Environmental Health Perspectives
Vol. 92, pp. 63–70, 1991

Effects of Vitamins on Chromium(VI)-Induced Damage

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The effects of vitamin E and vitamin B_2 on DNA damage and cellular reduction of chromium(VI) were investigated using Chinese hamster V-79 cells. Pretreatment with α-tocopherol succinate (vitamin E) resulted in a decrease of DNA single-strand breaks produced by Na_2CrO_4, while similar treatment with riboflavin (vitamin B_2) enhanced levels of DNA breaks. In contrast, levels of DNA-protein crosslinks induced by Na_2CrO_4 were unaffected by these vitamins. Electron spin resonance (ESR) studies showed that incubation of cells with Na_2CrO_4 resulted in the formation of both chromium(V) and chromium(III) complexes, and cellular pretreatment with vitamin E reduced the level of the chromium(V) complex, whereas pretreatment with vitamin B_2 enhanced the level of this intermediate. However, the levels of chromium(III) were unchanged by these vitamins. The uptake of chromate was not affected by vitamin E or vitamin B_2, nor were the levels of glutathione or glutathione reductase activity, which are both capable of reducing chromate. ESR studies demonstrated that a chromium(V) species was formed by the reaction of Na_2CrO_4 with vitamin B_2 and that vitamin B_2 enhanced the formation of hydroxyl radicals during the reaction of Na_2CrO_4 and hydrogen peroxide. Treatment of cells with Na_2CrO_4 resulted in a decrease of glutathione reductase activity, and pretreatment with vitamin E restored the enzyme activity suppressed by this metal. However, pretreatment with vitamin B_2 enhanced the inhibition of this enzyme by Na_2CrO_4. Using a colony-forming assay, pretreatment with vitamin E dramatically decreased the cytotoxicity of Na_2CrO_4, while pretreatment with vitamin B_2 was found to result in only a decrease of cell lethality of this metal. These results indicate that vitamin E and vitamin B_2 are capable of altering the biological effects of carcinogenic chromium(VI) compounds, possibly through their abilities to modify levels of chromium (V) in cells. The results also suggest that chromate-induced cytotoxicity may not be directly correlated with the genotoxic effects of this metal. The importance of the role of vitamins in chromate-induced toxicity is discussed.

Introduction

It is well known that chromium(VI) is more toxic and carcinogenic than chromium(III) (*1–3*) because, as shown in Figure 1, in contrast to chromium(III), chromium(VI) actively enters cells by the sulfate transport system (*3–5*). However, once inside the cells, chromium(VI) is readily reduced to chromium(III) (*3–5*). Therefore, the cellular metabolism of chromium(VI) may play a role in the induction of chromate toxicity and carcinogenicity. Chromium(VI) compounds have been shown to produce DNA single-strand breaks and DNA-protein crosslinks (*3,6–12*) and to selectively inhibit the activity of glutathione reductase (*13–15*).

Chromium(VI) can be reduced by biological reductants including glutathione (*16–19*), cysteine (*19*), ascorbate (*20*), hydrogen peroxide (*21*), and flavoenzymes (*22–24*) such as glutathione reductase (*13,14,25*). It is very interesting to note that many of these biological reductants are associated with vitamins. For instance, ascorbate (vitamin C) is capable of reducing chromium(VI), and riboflavin (vitamin B_2) is essential for the synthesis of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which are coenzymes for chromate-reducing flavoenzymes. In addition, these flavoenzymes need nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) as coenzymes, and these are formed with the vitamin nicotinamide. On the other hand, free radical scavengers such as vitamin E have been shown to protect cells from various oxidative damages (*26–28*) and to be effective in preventing the carcinogenic and/or mutagenic activity of chemical agents and ionizing radiation (*29–33*).

