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Introduction

There is no doubt that chromium has been the most extensively investigated metal with respect to the assessment of long-term effects and to the evaluation of the underlying mechanisms. Notwithstanding the huge data-base available in the literature, many aspects, bearing both scientific and practical interest, are still unclear or controversial. This paper will briefly discuss some relevant problems, including carcinogenicity and genotoxicity of chromium compounds as related to their valency and solubility, interactions with other compounds, bioavailability to target cells and molecules, interconversion processes between different oxidation states in various body areas and cell compartments, mechanisms of chromium metabolism and carcinogenicity, and existence of thresholds in chromium carcinogenesis.

Carcinogenicity of chromium in humans and experimental animals

The epidemiological evidence for chromium carcinogenicity results from studies in occupationally exposed individuals, mainly among workers in the bichromate-producing industry and in chromate-pigment manufacturing. Conversely, the epidemiologic evidence is not conclusive for other occupational groups, including workers in the ferrichromium industry or in the plating industry, painters and welders (IARC 1950 and 1987; Norseth 1986; Bidstrup and Davies 1986). It should be considered that the above occupational activities involve a combined exposure to mixtures not only of different chromium compounds but also of other toxic metals, such as lead in the pigment production industry or nickel in welding fumes. It is also noteworthy that the statistical analysis of data concerning workers of the bichromate producing industry in Germany, England

Abbreviations: Cr(0), Cr(II), Cr(III), Cr(IV), Cr(V), Cr(VI), chromium With oxidation state 0, +2, +3, +4, +5, +6: ELF, epithelial-lining fluid; GSH. reduced glutathione; PAM, pulmonary alveolar macrophages

and USA has shown a significant decline in lung cancer incidence since 1960, thanks to the improved measures of industrial hygiene (Bidstrup and Davies *1986).*

The general view on chromium valency is that no epidemiological studies so far have documented increased cancer risk in populations exposed to Cr(III) alone (IARC 1987; Langård 1988). Also, the evidence for chromium carcinogenicity is limited to lung cancer, whereas excess ratios for cancer at other sites arc an unusual finding and the small numbers reponed never did reach statistical significance (IARC 1987).

The carcinogenicity assays of chromium in experimental animals would have been expected to clarify the relative contribution of individual compounds in chromium carcinogenesis. Unfortunatley, these assays confirmed the main conclusion of the epidemiological studies, i. e. sufficient evidence of carcinogenicity was limited to certain Cr(VI) compounds (IARC 1987), but the resulting information was rather poor. Indeed, one of the most intriguing aspects is the discrepancy between the well-established evidence for carcinogenicity of Cr(VI) compounds in humans, mainly accumulated when high exposure occurred in the workplace, and the weak effects observed in experimental animals (Langård 1988).

Many carcinogenicity data have been generated by giving chromium compounds by unnatural administration routes, which do not reproduce human exposure patterns and by-pass protective mechanisms based on pharmacokinetic and metabolic processes. For instance, several Cr(VI) compounds have been shown to produce injection-site tumors following intramuscular, subcutaneous or intrapleural implantation (IARC *1980* and 1987; Langård 1988). The intrabronchial implantation of 21 chromium-containing materials loaded on inert pellets produced lung squamous carcinomas or bronchial carcinomas only in the case of some sparingly soluble chromate materials (Levy et al 1986). So far, inhalation studies have not led to definitive conclusions (Langård 1988).

In general, calcium chromate and some relatively insoluble Cr(VI) compounds, such as sintered calcium chromate, lead chromate, strontium chromate, sintered chromium trioxide and zinc chromate, have been the most commonly incriminated chromium compounds (IARC 1980). However, it is noteworthy that solutions of calcium chromate and of the highly soluble sodium dichromate had comparably weak carcinogenic effects when administered in high doses by intratracheal instillations to rats (Steinhoff et al 1986).

