Less than additive interaction between cigarette smoke and chromium(VI) in inducing clastogenic damage in rodents

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A combination of tobacco smoking with certain agents has been shown to exert synergistic carcinogenic effects. On the other hand, antagonism betweeen smoke and other pulmonary carcinogens has also been documented by both epidemiological and experimental data. In spite of a very large number of studies carried out for decades in workers exposed to hexavalent chromium, the influence of smoking habits on lung carcinogenesis induced by this metal has not been clarified. For this reason, we performed two studies evaluating clastogenic effects in rodents. In the first one, BDF$_1$ mice were exposed whole-body to mainstream cigarette smoke for 5 days and, on the last day, they received an i.p. injection of potassium dichromate. In the second study, Sprague–Dawley rats were exposed whole-body to environmental cigarette smoke for 18 consecutive days and for the same period of time they received daily intra-tracheal instillations of sodium dichromate. Individually, the two hexavalent chromium salts and cigarette smoke, either mainstream or environmental, enhanced the frequency of micronuclei in bone marrow polychromatic erythrocytes of both mice and rats. Moreover, individual exposure to either environmental cigarette smoke or sodium dichromate enhanced the frequency of micronuclei and multiple nuclei in pulmonary alveolar macrophages of rats. In both studies, combined exposure to cigarette smoke and hexavalent chromium produced less than additive clastogenic effects. These results are consistent with our previous data, showing that hexavalent chromium and either benzo[a]pyrene or cigarette smoke condensate behave antagonistically in in vitro mutagenicity test systems and that the chromium reducing capacity of human pulmonary alveolar macrophages and peripheral lung parenchyma is enhanced in smokers. Taken together, in the absence of any epidemiological evidence, these findings rule out any occurrence of synergism between cigarette smoke and hexavalent chromium, at least in certain stages of the carcinogenesis process.

Abbreviations: CA, chromosomal aberrations; CS, cigarette smoke; ELF, epithelial lining fluid; ECS, environmental cigarette smoke; GSH, reduced glutathione; i.t., intra-tracheally; MCS, mainstream cigarette smoke; MN, micronucleated; NCE, normochromatic erythrocytes; PAM, pulmonary alveolar macrophages; PBS, phosphate-buffered saline; PCE, polychromatic erythrocytes; PN, polynucleated; SCE, sister chromatid exchanges; TPM, total particulate matter.

Introduction

Although it is well established that tobacco smoking causes lung cancer, cancer at other sites and several other adverse health effects, less information is available concerning the outcome of combined exposure to cigarette smoke (CS), either mainstream (MCS) or environmental (ECS), and other agents (1). As to carcinogenic risk, there is evidence for synergism between CS and exposure to arsenic, asbestos, ethanol, silica and radiation (radon, atomic bomb emissions and X-rays), whereas there is antagonism with chloromethyl methyl ether and bis(chloromethyl) ether (2,3).

For many other carcinogens, however, the power of epidemiological studies is insufficient to pinpoint interactions with CS. Indeed, CS represents a major confounding factor, especially when evaluating the health effects of other airborne agents. The situation is complicated by the fact that CS is a complex mixture. More than 3040 chemical compounds have been isolated from processed tobacco leaf and MCS contains nearly 4000 identified chemicals plus an unknown number of unidentified chemicals (4). Since many of these compounds, which belong to a variety of chemical families, have toxicological relevance and work by different mechanisms (3), it is difficult to assess the overall impact on human health of an interaction between CS and other chemicals, even in the workplace, where in principle exposures are more accurately defined.

This is the case for the interaction between CS and Cr(VI). At variance with metallic chromium and Cr(III), there is sufficient evidence for the carcinogenicity of Cr(VI) in the human lung, at least at the high doses which are encountered in certain occupational settings (5,6). In spite of a very large number of studies on Cr(VI)-exposed workers which have been carried out world wide for several decades, epidemiological studies have not clarified whether combined exposure to CS and Cr(VI) has synergistic, additive or antagonistic effects (7).