As illustrated in Figure 1, it is speculated that free radical species such as hydroxy radicals and glutathionyl radicals are generated during the reduction of chromium(VI) in cells (*18,21,25*). However, in spite of these evidences, the effects of cellular vitamins on chromate-

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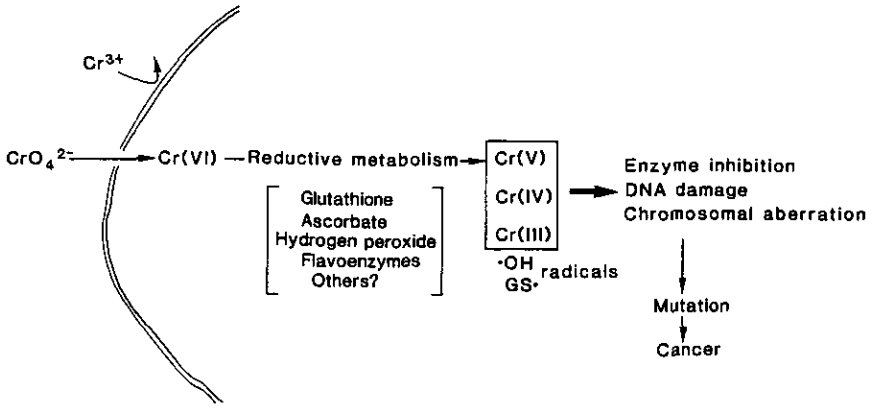

FIGURE 1. Cellular reduction of chromium(VI).

induced damages have not been studied. As knowing the effects of vitamins on chromate-induced damages may help elucidate both the mechanism of protection and the mechanism of chromium carcino-genicity, we examined whether all vitamins have similar effects on chromate-induced damage. In this paper, using cultured Chinese hamster V-79 cells, the action of vitamins, in particular vitamins E and B_2, on selected chromate-induced damage is summarized based upon our recent studies (*34–38*).

Materials and Methods

Cell Culture

V-79 cells were grown as described previously (*37*). Cells were pretreated with vitamins or with the solvent dimethyl sulfoxide (DMSO) alone in complete growth medium at the time they were plated. Twenty-four hours after plating, logarithmically growing cells were treated for 2 hr with Na_2CrO_4 in salts-glucose medium (SGM; 50 mM Hepes buffer [pH 7.2] with 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 5 mM glucose) (*34–38*).

DNA Damage

The alkaline elution technique for analysis of DNA lesions was performed as described (*9*). To quantify the extent of DNA single-strand breaks and DNA protein crosslinks, the strand scission factor and crosslink factor were calculated from the alkaline elution patterns (*34–36*).

ESR Spectroscopy

The formation of paramagnetic chromium in cells was determined by electron spin resonance (ESR) analysis (*36,37*). Briefly, 40×10^6 cells were placed into an ESR tube and were rapidly frozen in liquid nitrogen. ESR measurements were made at temperatures of 153 K using a JES-FE3X spectrometer with 100 KHz modulation, 8 mW microwave power, and 4.0 G modulation amplitude.

The ESR spin trapping studies were carried out at room temperature in samples containing 100 mM Tris-HCl (pH 8.0), 5 mM Na_2CrO_4, 25 mM H_2O_2, 200 μM vitamin B_2, and 100 mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as spin trapping agents (*38*).

Glutathione Reductase and Glutathione, Flavin Derivatives, and Cytotoxicity

Glutathione reductase was measured according to the method of Staal et al. (*39*) as detailed in Sugiyama et al. (*36*). The total glutathione (oxidized and reduced) was determined as described by Tietze (*40*) with minor modifications (*34*).

Flavin derivatives were extracted from cells and were measured by means of high pressure liquid chromatography (HPLC) following the procedure detailed in Sugiyama et al. (*37*).

Chromate-induced cytotoxicity was estimated by colony-forming assay as described in Sugiyama et al. (*36,37*).

Results and Discussion

In the present study, cells were pretreated with vitamins for 24 hr before treatment with chromate because if the cells are treated with vitamins in the presence of chromate, it is difficult to tell whether the action of the vitamins occurs in extracellular or intracellular systems. Thus, first of all, the effect of 24-hr vitamin treatment on cell growth was examined. As shown in Table 1, treatment with vitamin E at 50 μM resulted in an inhibition of cell growth, whereas similar treatment with vitamin B_2 did not change cell growth even at 200 μM. The concentration at which cells were able to grow at rates similar to those of the controls was less than 25 μM for vitamin E and less than 200 μM for vitamin B_2.

Table 1. Effects of vitamin E and vitamin B_2 on growth of V-79 cells.[a]

Treatment	Concentration, μM	Cell number (× 10^6), 24 hr
Control	—	1.1 ± 0.2
Vitamin E	10	1.3 ± 0.1
	25	1.0 ± 0.1
	50	0.7 ± 0.1
Vitamin B_2	10	1.1 ± 0.1
	50	1.1 ± 0.2
	200	1.1 ± 0.2

[a]Cells were plated at a density of 0.5 × 10^6 cells in complete growth medium containing various concentrations of vitamins. After 24 hr, cell number was determined. Each value is the mean ± SD of at least two experiments in triplicate. Modified from Sugiyama et al. (*34*).

