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Nasal cell micronuclei, cytology and clinical symptoms in stainless steel production workers exposed to chromium

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The objective of the present study was to determine whether workers in stainless steel production with low exposure to various forms of chromium show an increase in micronucleated nasal cells or an excess of nasal symptoms or disease. Altogether, 48 workers employed in a stainless steel production chain were studied, 29 of them in the steel melting shop with exposure to hexavalent chromium (Cr^{6+}), 14 in the sintering and crushing departments of the ferrochromium plant with exposure to trivalent chromium (Cr^{3+}) and five in the mine with exposure to chromite ore (Cr^{3+}). Thirty-nine workers from the cold rolling mill, with very low exposure to chromium, served as referents. All the subjects were never smokers with a minimum of 14 years employment in the same department. There were no significant differences between the exposure groups and the referents regarding the mean frequency of centromere-negative or centromere-positive micronuclei (studied by pancentromeric fluorescence *in situ* hybridization), nasal diseases and symptoms or mucociliary clearance of the nasal cavity. No statistically significant differences in the incidence of cell atypia or inflammatory cells were detected between the exposed workers and the reference group, except for an increase in lymphocytes among the chromite ore workers. Anterior rhinoscopy indicated slight inflammatory changes in nasal mucosa and secretion more often in the Cr^{6+} and Cr^{3+} groups than in the referents, the Cr^{6+}-exposed workers showing more livid or oedemic epithelium. In conclusion, the stainless steel production workers, with low exposure to dusts or fumes containing hexavalent or trivalent chromium, did not show clinical changes in the nasal mucosa or an increase in nasal cell micronuclei or symptoms of nasal diseases, except for slight changes in the nasal epithelium and secretion.

Introduction

Occupational exposure to hexavalent chromium (Cr^{6+}) in the production of chromates and chromate pigments and in chromium plating has been associated with an increased risk of lung cancer (Sorahan *et al.*, 1988; International Agency for Research on Cancer, 1990; Rosenman and Stanbury, 1996; De Flora, 2000). Chromium plating workers have also been described as being at an elevated risk of perforation of the nasal septum, ulceration of the nasal mucosa (Kuo *et al.*, 1997), atypical nasal epithelium (Bolla *et al.*, 1990) and nasal cancer (International Agency for Research on Cancer, 1990; De Flora, 2000). In other professions with exposure to chromium, including the production of ferrochromium and stainless steel, data on cancer risk have been equivocal (IPCS, 1988; International Agency for Research on Cancer, 1990; Langård *et al.*, 1990; Moulin *et al.*, 1990, 1993, 1995, 2000; Simonato *et al.*, 1991; Moulin, 1997; Danielsen *et al.*, 2000).

The carcinogenicity of Cr^{6+} may partly be mediated through genotoxic mechanisms. Cr^{6+} was shown to be genotoxic in various short-term tests (Shindo *et al.*, 1989; Yang *et al.*, 1992; Katz and Salem, 1993; Wise *et al.*, 1993; Beyersmann, 1994; Manning *et al.*, 1994; Hartwig, 1995; De Flora, 2000). The frequencies of chromosome aberrations, sister chromatid exchanges (SCEs) and micronuclei (MN) have been found to be elevated in peripheral lymphocytes of chromium electroplating workers (Sarto *et al.*, 1982; Stella *et al.*, 1982; Lai *et al.*, 1998; Vaglenov *et al.*, 1999; Wu *et al.*, 2000; Benova *et al.*, 2002).

We have studied a unique production chain of stainless steel, in which the chromite ore mine, ferrochromium works and stainless steel production units are located in the same region (Huvinen *et al.*, 1993, 1996, 1997, 2002). An exposure study was carried out for the entire production chain in 1987 (Huvinen *et al.*, 1993). Exposure to Cr^{6+} was detected in the steel melting shop, where the median Cr^{6+} concentration in the air was 0.5 $\mu g/m^3$. The highest personal values were recorded during the handling of molten metal by the arc furnace (6.6 $\mu g/m^3$). Cr^{6+} was also present in personal air samples collected in the furnace department of the ferrochromium plant, with average levels of 0.075–0.45 $\mu g/m^3$; the highest concentrations were detected during tapping in the vicinity of the tap hole, where the proportion of Cr^{6+} was 10-fold higher than the level in other areas. Workplace exposure levels have been monitored on a regular basis, and the trend has been towards a decrease (Huvinen *et al.*, 2002).