Due to the lack of information provided by epidemiological studies and to the fact that, for different reasons, it is very difficult to reproduce the lung tumorigenicity of both Cr(VI) (5) and CS (3,8) in animal models, we designed a study aimed at evaluating the interaction between CS and Cr(VI) in inducing clastogenic effects in rodents. With this goal in mind, we performed two separate experiments. In the first one, we exposed whole-body BDF$_1$ mice to MCS and/or to Cr(VI), given i.p., and measured the frequency of micronucleated (MN) polychromatic erythrocytes (PCE) in bone marrow. In the second experiment, we exposed whole-body Sprague–Dawley rats to ECS and/or Cr(VI), given intra-tracheally (i.t.), and measured the frequency of MN PCE in bone marrow and of MN and polynucleated (PN) pulmonary alveolar macrophages (PAM) collected by bronchoalveolar lavage. The results reported herein show that individually both CS and Cr(VI) exert clastogenic effects, but the damage produced by their combination is less than additive.

Materials and methods

Animals

The experiments in mice and rats were carried out in the Sofia laboratory and the Genoa laboratory, respectively. Male BDF$_1$ (C57BL×DBA$_2$) mice, aged 8–12 weeks and weighing 23–25 g, were from the Animal Laboratory, National Centre of Oncology (Sofia, Bulgaria). Male Sprague–Dawley rats, aged 6–8 weeks and weighing 180–200 g, were purchased from Harlan Italy (Correzzana, Milan, Italy).

The animals were housed in plastic cages on sawdust bedding and maintained on standard rodent chow and tap water *ad libitum*. The temperature of the animal rooms was 23 ± 2°C, with a relative humidity of 55% and a 12 h day/night cycle. The housing and treatment of animals were in accordance with national and institutional guidelines.

Treatment of mice

After acclimatization for 2 weeks, BDF$_1$ mice were divided into four groups, each composed of 10 animals, and treated as follows: (i) sham-exposed mice, kept in filtered air and receiving an i.p. injection of 200 μl of 0.15 M NaCl after 5 days; (ii) mice exposed to MCS for 5 days; (iii) mice receiving a single i.p. injection of potassium dichromate (K$_2$Cr$_2$O$_7$) (E. Merck, Darmstadt, Germany), dissolved in 200 μl of 0.15 M NaCl at a dose of 50 mg/kg body wt; (iv) mice exposed to MCS for 5 days and, on day 5, receiving a single i.p. injection of potassium dichromate. A whole-body exposure to MCS was obtained, as previously described (9), using filter-tipped commercial cigarettes (Arda-Bulgartabac) that have a declared content of 31.5 mg tar and 1.6 mg nicotine. Briefly, each of the groups of mice undergoing this treatment was placed in a 22.5 l sealed glass chamber that was subsequently filled by means of a 50 ml syringe with the MCS generated by one cigarette. The chamber was opened after 10 min and, after a 1–2 min interval needed to renew the air, filled again with fresh smoke for a total of nine times, for 5 consecutive days. The concentration of total particulate matter (TPM) in the exposure chamber was, on average, 533 mg/m^3 air.

Treatment of rats

After acclimatization for 1 week, Sprague–Dawley rats were divided into four groups, each one composed of eight animals, and treated as follows: (i) sham-exposed rats, kept in filtered air and receiving daily, for 18 consecutive days, an i.t. instillation of 100 μl phosphate-buffered saline (PBS), pH 7.4; (ii) rats exposed to ECS for 18 consecutive days; (iii) rats receiving daily, for 18 consecutive days, an i.t. instillation of sodium dichromate (Na$_2$Cr$_2$O$_7$·2H$_2$O$_2$) (E. Merck), dissolved in 100 μl of PBS, pH 7.4, at a dose of 0.25 mg/kg body wt; (iv) rats exposed to both ECS and sodium dichromate. ECS was generated from Kentucky 2R1 reference cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY) having a declared content of 44.6 mg TPM and 2.45 mg nicotine each, with a 23 mm butt remaining after smoking. The cigarettes were kept for 48 h before use in a standardized atmosphere humidified with a mixture of 70% glycerol and 30% water. Whole-body exposure of rats to ECS was achieved using a smoking machine (model TE-10; Teague Enterprises, Davis, CA). This machine (10) is designed to produce sidestream smoke, mainstream smoke or a combination of the two by burning 2, 5 or 10 cigarettes at one time. We used a mixture of sidestream (89%) and mainstream smoke (11%), mimicking exposure to ECS. Based on preliminary experiments, we decided to burn 10 2R1 cigarettes at one time, 6 h/day, divided in two rounds with a 3 h interval. This accounted for a whole-body exposure to the smoke generated by 4320 cigarettes. Under these conditions, the TPM in the exposure chambers was 120 mg/m^3 air.