Therefore, these concentrations were used throughout the subsequent experiments (*34*).

Effects of Vitamins on DNA Damage and Cellular Reduction of Chromium(VI)

Table 2 shows the effects of vitamins on Na_2CrO_4-induced DNA damages in V-79 cells. Pretreatment with nontoxic levels of vitamin E resulted in a significant decrease of DNA single-strand breaks produced by chromate. In contrast, similar pretreatment with vitamin B_2 enhanced the number of breaks by about four times above that seen with chromate alone. As shown in Table 2, these effects were not due to changes in the cellular uptake of chromate.

In contrast to DNA breaks, there was no change from initial levels of Na_2CrO_4-induced DNA-protein crosslinks in cells pretreated with vitamin E or vitamin B_2 (Table 2). Although the formation of DNA-protein crosslinks has been shown to require time (*7,41*), the level of crosslinks 4 hr after chromate treatment was not influenced by these vitamins (data not shown). Thus, cellular pretreatment with vitamin E or vitamin B_2 has a specific effect on the formation of DNA breaks but not that of protein crosslinks induced by chromate.

Since chromium(VI) easily passes through the cell membrane and is then reduced to chromium(III) (Fig. 1), the formation of the intermediate oxidation states such as chromium(V) and (IV) may play a role in the induction of DNA damage. Thus, the effects of vitamin E and vitamin B_2 on the production of paramagnetic chromium in cells were investigated directly by ESR spectrometry. Figure 2 shows the ESR spectra of both chromium(V) (sharp spectra) and chromium(III) (broad spectra) in V-79 cells treated with Na_2CrO_4. The formation of a chromium(V) signal was confirmed with an anisotropy at $g = 2.016$ and $g = 1.989$ and the line width of the maximum absorption peak was 12 to 13 G (*36,37*). On the other hand, the ESR signal due to chromium(III) complex was characterized by a g value of about 2.03 and a line width of 700 to 800 G (*37*). These levels of chromium(V) and (III) increased in a concentration-dependent manner (50–500 μM).

As shown in Table 3, when cells were pretreated with vitamins, the ESR signal intensity of chromium(V) (g = 1.989) was significantly decreased by vitamin E, while similar treatment with vitamin B_2 resulted in an approximately 2-fold increase of chromium(V) compared to un-pretreated Na_2CrO_4-treated cells. These results were correlated with the effects of vitamin E and vitamin B_2 on chromate-induced DNA breaks (Table 2).

Table 2. Effects of vitamin E and vitamin B_2 on cellular uptake of ^{51}Cr, DNA single-strand breaks, and DNA-protein crosslinks induced by Na_2CrO_4.[a]

Pretreatment, μM[b]	Na_2CrO_4, μM	^{51}Cr uptake, %[c]	DNA breaks (SSF)[d]	DNA-protein crosslinks (CLF)[d]
None	50	100	0.12 ± 0.01	2.23 ± 0.15
Vitamin E, 25	50	116	0.05 ± 0.01*	2.39 ± 0.13
Vitamin B_2, 200	50	86	0.46 ± 0.04†	2.68 ± 0.25

[a]Modified from Sugiyama (*34*) and Sugiyama et al. (*35*).

[b]Cells were pretreated for 24 hr with vitamins and then treated for 2 hr with Na_2CrO_4. Following treatment, cellular DNA was analyzed by alkaline elution.

[c]Cellular uptake of chromate was measured by radioisotope ^{51}Cr analysis as described (*36*).

[d]Each value is the mean ± SE for at least four determinations. SSF, strand scission factor; CLF, crosslink factor.

*$p < 0.01$.

†$p < 0.001$ compared to un-pretreated chromate-treated values.

