DNA-protein crosslinks, and DNA-chromium adducts [Sugiyama et al., 1986; Shi and Dalal, 1990; Borges et al., 1991; Aiyar et al., 1991; Costa, 1991; Manning et al., 1992]. The most persistent form of damage was DNA-chromium adducts, which remained unrepaired for at least 32 h after chromium(VI) treatment in CHO cells [Manning et al., 1992]. These unrepaired lesions could interfere with the expression of inducible genes [Hamilton and Wetterhahn, 1989; Manning et al., 1992] or result in mutations [Yang et al., 1992]. Unlike the selection of pre-existed chromium(VI) resistant mutants by the treatment of a single and lethal dosage of chromium(VI) [Campbell et al., 1981], mutation events may occur during our experimental process. One may obtain chromium resistant cells that also posses altered cellular defense systems. However, analysis of two other subclones, Cr16-4 and Cr16-9, revealed that these cells have a similar characterization as Cr16-1 (Table II and unpublished data). Therefore, obtaining a mutant with altered cellular defense systems under our experimental conditions may be relatively difficult.

CHO cells resistant to ROS generating chemicals, i.e., H_2O_2, and oxyanion, i.e., AsO_2^-, have elevated levels of GSH/catalase [Sugiyama et al., 1993] and GSH/GST [Lee et al., 1989], respectively. These H_2O_2 and arsenic resistant cells are coresistant to some metals but not to chromium(VI), possibly due to the fact that their sulfate transport system for chromium uptake remains functional.

CHO mutants deficient in sulfate uptake have been demonstrated to occur through an SO_4^{2-}/Cl^- anion transport system and the cells are resistant to chromium(VI) [Esko et al., 1986]. This anion exchanger may regulate the intracellular pH in CHO cells [Elgavish et al., 1988]. The function of this anion transport system in CHO cells appears to be similar to the band 3 anion enchanger in erythrocytes [Elgavish et al., 1988]. Our results showed that extracellular Cl^- reduced sulfate influx in the parental CHO-K1 but had no effect in Cr16-1 cells. This suggests that the activity of the anion transporter in chromium resistant cells may be similar to that described by Elgavish et al. [1988]. Anion exchangers with similar activities have been demonstrated in many types of cells, including lung and skin fibroblasts, and epithelial plasma membranes, such as renal brush border membranes and tracheal apical membranes [Elgavish et al., 1988]. Treatment of rats with chromium(VI) causes a depression of glucose transport by renal brush border membrane vesicles [Ansari et al., 1991]. This effect has been shown to be related to the production of acute tubular necrosis (clinically evident as a marked reduction in urine flow rate) and irreversible low molecular weight proteinuria in the kidneys in chromium workers [Wedeen and Qian, 1991]. Our current study provides an in vitro example that long-term exposure of cells to low dosages of chromium(VI) could induce defect in a membrane sulfate anion transport system. In addition, the cDNA of the erythroid band 3 anion transport protein has been isolated [Kopito and Lodish, 1985]. The cDNA should allow one to investigate the relationship of the erythroid band 3 gene to the sulfate anion transport gene in our chromium(VI) resistant cells.

Cystic fibrosis (CF) is another well-known example of human disease in which defects are related to the anion transport system [Boat et al., 1989; Drumm et al., 1990; Anderson et al., 1991]. The predominant clinical manifestations of this disease are related to the abnormal electrolyte composition of epithelial secretions and mucus secretions, thereby resulting in obstruction and infection within the lung, intestine, pancreas, biliary tract, salivary glands, and genitourinary tract [Boat et al., 1989]. CF is known to be caused by mutations in the gene coding for cystic fibrosis transmembrane conductance regulator [Boat et al., 1989; Drumm et al., 1990; Anderson et al., 1991]. Alternations in the Cl^- conductance and sulfate transport abilities have been shown in CF cells [Elgavish and Meezan, 1992]. Finding out whether CF cells are resistant to chromium(VI) would be a worthwhile topic of further study. In addition, a cohort or case-control investigation from this perspective could also be a relevant subject matter.

ACKNOWLEDGMENTS

The authors thank Dr. T.C. Lee for his advice, providing CHO-K1 cells, and use of the atomic

absorption spectrophotometry. Dr. T.C. Wang is also appreciated for his advice. The excellent technical advice and support from Dr. H. Huang and Dr. L.Y. Lin are gratefully acknowledged. The authors also thank the National Science Council, Republic of China, for financial support of this manuscript under contract NSC82-0421-B-007-017-Z.

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