Cytogenetic analyses

Twenty-four hours after the last treatment, the animals were anesthetized with ethyl ether and killed by cervical dislocation. Immediately after killing the rats, bronchoalveolar lavage was performed by lavaging the lungs with two 5 ml aliquots of cold (4°C) 0.15 M NaCl infused via a cannula inserted into the trachea. The cells were washed twice with RPMI 1640 and then spun onto slides in a cytocentrifuge and fixed with methanol. Bronchoalveolar lavage cells were stained with 10% Giemsa solution for 10 min. A total of 2000 PAM/sample were scored for MN and PN cells.

The left femurs from both mice and rats were removed and dissected. Bone marrow cells were collected, smeared on duplicate slides, air dried and stained with May–Grünwald–Giemsa according to Schmid (11) (mouse bone marrow) or with hematoxylin and eosin according to Albanese and Middleton (12) (rat bone marrow). From each animal, 200 erythrocytes were scored for the evaluation of toxic effects, as inferred from the PCE/normochromatic erythrocytes (NCE) ratio, and 2000 PCE were scored for the presence of MN cells.

Statistical analysis

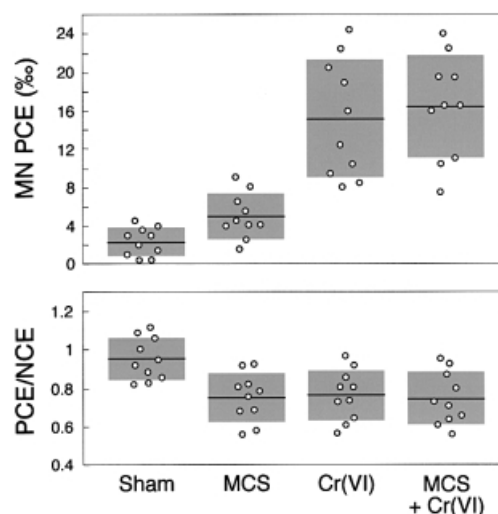Comparisons of mean values between treatment groups were made by Student's *t*-test for unpaired data.

Fig. 1. Frequency of MN PCE and PCE/NCE ratio in bone marrow cells of male BDF$_1$ mice, either sham-exposed (controls) or exposed whole-body to MCS for 5 consecutive days or receiving a single i.p. injection of potassium dichromate [Cr(VI)] or exposed to both agents [MCS + Cr(VI)]. Each symbol indicates the value observed in each animal. Mean ± SD values within each group of 10 mice are also shown.

Results

Clastogenic damage of mainstream cigarette smoke and/or chromium(VI) i.p. in BDF$_1$ mice

The results of this experiment are shown in Figure 1. The whole-body exposure of mice to MCS for 5 consecutive days resulted in a 2.1-fold increase in the frequency of MN PCE in bone marrow, as compared with sham-exposed mice ($P <$ 0.01). A single i.p. injection of a high dose of potassium dichromate (50 mg/kg body wt) resulted in a considerable clastogenic effect in bone marrow cells, with a 6.4-fold increase in the frequency of MN PCE ($P <$ 0.001). When potassium dichromate was administered at the end of the 5 day period of exposure to MCS, the clastogenic effect was similar to that induced by Cr(VI) alone, i.e. a 7.4-fold increase in the frequency of MN PCE ($P <$ 0.001).