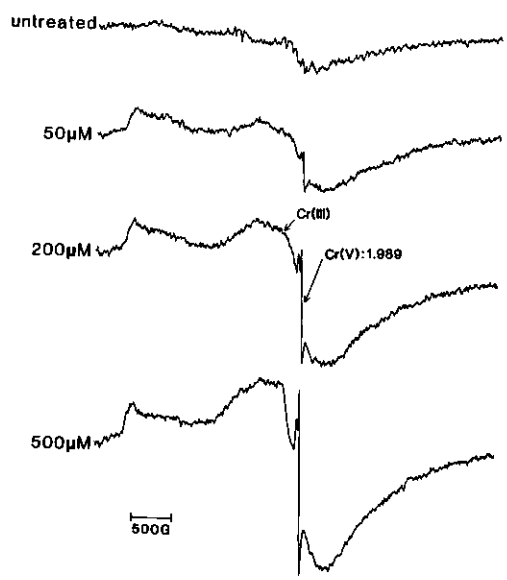

FIGURE 2. ESR spectra of chromium(V) and chromium(III) complex at 153 K. Cells were treated for 2 hr with various concentrations of Na_2CrO_4. Following treatment, an ESR signal was obtained from the cells as described (*37*).

Table 3. Effects of vitamin E and vitamin B_2 on formation of cellular chromium(V) and chromium(III) complex.[a]

Pretreatments, μM	Na_2CrO_4, μM	ESR signal intensity, %	
		Cr^V	Cr^{III}
None	200	100	100
Vitamin E, 25	200	62 ± 11	96 ± 7
Vitamin B_2, 200	200	227 ± 11	99 ± 3

[a]Cells were pretreated for 24 hr with vitamins and treated for 2 hr with 200 μM Na_2CrO_4. Following treatment, an ESR signal was obtained from cells. Each value is the mean ± SD for at least three separate experiments. Modified from Sugiyama et al. (*36,37*).

Several recent studies using ESR spectroscopy have reported that the reactive chromium(VI) is relatively long lived (*16,17,23*) and that it causes DNA breaks *in vitro* (*21,42,43*), indicating that the production of DNA breaks might be closely related to the level of this reactive intermediate. Therefore, these results suggest that the protective effect of vitamin E and the enhancing effect of vitamin B_2 on chromate-induced DNA breaks may be due to modification of the formation of chromium(V) in cells.

Recently, isolated chromium(V) intermediates have been shown to potentially induce mutation in bacterial cell systems (*43*). Our results show that cellular levels of chromium(V) were reduced by vitamin E. In addition, under similar conditions, vitamin E was found to suppress the clastogenic and mutagenic action of chromate compounds (unpublished observation). Collectively, these results suggest that chromium(V) might be the critical form that is responsible for the genotoxic and clastogenic, as well as the mutagenic, activity of chromate.

On the other hand, the formation of chromium(III) was not affected by pretreatment with vitamin E or vitamin B_2 (Table 3). Several *in vitro* studies have shown that only chromium(III) can form a ternary complex with DNA and protein (*3,5,10*), and present results show that DNA-protein crosslinks induced by chromate were not influenced by vitamins (Table 2). Therefore, these results indicate that cellular levels of chromium(III) should play a role in the induction of DNA-protein crosslinks. However, it is not clear why these vitamins have an ability to change the chromium(V) but not the chromium(III) complex in cells. With respect to DNA damage, DNA breaks induced by chromate have been reported to be associated with cellular levels of glutathione and the activity of cytochrome P-450 reductase, whereas protein crosslinks were not dependent upon these factors (*12*). Furthermore, our previous studies have shown that in three different cell lines of human, mouse, and hamster origin, the order of sensitivity to DNA-protein crosslinks was not consistent with the sensitivity to formation of DNA breaks by chromate (*8*), suggesting that chromium-induced DNA-protein crosslinks may be formed by a different mechanism than that for single-strand breaks. Therefore, it is possible that the formation of chromium(III) complexes including DNA-protein crosslinks might be controlled by a different cellular components than for chromium(V).

Since ascorbate (vitamin C) is capable of reducing chromium(VI) directly to chromium(III) (*37*), we are investigating the effect of pretreatment with this vitamin. The results show that cellular levels of ascorbate were increased, resulting in a decrease of chromium(V) and an increase of chromium(III) in V-79 cells (unpublished observation). Thus, further study with vitamin C could lead to a better understanding of the role of intracellular paramagnetic chromium on DNA damage induced by chromate.