A study of the respiratory health of ferrochromium and stainless steel production workers with an average exposure time of 18 years indicated no respiratory changes detectable by lung function tests or radiography and no increase in symptoms of respiratory diseases (Huvinen *et al.*, 1996). Chromite miners had a decreased lung function and an increased occurrence of radiological findings, possibly partly attributable to high dust exposures in the past and to the fibrous components of the dust.

A magnetopneumographic study of the lungs suggested low exposure to magnetic dust (Huvinen *et al.*, 1997). Miners and workers from the concentrator and sintering plants and the cold rolling mill showed a remnant magnetic field (RMF) comparable to that of the referents. Among workers in the ferrochromium and steel melting shops, the RMF was slightly

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Table I. Number, mean age and mean years of employment of the subjects in the study groups

Group of workers	Department	No. of subjects	Mean age (range) (years)	Mean duration of employment (range) (years)
Cr^{6+}	Steel melting shop	29	48.1 (35–62)	21.4 (14–30)
Cr^{3+}	Ferrochromium works; sintering and crushing	14	48.4 (34–56)	24.4 (14–30)
Chromite	Chromite ore mine	5	49.2 (39–58)	27.6 (19–31)
Referents	Cold rolling mill	39	44.6 (35–59)	20.2 (17–22)
All		87	46.6 (34–62)	21.7 (14–31)

elevated, although lower than levels observed in a welding or repair shop. There was a relationship between the RMF and the urinary chromium concentration. Sintering plant workers and possibly also miners and concentrator workers showed a retarded RMF relaxation rate. The duration of exposure correlated weakly but significantly with the relaxation rate.

A follow-up study in 1998 confirmed the results of the previous cross-sectional study in 1993. An average exposure time of 23 years in modern ferrochromium and stainless steel production and low exposure to dusts and fumes containing Cr^{6+} and Cr^{3+}, nickel and molybdenum did not lead to respiratory changes detectable by lung function tests or radiography or to an increase in symptoms of respiratory disease. The workers exposed to Cr^{3+} still had more respiratory symptoms than those in the reference group and the workers in the chromite mine had still lower lung function test results than did the reference group, but the difference was smaller than 5 years earlier.

The purpose of our present study was to determine whether genotoxic exposure to low levels of Cr^{6+}, Cr^{3+} or chromite ore (Cr^{3+}) in the stainless steel production chain could induce an increase in micronucleated nasal cells. The micronuclei (MN) detected were characterised using fluorescence *in situ* hybridisation (FISH), to distinguish the micronucleation of chromosome fragments from that of whole chromosomes. We also studied whether long-term occupational exposure in the various steps of the chain causes nasal diseases detectable by clinical or cytological methods or an excess of nasal symptoms.

Materials and methods

Subjects and their exposure

At the time of this study, April–May 1998, there were 1050 workers in the production departments. Only never smokers with a minimum of 14 years employment in the same department were included in the study. Altogether 97 workers met these criteria, and 88 participated in the study. All the subjects were men with similar ethnic and socio-economic backgrounds. One participant had to be omitted from the analyses because of a failure in the preparation of his cell sample.

The reasons for not agreeing to participate among the nine workers who otherwise met the criteria were temporary work abroad, hospital examinations other than for otorhinolaryngological reasons, holiday, impossibility to match work shifts with the study appointment and no specific reason (five subjects).

The subjects were divided into four groups according to exposure to different chromium compounds. The Cr^{6+} group included 29 workers from the steel melting shop. The previous exposure study (Huvinen *et al.*, 1993) had indicated an average exposure of 1.8 mg/m^3 total dust. The dust contained 2–4% chromium and the median Cr^{6+} concentration was 0.5 μg/m^3. The highest values were detected in personal samples from the handling of molten metal by the arc furnace (6.6 μg/m^3). As judged from stationary samples, Cr^{6+} seemed to be present at low concentrations throughout the steel melting shop, although it exceeded the detection limit of 0.5 μg/m^3 in only some of the samples. In 1999 the median concentrations in the steel melting shop were 0.70 mg/m^3 total dust, 5.3 μg/m^3 total chromium, 0.3 μg/m^3 Cr^{6+}, 3 μg/m^3 nickel and 0.6 μg/m^3 molybdenum (Huvinen *et al.*, 2002).