Activity of chromium compounds in short-term test systems

A great variety of short-term test systems, mostly evaluating genetic effects in *vitro* or *in vivo* targets, have been used in order to assess the activity of individual chromium compounds and to clarify some of the mechanisms involved in chromium carcinogenesis. A large number of studies have shown that Cr(VI) compounds, once in solution, are positive in virtually all the experimental systems where they have been tested. Cr(III) compounds have been found to produce genetic changes in acellular or subcellular systems, such as purified nucleic acids or isolated cell nuclei, but, with few exceptions, they arc considered to be

devoid of activity in cellular systems (Rianchi and Levis 1984 and 1987; De Flora et al 1984a and 1988; Petrilli et al 1986a; Beyersmann and Köster 1987; IARC 1980 and 1987).

The selective effects of different chromium species are generally ascribed to their different abilities to cross cell membranes. In fact, while Cr(VI) readily penetrates into living cells, Cr(III) has a limited uptake (Bianchi and Levis 1987; Beyersmann and Köster 1987). The positive responses with Cr(III) in short-term tests have been ascribed either to contamination with Cr(VI) (IARC 1987) or to special conditions allowing its penetration into cells. For instance, genetic effects in bacteria (De Flora et al 1988) or yeast (Galli et al 1985) occur only when a high molarity of phosphate, largely exceeding physiological conditions, are present in the medium. It has been also demonstrated, in both prokaryotic (Warren et al 1981) and eukaryotic cells (Beyersmann and Köster 1987), that complexes of Cr(III) with certain hydrophobic ligands are genotoxic. However, it is not clear whether similar complexes may occur under natural conditions or whether the organic moiety may be also responsible for some genetic damage. It is noteworthy that, at variance with synthetic complexes having a similar chemical composition, the glucose tolerance factor (GTF) is nonmutagenic in bacteria (De Flora et al 1988). GTF is a natural complex of Cr(III) with nicotinic acid, glycine, glutamic acid and cysteine, and is used as a dietary supplement in case of deficiency of Cr(III) intake with food and of impaired glucose tolerance.

Metabolic interconversions between Cr(VI) and Cr(III)

Chemical and biochemical interconversions between Cr(VI) and Cr(III) have been investigated by using body fluids (i.e. saliva, gastric juice, blood plasma, epithelial-lining fluid) and various kinds of cell or tissue preparations [i.e. unfractionated cell homogenates, mitochondria, post-mitochondrial (S9 or S12), cytosolic (S105) and microsomal fractions) from various animal species, including humans (see Table 1). Metabolism was investigated either in healthy and untreated individuals or under the influence of diseases (i.e. lung cancer, primary hepatocellular carcinoma, viral hepatitis, peptic ulcer), drug administration (i.e. antiulcer drugs) enzyme inducers (i.e. Aroclor 1254, phenobarbital, 3-methylcholanthrene), glutathione (GSH) depletors (i.e. diethylmaleate, buthionine sulfoximine) or analogs and precursors (i.e. N-acetylcysteine), special diets (i.e. cirrhogenic diet) or exposures (i.e. cigarette smoking, narcosis with ether, intratracheal instillation of NaCl or sodium dichromate).

On the whole, Cr(III) oxidation to mutagenic Cr(VI) was only produced by oxidizing chemicals but not by metabolic systems, which rules out the hypothesis that oxidative phenomena of Cr(III) may occur in the organism (Petrilli and De Flora 1978b). On the other hand, a metabolic reduction of Cr(VI) could be reproduced in the presence of a variety of tissue or cell preparations. Reference is made to the papers indicated in Table 1 and to next sections for details.

Selective reduction of Cr(VI) and its relationship with carcinogenicity targets

Previous studies showed that the rank of Cr(VI)-reducing ability of post-mitochondrial fractions from various tissues of Aroclor-pretreated rats was the following: liver, adrenals, kidney, testis, stomach and lung. No decrease of Cr(VI) mutagenicity was produced by preparations of skeletal muscle, spleen, bladder and colon (Petrilli and De Flora 1980 and 1986b; De Flora 1982). Recently, additional experiments were carried out by comparing the ability of S12 fractions of liver, skin, subcutis and skeletal muscle from Aroclor-pretreated rats in decreasing the mutagenicity of Cr(VI) (Fig. 1). Even at equivalent protein concentration, liver preparations were quite efficient, whereas those of skeletal muscle had a negligible Cr(VI)-reducing activity, which confirms our previous findings. This is of interest because, as discussed later, skeletal muscle is a typical target of implantation-site sarcomas induced by several Cr(VI) compounds.