Mechanism of Action of Vitamins

To examine whether vitamin E and vitamin B_2 had an effect on chromate-reducing flavoenzymes, glutathione reductase was examined following treatment with vitamins. However, as shown in Table 4, no alteration of glutathione reductase activity was observed in V-79 cells treated with vitamin E or vitamin B_2 (*36,37*). Among cellular components, glutathione has been shown to be one of major reductants of chromate (*16–19*), but cellular treatment with these vitamins did not affect the content of glutathione (Table 4) (*34*). Since riboflavin is a precursor molecule for FAD and FMN, and all the chromate-reducing enzymes have been shown to be flavoenzymes, it might be possible that the increase of chromium(V) by vitamin B_2 is related to other chromate-reducing flavoenzymes activated by FAD and FMN. However, the treatment with Vitamin B_2 did not influence the content of FAD and FMN in V-79 cells (Table 5) (*37*). All of this suggests that the effects of vitamins on the formation of chromium(V) might not be due to the modification of glutathione and chromate-reducing flavoenzyme activity.

The antioxidant effect of vitamin E is well documented in the literature, and this effect may be due in part to efficient radical scavenging (*26,27,30*). Thus, the protective mechanism of vitamin E in preventing DNA breaks produced by chromate might be due to its scavenging of paramagnetic chromium(V) during reduction of chromium(VI) in cells. In fact, cellular treatment with vitamin E resulted in a 10-fold increase of α-tocopherol

Table 4. Effects of vitamin E and vitamin B_2 on glutathione reductase activity and cellular glutathione content.[a]

Pretreatment, μM	Glutathione reductase, nmole/min/mg protein	Glutathione, μg/mg protein
None	25.6 ± 4.7	4.79 ± 0.50
Vitamin E, 25	27.5 ± 5.9	5.22 ± 1.10
Vitamin B_2, 200	26.3 ± 3.2	4.94 ± 0.54

[a]Cells were plated in complete growth medium containing vitamins. Following 24 hr treatment, cellular glutathione reductase activity and glutathione content were determined. Each value is the mean ± SD for at least two separate experiments. Modified from Sugiyama et al. (*34,36,37*).

Table 5. Effect of vitamin B_2 on cellular content of flavin derivatives.[a]

Treatment, μM	Flavin derivatives, nmole/g protein		
	FAD	FMN	Riboflavin
None	13.8 ± 2.8	6.0 ± 0.6	0.10 ± 0.03
Vitamin B_2, 200	12.7 ± 4.4	6.9 ± 0.8	2.17 ± 1.10

[a]Cells were treated for 24 hr with 200 μM vitamin B_2, and cellular content of flavin derivatives was measured by HPLC. Each value is the mean ± SD for three separate experiments. FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide. Modified from Sugiyama et al. (*37*).

as determined by HPLC analysis (unpublished observation). Since chromium(VI) has been shown to be metabolized to chromium(V) with simultaneous formation of active oxygen (*21,25*) and glutathione radicals (*18*), it is difficult to exclude the possibility of the scavenging effects of vitamin E against these radical species.

In the case of vitamin B_2, as indicated in Table 5, cellular pretreatment with this vitamin resulted in a marked increase of riboflavin content. Thus, we further examined the direct interaction of vitamin B_2 and chromate *in vitro* using ESR spectrometry. As shown in Figure 3, neither vitamin B_2 nor Na_2CrO_4 alone could produce an ESR signal. However, a new signal with *g*-value of 1.977 was detected during the reaction of chromate with vitamin B_2 (*38*), indicating that chromate reacts with vitamin B_2 to form chromium(V) species. These *in vitro* studies suggest that the enhancement of chromium(V) formation by vitamin B_2 might be due to the increase in cellular riboflavin.

With respect to DNA breaks, chromium(VI) reacts with hydrogen peroxide to form chromium(V), leading to the generation of hydroxyl radicals, which caused DNA breaks *in vitro* (*21*). As shown in Figure 4, The ESR spin trapping study demonstrated that the formation of DMPO spin adduct with intensity 1:2:2:1 represents the adduct of the hydroxyl radical during the reaction of Na_2CrO_4 and hydrogen peroxide (*38*). When vitamin B_2 was added to this reaction mixture, a significant increase in DMPO-OH adduct was detected, indicating the enhancement of hydroxyl radical formation. During the reaction of chromate and hydrogen peroxide, a tetraperoxochromate(V) was also formed and addition of vitamin B_2 resulted in an increase of this chromium(V) species (data not shown) (*38*). The hydroxyl radical has been shown to be particularly active, reacting with and breaking DNA (*44*). Thus, these results suggest that one possible mechanism of enhanced chromate-induced breakage by vitamin B_2 might involve an increase of chromium(V)-related hydroxyl radical formation. Further studies are necessary to elucidate how hydrogen peroxide as well as hydroxyl radicals would become available for chromate-induced DNA breaks to occur *in vivo*.