All 14 workers in the Cr^{3+} group were working in the sintering and crushing departments of the ferrochromium works, where the average dust level was 2.4 mg/m^3 (Huvinen *et al.*, 1993). The average exposure level to total chromium was 248 μg/m^3 (Huvinen *et al.*, 1993). During the sintering process reduced remains in the trivalent oxidation state, as in chromite, until it is in the arc furnace.

The chromite group included five chromite ore miners. The average dust concentration in the air of the miners was 1 mg/m^3 and the median personal exposure level to chromium was 22 μg/m^3; Cr^{6+} was not detected in any of the samples (Huvinen *et al.*, 1993).

The reference group consisted of 39 workers from the cold rolling mill (the Sendzimir rolling mill, the skin pass mill and the splitting and cutting line) where the level of exposure to dust in general, and to chromium in particular, was very low. Throughout the cold rolling mill the total dust content averaged 0.3–0.5 mg/m^3 (Huvinen *et al.*, 1993). The chromium content of the air in this facility was generally below the detection limit (10 μg/m^3) of the measurement method.

The groups studied were rather similar with respect to age and years of employment in their present departments (Table I). The exposed workers had been employed in the same department, on average, for 23 years, whereas the reference group had been employed by the company for 20 years. The reference group was, on average, slightly younger (almost 4 years) than the other three groups.

Clinical examination

The subjects filled out a detailed questionnaire assessing nasal symptoms and their relation to work conditions. The physical examination included anterior rhinoscopy and rigid nasoendoscopy (Hopkin's endoscopes, diameter 2.3 mm, 0° and 30°). The mucociliary clearance of the nasal cavity was determined using the saccharine test. A particle of saccharine (diameter 3 mm) was placed on the antero-medial portion of the right inferior turbinate. The time between application and the patient's subjective sensation of a saccharine taste in the pharynx was recorded.

Sample collection

A cytological sample was collected from the middle meatus with an endocervical brush (Accellon Multi; Medscan Medical AB, Malmö, Sweden). After sampling, the sample brush was rinsed in 15 ml of physiological (0.9%) NaCl for ~1 min and the resulting cell suspension was divided into two parts, 6 ml for the cytological examinations and 9 ml for MN analysis.

Cytological examination

The nasal cell suspensions were centrifuged, the supernatant was removed and the cells were fixed in 2.5% (w/v, final concentration) polyethylene glycol and 50% (v/v) ethanol. For each subject, the cells were centrifuged on two glass slides in a Cyto-tek (Miles Scientific, Electra Industries, USA) cytological centrifuge. Subsequently, one slide was stained with Papanicolau's stain and the other (for the detection of eosinophils) with Wright's stain. For each subject, 200 cells were classified according to cell type, to determine the relative proportion of various types of cells in the sample. The types included columnar cells (subgrouped as surface, mid-level or basal cells), goblet cells, squamous cells, neutrophils, lymphocytes and eosinophils. In addition, the presence of erythrocytes was recorded. The cells were also screened for cytological changes, including metaplasia and dysplasia.

Micronucleus assay

Microscopical slides (5–15 per sample) for the analysis of MN were prepared at the site of the sampling. Each 9 ml cell suspension was centrifuged (2000 r.p.m., Hermle Z 230, angle rotor), the supernatant was removed and a small amount of physiological NaCl was added to obtain a milky cell suspension. The slides (5–15 per sample) were prepared using a cytocentrifuge (50 μl sample/slide, 1500 r.p.m. for 5 min; Cytospin 3; Shandon Scientific Ltd, Astmoor, UK). The cell density on the slides was checked under a microscope and, if necessary, the suspension was further diluted to obtain optimal preparations. At the cytogenetic laboratory the slides were allowed to air dry for 24–45 h. They were then fixed in 80% methanol at 4°C for 24–48 h, air dried and stored at –20°C until FISH. A modification of previously

Table II. Number of subjects showing infected, livid/oedemic and atrophic nasal epithelium or atypical nasal secretion in anterior rhinoscopy