Exposure to MCS, injection of Cr(VI) and their combination were all moderately toxic to bone marrow cells, as shown by a slight but significant decrease in the PCE/NCE ratio ($P <$ 0.01 in all cases).

Clastogenic damage of environmental cigarette smoke and chromium(VI) i.t. in Sprague–Dawley rats

The results of this experiment are shown in Figures 2 (PAM) and 3 (bone marrow cells). The whole-body exposure of Sprague–Dawley rats to ECS for 18 consecutive days resulted in a 2.3-fold increase ($P <$ 0.05) in MN PAM and a 2.4-fold increase ($P <$ 0.001) in PN PAM recovered by bronchoalveolar lavage, as compared with sham-exposed rats. The daily i.t. instillation of sodium dichromate (0.25 mg/kg body wt) for the same time period resulted in a 3.2-fold increase ($P <$ 0.001) in MN PAM and a 2.3-fold increase ($P <$ 0.001) in PN PAM. The combined exposure to ECS and Cr(VI) resulted in a 3.9-fold increase ($P <$ 0.001) in MN PAM and a 2.6-fold increase ($P <$ 0.001) in PN PAM.

The clastogenic effect produced by the two combined agents was less than additive. In fact, on subtracting the values observed in controls, the frequency of MN PAM was 1.6‰ in ECS-exposed rats and 2.8‰ in Cr(VI)-treated rats. Hence, the
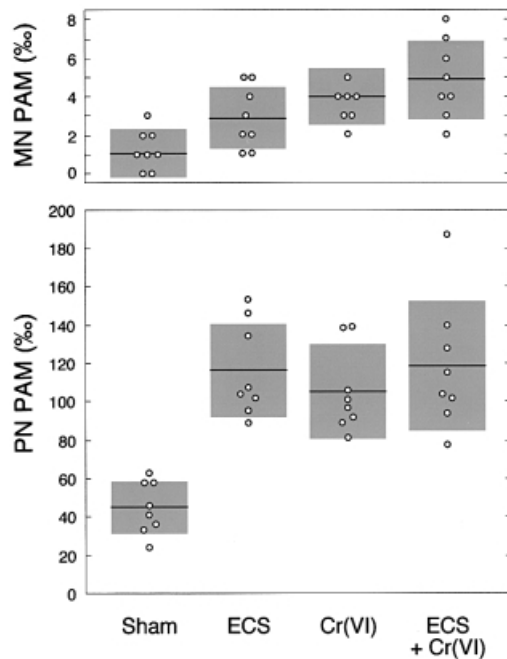
Fig. 2. Frequency of MN and PN PAM collected by bronchoalveolar lavage from male Sprague–Dawley rats, either sham-exposed (controls) or exposed whole-body to ECS for 18 consecutive days or receiving daily i.t. instillations of sodium dichromate [Cr(VI)] for the same time period or exposed to both agents [CS + Cr(VI)]. Each symbol indicates the value observed in each animal. Mean ± SD values within each group of eight rats are also shown.
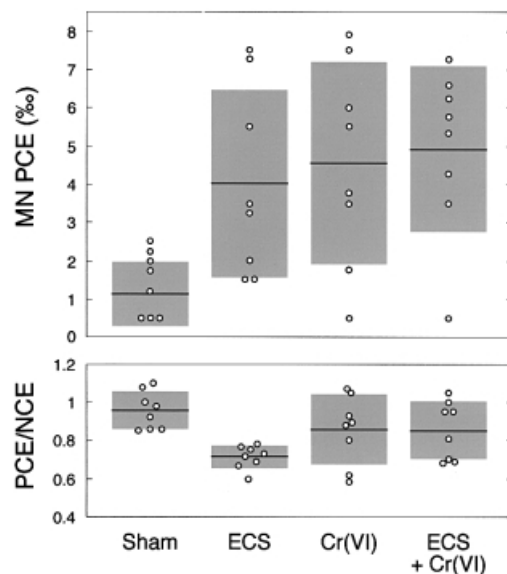