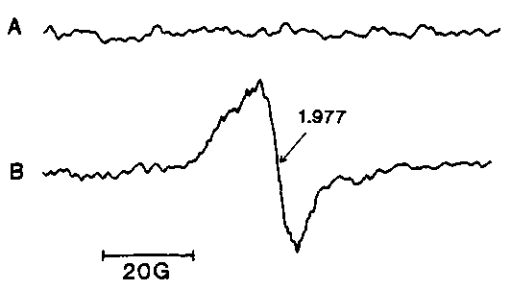

FIGURE 3. ESR spectra of a reaction mixture containing 100 mM Tris-HCl (pH 8.0) and 200 μM vitamin B_2 in the absence (*A*) or presence (*B*) of 5 mM Na_2CrO_4 after 8 min mixing. Modified from Sugiyama et al. (*38*).

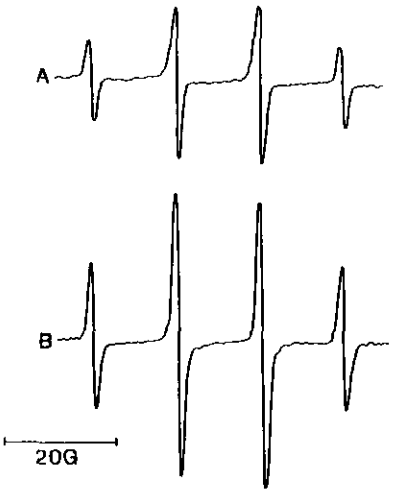

FIGURE 4. ESR spectra of a reaction mixture containing 100 mM Tris-HCl (pH 8.0), 5 mM Na_2CrO_4, 25 mM H_2O_2, and 100 mM DMPO in the absence (*A*) or presence (*B*) of 200 μM vitamin B_2 after 8 min mixing. Modified from Sugiyama et al. (*38*).

Effects of Vitamins on Chromate Inhibition of Glutathione Reductase

Previous studies have shown that chromate compounds selectively inhibit the activity of glutathione reductase not only in erythrocytes (*13,14*), but also in the liver (*15*), and that this inhibition was prevented by antioxidants such as vitamin C (*13*) and *N*-acetylcysteine (*15*). Thus, the effects of vitamin E and vitamin B_2 on chromate inhibition of glutathione reductase was examined in V-79 cells treated with Na_2CrO_4. As shown in Figure 5, the treatment of cells with chromate decreased glutathione reductase activity in a concentration-dependent fashion (5–15 μM), whereas pretreatment with vitamin E resulted in a recovery of this enzyme activity suppressed by chromate (*36*). On the other hand, similar pretreatment with vitamin B_2 enhanced this inhibition (*37*). The mechanism of chromate inhibition of this enzyme remains obscure. However, other studies

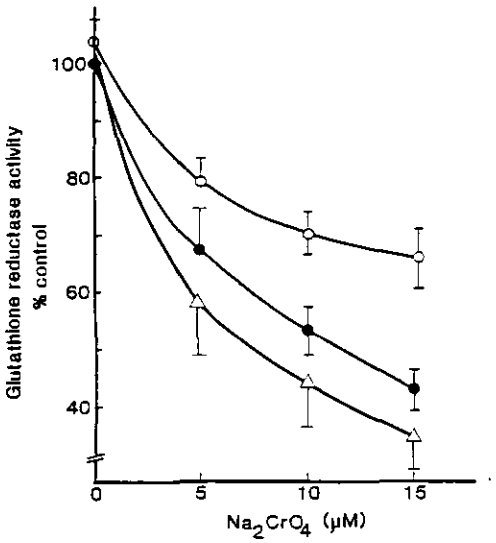

FIGURE 5. Effects of vitamin E and vitamin B_2 on glutathione reductase activity suppressed by Na_2CrO_4. Cells were pretreated for 24 hr with vitamin E (○), vitamin B_2 (△), or with DMSO alone (●) and then treated for 2 hr with Na_2CrO_4. Glutathione reductase activity was expressed as the percentage over control. Modified from Sugiyama et al. (*36,37*).