Group	No. of subjects	Subjects with atypical nasal epithelium								Subjects with atypical nasal secretion	
		Infected		Livid/oedemic		Atrofic		Total			
		%	RR (CI)	%	RR (CI)	%	RR (CI)	%	RR (CI)	%	RR (CI)
Cr^{6+}	29	28	1.5 (0.64–3.7)	38	3.7 (1.3–10.6)	7	2.7 (0.36–20.2)	72	2.4 (1.4–4.1)	72	1.8 (1.1–2.8)
Cr^{3+}	14	43	2.4 (0.95–5.7)	14	1.4 (0.31–5.8)	14	5.6(0.75–40.9)	71	2.3 (1.3–4.1)	86	2.1 (1.3–3.3)
Chromite	5	60	3.3 (1.1–8.0)	0	NA[a] (up to 5.5)	0	NA (up to 26.4)	60	2.0 (0.68–4.0)	40	0.98 (0.27–2.3)
Referents	39	18	1.0	10	1.0	3	1.0	31	1.0	41	1.0

For the chromium-exposed groups, risk ratios (RR) and 95% confidence intervals (CI) are shown in comparison with the referents.
[a]NA, not available; upper bound for confidence interval shown in parentheses.

described methods (Titenko-Holland *et al.*, 1994, 1996; Surrallés *et al.*, 1997) was used in the FISH staining. Briefly, a digoxigenin-conjugated α satellite DNA probe (All Human Centromeres Probe; ONCOR, Gaithersburg, MD) was hybridized with the cells and was detected with anti-dixoxigenin (Sigma, St Louis, MO) and fluorescein-conjugated secondary antibodies (Boehringer Mannheim, Mannheim, Germany). The slides were counterstained with propidium iodide (95–98%; Sigma) and 4′,6'-diamidino-2-phenylindole (DAPI) (98%; Aldrich Chemical Co., Milwaukee, WI). Thereafter, the slides were coded and mounted in antifade solution (Vectashield Mounting Medium; Vector Laboratories, Burlingame, USA) and analysed by one scorer using a Leica Dialux 22 fluorescence microscope with a 100× objective and 12.5-fold ocular lens. For each subject, 4000 cells were scored for the presence of MN at UV excitation (filter block A, detection of DAPI). The MN found were characterized at blue and green excitation (filter blocks I3 and N2.1, detection of fluorescein and propidium iodide) as centromere-positive (C^+) (fluorescein label present), centromere-negative (C^-) (fluorescein label absent) or not classifiable (fluorescein label not unequivocal). All the MN (including those not classifiable as to centromere content) were used in calculating the frequency of micronucleated cells. The percentages of C^+ and C^- MN were used to calculate the frequencies of C^+ and C^- MN. When all the analyses were ready, the code was broken.

Statistical methods

For comparing the prevalence of categorical variables (such as nasal discharge and the condition of the mucous membrane) between each exposed group and the reference group, likelihood-based risk ratios (Miettinen and Nurminen, 1985) and their 95% confidence intervals were used. The same method was applied to study the effect of atypical columnar cells on the amount of MN.

In the case of the continuous variable (saccharine test), the Kolmogorov–Smirnov test was applied. This test and the Wilcoxon two-sample test were used to study the differences in the distributions of nasal cell types between each exposed group and the reference group.

The frequencies of the C^+ and C^- MN were determined separately, because they represent different biological entities. The effect of occupational exposure and the atypia of columnar epithelium were analysed by the Kolmogorov–Smirnov test and the Wilcoxon test. The linear interdependence between the MN frequencies and different nasal cell types were studied using Spearman rank correlation coefficients.

Most of the statistical analyses were performed using the Statistical Analysis System v.6.12 software (SAS Institute Inc., 1989). However, the risk ratios were calculated by the special Rate Analysis program (Finnish Institute of Occupational Health, Helsinki).

Results

Clinical findings

There were no marked differences between the groups regarding previous nasal diseases such as nasal polyps, sinusitis or nasal obstruction symptoms. None of the subjects had a nasal tumour, chronic ulceration or septal perforation. Nasal polyps were seen in only one person (Cr^{6+} group). Some of the exposed workers showed drying of the nasal epithelium and metal dust in the nasal vestibulum and in the middle meatus. In the anterior rhinoscopic examination the nasal epithelium more often showed changes in the Cr^{6+} and Cr^{3+} groups than in the reference group (Table II). In these groups nasal secretion was commoner than in the referents (Table II). There were no significant differences in the results of the saccharine test between the groups (data not shown).