As shown in Fig. 1 and Table 1, subcutis and even more skin preparations had a clearly detectable activity in decreasing Cr(VI) mutagenicity. It is noteworthy that, together with inhalation and ingestion, skin contact is one of the possible routes of exposure of humans to chromium. However, no cases of skin cancer induced by contact with chromium have been ever reported, only acute irritative dermatitis and allergic eczematous dermatitis being known to occur in chromate-exposed workers. Contact hypersensitivity has been ascribed to Cr(VI) reduction to Cr(III) in the skin, chiefly by sulfhydryl groups of aminoacids, resulting in Cr(III) conjugation with autologous proteins (IARC 1980). However

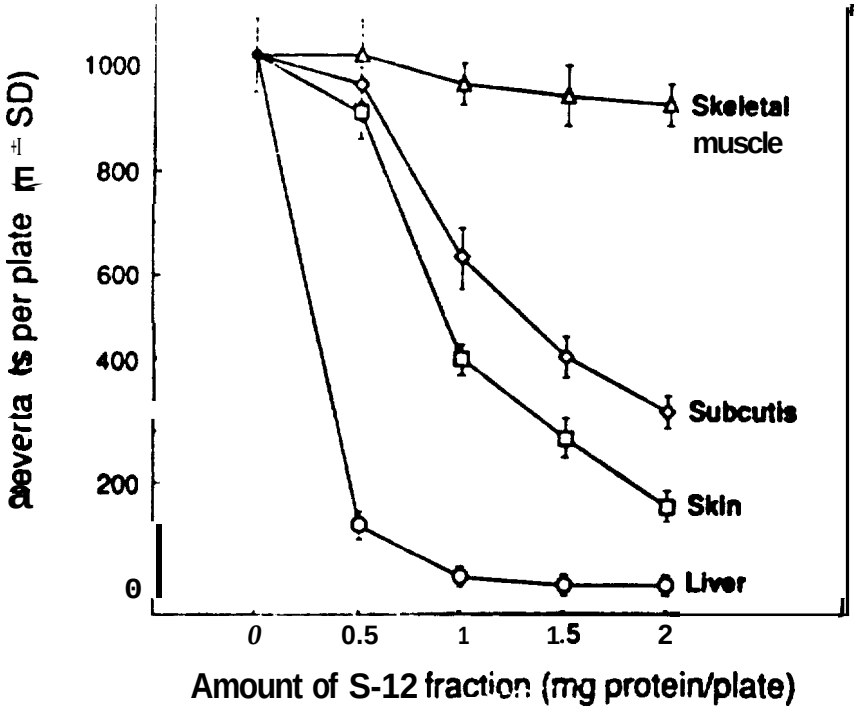

Fig. 1 Effect of increasing amounts of tissue S12 fractions pooled from 5 male Sprague-Dawley rats, tested at equivalent protein concentration, on the mutagenicity of sodium dichromate (30 μg/plate) in strain TA100 of S. *typhimurium*

Table 1. Efficiency of tissue or cell preparations in reducing the mutagenicity of Cr(VI) in the Ames reversion test, as assessed in studies performed in this laboratory

Metabolic Cr(VI)-reducing efficiency	Tissue or cells	Animal species	References
High	Liver	Humans	De flora, 1982
		Rat	De Flora, 1978: Petrilli et al., 1986b; this study
		Mouse	De flora, 1982
		Hamster	De Flora et al., 1985b
		Woodchuck	De Flora et al., 1987a
		Chicken	De Flora et al., 1985b
		Pekin duck	Unpublished data
		Rainbow trout.	De Flora et al., 1982
	Pulmonary alveolar macrophages	Humans	Petrilli et al., 1986c
		Rat	De Flora et al., 1986
	Erythrocytes	Humans	Petrilli and De Flora, 1978a
Medium	Adrenals	Rat	Petrilli et al., 1986b
	Kidney	Rat	Petrilli et al., 1986b
	Testis	Rat	Petrilli et al., 1986b
	Stomach	Rat	Petrilli et al., 1986b
	Skin	Rat	This study
	Subcutis	Rat	This study
	Lung parenchyma	Rat	Petrilli et al., 1985
		Mouse	De Flora, 1982
		Humans	De Flora et al., 1984b and 1987b
	Bronchial tree	Humans	De Flora et al., 1984b
Negligible	Skeletal muscle	Rat	Petrilli et al., 1978a
	Colon	Rat	Petrilli et al., 1986b
	Bladder	Rat	Petrilli et al., 1986b
	Spleen	Rat	Petrilli et al., 1986b