Fig. 3. Frequency of MN PCE and PCE/NCE ratio in bone marrow cells of male Sprague–Dawley rats, either sham-exposed (controls) or exposed whole-body to ECS for 18 consecutive days or receiving daily i.t. instillations of sodium dichromate [Cr(VI)] for the same time period or exposed to both agents [CS + Cr(VI)]. Each symbol indicates the value observed in each animal. Mean ± SD values within each group of eight rats are also shown.

expected additive frequency would have been 4.4‰, versus the observed 3.6‰ in the combined treatment group. Similarly, the net frequency of PN PAM was 71.4‰ in ECS-exposed rats and 60.5‰ in Cr(VI)-treated rats. Hence, the expected additive frequency would have been 131.9‰, versus the observed 73.6‰ in the combined treatment group.

The same conclusion was drawn by testing bone marrow cells from the same animals (Figure 3). In fact, exposure to ECS, administration of Cr(VI) and the combined treatment yielded very similar increases in the frequency of MN PCE, i.e. 3.5-, 4.0- and 4.3-fold, respectively. Of the three treatment groups, only that including ECS-exposed rats exhibited a slight but significant ($P < 0.01$) decrease in the PCE/NCE ratio.

Discussion

The results of the present study highlight the ability of both CS and Cr(VI) to induce clastogenic damage in animal models. At the same time, they rule out any occurrence of synergistic effects of the combined agents in affecting this type of early biological marker.

In spite of some negative findings, there is overwhelming evidence which supports the ability of CS to induce clastogenic effects in rodents. Thus, no significant effect was observed in studies evaluating sister chromatid exchanges (SCE) and chromosomal aberrations (CA) in bone marrow cells of CS-exposed Chinese hamsters (13), SCE in peripheral lymphocytes from Wistar II rats exposed whole-body to mainstream CS (14) and SCE, CA and MN in bone marrow cells of Sprague–Dawley rats exposed nose-only to mainstream CS (15). In contrast, an elevation of SCE was observed in bone marrow cells of both BC3F1/Cum mice (16) and B6C3F1 mice (17) following nose-only exposure to CS. Both structural and numerical CA were enhanced in PAM of Fischer 344/N rats exposed to CS, either nose-only or whole-body (18). An increased frequency of MN and binucleated PAM was observed in CBA/H mice exposed whole-body to CS (19). Furthermore, a CS condensate enhanced MN PCE in the bone marrow of NMRI mice treated i.p. (20) and, interestingly, sidestream smoke was more potent than mainstream smoke in producing this clastogenic effect (21).

In our laboratories, the whole-body exposure of BDF_1 mice to mainstream CS had previously been found to increase the frequencies of MN PCE in bone marrow (22), of MN NCE in peripheral blood (23) and, after transplacental passage, of MN PCE in fetal liver and in the liver and peripheral blood of newborn mice (24). Moreover, whole-body exposure to MCS enhanced the frequency of both MN PAM and MN PCE in Sprague–Dawley rats (25), BD_6 rats (26, 27) and Balb/c and BDF_1 mice (27). Recently, we investigated the time course of induction and persistence of cytogenetic alterations in cells of BDF_1 mice exposed whole-body to mainstream CS for 3 weeks. The frequency of MN PCE increased early in exposure but declined to background levels upon discontinuation of exposure to CS. The appearance of MN NCE in peripheral blood was slightly delayed but persisted for an additional 3 weeks and formation of MN PAM and binucleated PAM required 2–3 weeks to reach a plateau, which persisted until 11 months after discontinuation of exposure to CS (9).

The findings of the present study are in line with our previous experience in BDF_1 mice exposed to MCS and, in addition, they show the occurrence of cytogenetic alterations in Sprague–Dawley rats exposed to ECS. In particular, CS induced in both mice and rats a significant increase in MN PCE and was slightly toxic to bone marrow cells, as shown by a modest but significant decrease in the PCE/NCE ratio. Moreover, ECS induced clastogenic damage in the lower respiratory tract of rats, which was documented by an enhancement of MN PAM and PN PAM. The latter type of alteration

is regarded as the result of a cytokinesis failure or block after completion of nuclear division, due to disruption of the cellular skeleton, rendering the production of additional genetic damage more probable (9). Under the same conditions and in the same rat strain, ECS induced the formation of nucleotide alterations, detected by ^{32}P-post-labeling in the tracheal epithelium, bronchoalveolar lavage cells, lung, heart, bladder and testis (28).