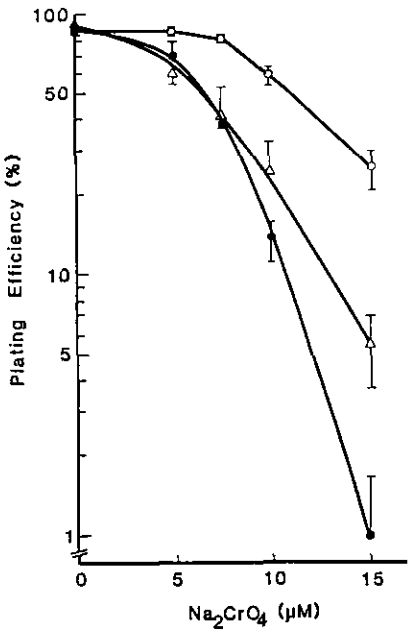

FIGURE 6. Effects of vitamin E and vitamin B_2 on Na_2CrO_4-induced cytotoxicity. Cells were pretreated for 24 hr with vitamin E (○), vitamin B_2 (△), or DMSO alone (●) and then treated for 2 hr with Na_2CrO_4. Following treatment, appropriate numbers of cells were plated and allowed to form colonies. Modified from Sugiyama et al. (*36,37*).

have shown that the enzyme inhibition was accompanied by reduction of chromium(VI) to chromium(III), and trivalent chromium could not inhibit this enzyme *in vitro* (*13*). The present results show that vitamins affected cellular levels of chromium(V) species but not those of chromium(III). These results strongly suggest that enzyme inhibition might be closely related to the cellular formation of chromium(V) during reduction of chromium(VI).

Effects of Vitamins on Chromate Cytotoxicity

Figure 6 shows the effects of vitamin E and vitamin B_2 on chromate-induced cytotoxicity using the colony-forming assay. Pretreatment with vitamin E resulted in a marked reduction of the cytotoxicity induced by Na_2CrO_4. On the other hand, pretreatment with vitamin B_2 had no effect on the cytotoxicity of sublethal concentrations (5–7.5 μM), but there was a significant decrease of cytotoxicity induced by a lethal concentration (15 μM) of chromate (*37*). This result was unexpected, because similar pretreatment with vitamin B_2 enhanced the formation of DNA breaks as well as the inhibition of enzyme activity induced by chromate. A recent study has shown that antioxidant enzymes such as catalase and superoxide dismutase are effective in reducing the formation of chromium(VI)-induced DNA breaks, but these antioxidants have no effect on the cytotoxicity caused by this metal (*45*). However, the present studies have demonstrated that vitamin E protected cells from chromate-induced DNA breaks as well as from cytotoxicity. Thus, these results indicate that DNA damage induced by chromate may contribute to the cytotoxicity but apparently is not the only lesion associated with cell death induced by this metal.

Conclusion

The effects of vitamin E and vitamin B_2 on chromate-induced damage as well as on the formation of chromium(V) and (III) were studied using V-79 cells. The results indicate that *a*) the level of chromium(V) in cells treated with chromate shows a strong correlation with the induction of DNA breaks and enzyme inhibition. Therefore, chromium(V) might be one critical ultimate form which is responsible for the toxic action of chromate; *b*) DNA-protein crosslinks are not as dependent upon chromium(V) formation as are DNA strand breaks, but may be associated with chromium(III) formation; *c*) chromium(VI)-induced cytotoxicity is not directly related to the induction of DNA damage by this metal, indicating that DNA damage might not be the only lesion required for cell death; *d*) in particular, vitamin E protected cells from all of the chromate-induced damages tested. Therefore, vitamin E might be a useful antitoxic agent for chromium compounds; and *e*) these vitamins are capable of altering the biological effects of chromate, indicating the importance of the action of vitamins on the chromate-induced toxicity.

Since the importance of vitamins in both human nutrition and cancer prevention has been well docu-

mented and since not all vitamins have similar effects on chromate-induced damage, studies are necessary to elucidate whether other vitamins have an effect on chromate-induced damage.

This work was supported in part by Fukuoka Cancer Society and Chiyoda Mutual Life Foundation.

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