experimental data provide evidence that, like in the liver, reduction of Cr(VI) mutagenicity by rat skin S12 fractions is mainly due to NADPH-dependent mechanisms (data not shown).

An efficient reduction of Cr(VI) occurs in the blood stream. A weak reducing activity was detectable in blood plasma (Korallus et al 1984), but the main process, as investigated in several laboratories using both analytical methods and mutagenicity test systems (e.g. Gray and Sterling *1950;* Petrilli and De Flora 1978a; Aaseth et al *1982;* Kitagawa et al 1982; Wiegand et al 1984), occurs in erythrocytes, where Cr(VI) is known to be selectively accumulated (Gray and Sterling 1950). Such reduction has been mainly ascribed to GSH (Aaseth et al 1982; Kitagawa et al 1982; Wiegand et al 1984), the concentration of which can be increased in erythrocytes by administering *in vivo* suitable precursors such as N-acetylcysteine (De Flora et al 1985d).

Cr(VI) reduction in the blood may explain the lack of carcinogenicity at a distance from administration sites. In fact, as already discussed, no significant

increase of cancer at sites other than the lung has been reported in humans (IARC 1987), and in animals the only report contrary to this assumption was the induction of renal carcinomas following intramuscular injection of lead chromate to rats (IARC 1980). However, such effects should be ascribed to lead, which typically produces kidney tumors in rodents, rather than to chromate (IARC 1980).

Secretions of the digestive tract, such as saliva and gastric juice, are capable of reducing Cr(VI) due to thermostable components (Petrilli and De Flora 1982; Petrilli et al 1986b; De Flora and Boido 1980). In particular, studies on the circadian Cr(VI) reduction by gastric juice in several individuals provided evidence for a basal activity (a few μg Cr(VI) reduced per ml) during interdigestive periods and, irrespective of pH variations, for peaks of activity (some tens μg Cr(VI) reduced per ml) after each meal. Taking into account that the daily secretion of gastric juice is in the range of liters, tens of mg Cr(VI) would be expected to be reduced daily in the gastric environment (De Flora et al 1987c).

Such a phenomenon renders very unlikely the occurrence of carcinogenic effects of Cr(VI) introduced by the oral route or swallowed following removal by the respiratory tract, also because Cr(III) is very poorly absorbed from the intestine (Donaldson and Barreras 1966; Langård 1982), and a further reduction is expected to occur in the blood of the portal vein and then in liver cells. This physiological mechanism also provides an efficient barrier towards the oral toxicity of Cr(VI). In fact, the lethal dose in humans is estimated to be in the range of grams (Langård 1980).

Also in the respiratory tract, in addition to the well-known specific defense mechanisms against inhaled foreign particles, Cr(VI) tends to be reduced prior to reaching target cells. First, some chemical reduction occurs in the lumen of terminal airways, due to the so-called epithelial-lining fluid (ELF) (Petrilli et al 1986c). Moreover, pulmonary alveolar macrophages (PAM) are very active in metabolically reducing Cr(VI) both in humans (Petrilli et al 1986c) and in rats (De Flora et al 1986). This mechanism is considered to be quite important (Langård 1988), because these phagocytizing cells have an extremely long life span (months to years) and are very efficiently removed by the muco-ciliatory escalator, accounting for 1 to 5 million PAM leaving the lung each hour to be expectorated or swallowed (Green et al 1987).