Hundreds of results document the ability of Cr(VI) compounds, once available to target cells, to induce genetic and related effects in a variety of *in vitro* test systems (reviewed in refs 29,30). In contrast, the majority of clastogenic surveillance studies among Cr(VI)-exposed workers yielded negative or inconclusive results (reviewed in ref. 31). The conflicting nature of these data can be interpreted by taking into account the ability of the host organism to reduce and detoxify Cr(VI). These mechanisms, which attenuate the potential toxicity and carcinogenicity of Cr(VI), have been evaluated in quantitative terms in human body compartments (32).

The majority of *in vivo* studies in rodents showed the ability of soluble Cr(VI) compounds to induce cytogenetic damage, but less consistently than in *in vitro* models (29). Moreover, most *in vivo* studies involved administration routes which do not mimic any human exposure and used 'excessive' doses which can overwhelm the host defence mechanisms. For instance, the highly soluble Cr(VI) salts potassium dichromate and potassium chromate increased MN PCE in bone marrow cells of Chinese hamster and various mouse strains following i.p. administration of tens of milligrams of these compounds per kilogram body weight (33–38). Calcium chromate, which is slightly less soluble, was negative in both hamster and mouse when tested in comparative experiments under identical conditions (34,35). Moreover, administration of up to 20 mg Cr(VI), in the form of potassium dichromate, either in drinking water or by gavage, failed to produce any effect in the mouse bone marrow micronucleus test (39). This can be ascribed to the potent detoxification of Cr(VI) in the gastrointestinal tract, especially due to reduction by gastric juice (32).

Since the main goal of our study was to evaluate the occurrence of interactions between Cr(VI) and CS, we intentionally chose experimental conditions which were expected to yield positive results. Thus, i.p. injection of potassium dichromate at 50 mg/kg body wt produced an increase in MN PCE in mouse bone marrow, in agreement with the findings of the above referenced studies (33–38). Note that this would correspond to 3.5 g potassium dichromate [1.24 g Cr(VI)] given i.p. to a man weighing 70 kg.

In the experiment using Sprague–Dawley rats, we administered sodium dichromate i.t., since this type of administration bypasses the highly developed nasal filter of rodents and favors delivery of test agents to the lower respiratory tract (40). The dose we selected (0.25 mg/kg body wt) was the same as has been shown to produce a uniform distribution of sodium dichromate in rat lung (41). Administration for 3 consecutive days caused DNA fragmentation, DNA–protein crosslinking, ^{32}P-post-labeled DNA modifications and an increase in 8-hydroxy-2′-deoxyguanosine in the lung but not in the liver (42). When given 5 days/week for 4 weeks, it autoinduced Cr(VI) metabolism in the lung (43). When given for life, it failed to induce lung tumors (44). Therefore, it appears that this treatment induces several biomarkers in rat lung, as confirmed in the present study, but is not carcinogenic.

The dose of 0.25 mg/kg body wt sodium dichromate, given i.t. for 18 consecutive days, would correspond to a daily intake of 17.5 mg via the respiratory route in a man weighing 70 kg and therefore it would mimic exposure of a man to air containing ~1 mg/m^3 sodium dichromate [0.37 mg Cr(VI)/m^3], which is a very high dose. The Cr(VI) reducing ability of PAM is ~2 µg/10^6 cells in both rats (45) and non-smoking humans (46). A further defence mechanism of the lower respiratory tract is provided by the epithelial lining fluid (ELF), which is particularly rich in antioxidants, such as reduced glutathione (GSH), vitamin C (ascorbic acid), vitamin E (tocopherol), superoxide dismutase, catalase, albumin, ceruloplasmin, transferrin, lactoferrin and other proteins (47). The human ELF, which has been estimated to have a total Cr(VI) reducing capacity of 0.9–1.8 mg (32), is particularly rich in GSH (48), whereas in rats the Cr(VI) reducing activity of this fluid has mainly been ascribed to ascorbic acid (49). The results obtained in the present study provide evidence that the exposure regimen to sodium dichromate (4.5 mg/kg body wt instilled over 18 days) was sufficient to produce cytogenetic damage in PAM, which were directly exposed, as well as in PCE, after transportation of Cr(VI) in amounts escaping detoxification in the blood.