In addition, Cr(VI) mutagenicity was decreased to an appreciable extent in the presence of preparations of mixed-cell populations, representative of bronchial tree in humans, and of peripheral lung parenchyma in both humans and rodents (see Table 1). As assessed by testing paired samples from 18 individuals, lung parenchyma was significantly more efficientthan bronchial tree in decreasing Cr(VI) mutagenicity, and also contained considerably higher concentrations of GSH (Petruzzelli et al 1989).

Interindividual variations in Cr(VI) reduction

As assessed by comparatively testing homogeneous series of tissue samples for their ability to decrease the mutagenicity of Cr(VI), it was possible to determine the extent of interindividual variations in chromium metabolism.

For instance, interindividual variations were investigated in humans by testing 15 samples of ELF and 23 of PAM (Petrilli et al 1986c), 24 of bronchial tree (De Flora et al 1984b) and 71 of peripheral lung parenchyma (De Flora et al 1987b). The only factor explaining the observed differences of efficiency of both PAM and lung parenchyma (healthy tissue) samples was that of smoking habits, whereas age or diseases, such as lung cancer, had no significant effect

The interindividual variability in the liver metabolism of Cr(VI) and other mutagens has been investigated in woodchucks, a rodent species representing a suitable animal model for virai hepatitis and primary hepatocellular carcinoma. In a study with 36 trapped wild animals (De Flora et al 1987a), the interindividual variations were not related to viral infection. This was confirmed in a subsequent study in 28 captive woodchucks (manuscript in preparation). In fact, contrary to the response with hepatocarcinogenic promutagens, the decrease of Cr(VI) mutangenicity was not affected by the viral infection and, in animals bearing liver cancer, there was no significant difference between cancer and noncancer (virus-infected) tissues. interestingly, in the same study, it was noted that the metabolism of Cr(VI) and other mutagens is significantly enhanced in pregnancy.

Inducibility and autoinducibility of chromium metabolism

The liver metabolism of Cr(VI) was stimulated by known enzyme inducers, with the following rank of activity: Arochlor 1254, phenobarbital and 3-methylcholanthrene (De Flora et al 1985a). Conversely, only Aroclor 1254 was successful in stimulating Cr(VI) reduction in the lung (Petrilli et al 1985). Moreover, the *in vivo* administration of N-acetylcysteine to rats resulted in stimulation of Cr(VI) metabolism in both liver and lung (De Flora et al 1985d), as well as in isolated PAM (De Flora et al *1986).*

Interestingly, the pulmonary reduction of three Cr(VI) compounds was also significantly stimulated by the repeated intratracheal administration of Cr(VI) itself to rats (sodium dichromate, 0.25 mg/kg b.w., 5 times per week for 4 weeks) (Petrilli et al 1985). Note that, under identical treatment conditions, dichromate failed to induce lung tumours in rats (Steinhoff et al 1986). On the other hand, single cumulative intratracheal instillations (1.25 mg/kg b.w., once per week for 4 weeks), following a schedule which was found to be weakly carcinogenic (Steinhoff et al 1986), did not produce any significant variation in the pulmonary metabolism of chromium (Petrilli et al 1985).

Biochemical mechanisms involved in the intracellular Cr(VI) reduction

Multiple mechanisms participate in the intracellular reduction of chromium. Comparative analyses showed that the efficiency of rat liver preparations in reducing Cr(VI) ranked as follows: unfractionated cell homogenates, post-mitochondrial fractions, cytosolic fractions and microsomal fractions (DeFlora et al 1985a). This indicates that reduction occurs in various all structures, including e.g. mitochondria, where reduction has been mainly ascribed to GSH (Ryberg and Alexander 1984), the endoplasmic reticulum, where cytochrome P-450 has been shown to work as a chromate reductase (Garcia et al 1981), and the cell cytosol. A minor mechanism responsible for Cr(VI) reduction in the cytosol is provided by electron donors, and chiefly by GSH. However, the major mechanism is enzyme-catalysed, and several lines of evidence indicate the participation of DT diaphorase activity (DeFlora et al 1985a, 1987d and 1988). A possible 2-electron reduction of Cr(VI) via this enzyme activity, as has been demonstrated with other substrates, such as some quinones and azo dyes (Lind et al 1982), would by-pass the formation of highly reactive Cr(V). An additional flavoprotein enzyme involved in Cr(VI) reduction is aldhyde oxidase, which differs from cytochrome P-450 reductase and DT diaphorase in that it does not require NAD(P)H as an electron donor (Banks and Cooke 1986).

Mechanisms of chromium genotoxicity and carcinogenicity

Once chromium has penetrated the target cells in its hexavalent form, it is a matter of discussion which species is responsible for the genotoxic effects and consequently for the initiation of cancer.

In bacteria, where DNA is in close spatial arrangement with the cell membrane, unreduced Cr(VI) itself may be the ultimate mutagen, as shown e.g. by the fact that the *S. typhimurium* strains reverted by oxidative mutagens are the most sensitive in revealing Cr(VI) mutagenicity (Bennicelli et al 1983). On the other hand, in compartmentalized eukaryotic cells, reduced forms are more likely candidates as ultimate mutagens. In fact, the stable reduced form, i.e. Cr(III), is known to react more readily *in vitro* with purified nucleic acid, as compared to Cr(VI) (Bianchi and Levis 1987). The intermediate reduction products, i.e. the reactive Cr(V) and Cr(IV), are also possible candidates (Jennette 1982). Cr(V) may also act through generation of reactive oxygen species, as shown by the finding that reaction of Cr(VI) with intracellular hydrogen peroxide leads to formation of tetraperoxochromate (V), the decomposition of which generates hydroxyl radicals. Under *in vitro* conditions, such reaction results in DNA alterations without significant chromium-DNA adduct formation (Kawanishi et al 1986; Wetterhahn 1988). However, due to the extremely high reactivity of the hydroxyl radical, disappearing in less than 1 nsec and traveling no farther than 1 nm from the site of formation (Simic 1988), a reaction of this type would be conceivable in the intact cell only if occurring very closed to target DNA molecules. Indeed, the difficulties in understanding these problems may reflect the possibility that multiple mechanisms are involved in chromium genotoxicity.

Conclusions

On the whole, metabolic factors appear to play a crucial role in chromium toxicity and carcinogenicity. Cr(VI) reductive processes in the extracellular environment (e.g. in saliva, gastric juice, blood plasma or epithelial-lining fluid) or in nontarget cells (e.g. in red blood cells or alveolar macrophages) are expected to detoxify chromium, thereby limiting its potential effects and selecting target tissues and cells. The intracellular Cr(VI) reduction may be viewed either as an activating process, leading to formation of reactive species (Cupo and Wetterhahn 1985), or as a detoxification process, due to trapping of the reduced forms by nucleophilic components of the cytoplasm (Petrilli and De Flora 1986b and 1988).

In any case, the described mechanisms support the epidemiological and experimental evidence indicating the lung as the only target of Cr(VI) carcinogenicity. However, even in the respiratory tract the described defense mechanisms are expected *to* limit the potential carcinogenicity of Cr(VI). The problem of thresholds in carcinogenesis is quite controversial, and should be faced case by case (Shubik et al *1984).* In the case of Cr(VI) and other compounds which undergo detoxification in the organism, the existence of thresholds can be hardly argued, because the protective machinery of the organism must be saturated by an excess of the compound, which implies the existence *of* a (hardly quantifiable) threshold dose. *As* a matter of fact, mutagens undergoing detoxification are often noncarcinogenic or borderline carcinogens (De Flora 1978 and 1985). Epidemiological and animal carcinogenicity data for chromium appear *to* meet all the criteria proposed for categorizing a given compound among carcinogens subjected to thresholds, such as *(a)* lack of carcinogenicity in some studies, or *(b)* carcinogenicity only at very high doses (often close to the maximum tolerated dose), or *(c)* without linear dose-response effects (so-called hockey stick-like curves), or *(d)* only when administered in a single cumulative dose rather than in fractionated doses, or *(e)* only close to administration sites, or *(f)* in cells lacking detoxification mechanisms (e.g., skeletal muscle cells) or where these mechanisms can be overwhelmed by an excess Cr(VI) (e.g., in cells of the respiratory tract) (De Flora 1985).

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