The results of the two experiments described herein show that the clastogenic effects produced either by MCS in combination with Cr(VI) i.p. in mice or by ECS in combination with Cr(VI) i.t. in rats are less than additive. This conclusion was drawn by analyzing both bone marrow cells and PAM, which were exposed to both agents at the high doses which are needed in experiments in animal models to yield positive results. These data agree with the finding that no synergistic effect between CS and stainless steel welding was observed in the induction of chromosome damage in lymphocytes (50). Smokers usually show higher urinary chromium levels than non-smokers (31), which might be ascribed to enhanced retention of particulates in the bronchial tree of smokers. An alternative interpretation is that stimulation of Cr(VI) reduction by CS leads to increased urinary excretion of Cr(III). In fact, in previous studies we showed that an MCS condensate attenuated the direct mutagenicity of Cr(VI) in strain TA100 of *Salmonella typhimurium*, presumably due to the presence of reducing agents in this material. At the same time, Cr(VI) inhibited the metabolic activation of both MCS condensate and benzo[*a*]pyrene to mutagenic derivatives in strain TA98 (51). An antagonistic effect between Cr(VI) and benzo[*a*]pyrene diolepoxide was recently shown by evaluating the frequency of *HPRT* mutants in cultured human fibroblasts (52).

The hypothesis that synergism may occur between CS and other agents, including carcinogenic metals, is often raised in the literature (1), but, with the previously reported exceptions, it is merely speculative and not substantiated by experimental or epidemiological data. It should be mentioned that antagonistic effects are also possible, as shown not only by epidemiological studies with methyl ethers (2,3), but also by animal studies evaluating the combination of MCS with urethane (53) and of ECS with either urethane or 3-methylcholanthrene (8) in inducing lung tumors in mice.

From a mechanistic point of view, it is noteworthy that two independent *ex vivo* studies in humans, both of them analyzing blind samples, demonstrated enhanced Cr(VI) reduction in the respiratory tract of smokers. In particular, the PAM from 11 current smokers exhibited a Cr(VI) reducing capacity which was significantly higher than that of either eight ex-smokers or four never-smokers (46). Similarly, preparations of peripheral lung parenchyma from 45 smokers were significantly

more efficient than those from 26 non-smokers in decreasing Cr(VI) mutagenicity (54). At the molecular level, a mixture of Cr(VI) and a CS condensate in an acellular system resulted in enhanced generation of OH· radicals and induction of single-strand breaks in plasmid DNA (55). Although of interest, this type of approach suffers from the limitation that generation of OH·, which does not live more than 1 ns and does not travel farther than 1 nm (56), was artificially achieved in a tube in close contact with DNA, rather than in a cell or in a whole organism.

In conclusion, we have evidence that: (i) CS and Cr(VI) behave antagonistically in an *in vitro* mutagenicity test system (51); (ii) the Cr(VI) reducing capacity of both human PAM (46) and peripheral lung parenchyma (53) is enhanced in smokers; (iii) the clastogenic effects of CS and Cr(VI), irrespective of the route of administration, are less than additive in PAM and bone marrow cells of rodents (this study). Thus, in the absence of any epidemiological evidence (1,7), both metabolic markers in humans and genotoxicity biomarkers in rodents suggest lack of synergism between CS and Cr(VI) in certain stages of the carcinogenesis process. We have implemented further studies aimed at evaluating the consequences of combined exposure on molecular biomarkers, such as formation of DNA adducts, oxidative DNA damage and occurrence of DNA–protein crosslinks in rat lung.

Acknowledgements

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