Cytology

In general, the commonest cell type (~75% of all cells) detected in the nasal samples represented columnar surface epithelium, other epithelial cells accounting for ~14% of all cells (Table III). No cytological evidence of dysplasia or other malignancy was detected. Benign atypia of the columnar epithelium was seen in 28 cases and squamous metaplasia was found in four subjects. On average, 11% of the cells were neutrophils, the highest individual value reaching 63% (in the chromite group). Eosinophils and lymphocytes were very rare (on average 0.07 and 0.2%, respectively). However, the five chromite ore workers had more lymphocytes in their nasal samples than did the referents (0.68 versus 0.18%, $P = 0.025$). Generally, there were no indications of major infectious or allergic events. When the results were analysed with regard to occupational exposure, we were unable to detect any significant differences in columnar epithelial cell atypia or inflammatory cells between the referents and workers exposed to Cr^{6+} or Cr^{3+}.

Micronuclei

Table IV shows that, on average, slightly over one-third of the MN found carried a centromeric label (C^+ MN) and were thus regarded as containing a whole chromosome. The majority of MN showed no centromeric signals (C^- MN) and were accordingly considered to consist of chromosome fragments.

The mean frequency of C^- MN was very similar in the study groups, suggesting no exposure effects (Table II). The mean frequencies of C^+ MN also showed no differences among the four groups. No evidence of a genotoxic effect of Cr^{6+} or Cr^{3+} on nasal cells was evident in the statistical analysis. The proportions of the different nucleated cell types or the presence of atypia, as derived from the cytological analysis, did not affect the frequency of MN.

Discussion

Sinonasal cancer is a rare disease. Exposure to chromates is among the few occupational exposures considered to be associated with this malignancy. The present study on a stainless steel production chain included a group of workers exposed to Cr^{6+} in a steel melting shop in addition to a group of ferrochromium workers primarily exposed to Cr^{3+} and a small subgroup of chromite (Cr^{3+}) miners. As the exposure to Cr^{6+} was generally low among the workers in the steel melting shop, it was no surprise that they showed no chronic ulcerations, septal perforations, nasal tumours or impairment of mucosal

Table III. Results of the cytological analysis of cells exfoliated from the nasal middle meatus

Group	No. of subjects	Mean percentage (range) of various types of nucleated cells				No. (%) of subjects with	
		Epithelial cells		Inflammatory cells		Atypia of columnar epithelium	Squamous metaplasia
		Surface columnar	Other types	Neutrophils	Other types		
Cr^{6+}	29	76.2 (43–94)	13.7 (4–32)	9.7 (1–39)	0.4 (0.0–2.1)	10 (34.5)	1 (3.5)
Cr^{3+}	14	74.9 (33–93)	12.6 (6–22)	12.0 (0–59)	0.4 (0–1.2)	5 (35.7)	0 (0.0)
Chromite	5	64.4 (22–88)	16.0 (7–32)	18.8 (1–63)	0.8 (0.1–2.2)	2 (40.0)	0 (0.0)
Referents	39	75.2 (32–92)	14.1 (6–29)	10.4 (0–52)	0.3 (0–2.6)	11 (28.2)	3 (7.7)

Table IV. Mean number of nasal cells containing MN with (C^+, whole chromosomes) or without (C^-, chromosomal fragments) a centromeric signal in 4000 cells scored per subject

Group of workers	No. of subjects	C^+ MN (SD, range)	C^- MN (SD, range)
Cr^{6+}	29	2.62 (2.65, 0–10)	4.41 (2.40, 1–9)
Cr^{3+}	14	2.29 (2.02, 0–6)	4.50 (2.03, 2–8)
Chromite	5	2.80 (3.27, 0–8)	4.80 (2.77, 2–8)
Referents	39	2.67 (2.22, 0–11)	5.18 (2.91, 0–14)
All	87	2.60 (2.36, 0–11)	4.79 (2.59, 0–14)

functions. Nasal epithelium lividity/oedema was increased among the Cr^{6+} group and alterations in the nasal epithelium were also commoner in the Cr^{3+} group than in the reference group. In both exposure groups nasal secretion deviated from normal more often than among the referents.

It is known that adenocarcinomas of nasal mucosa exclusively originate in the region of the middle turbinate and the adjacent ethmoidal region. Cytological examination of samples collected from these areas could thus provide additional information on exposure effects before any clinical signs are apparent. Therefore, the present study also included a cytological study of cells collected from the middle meatus.

Previous cytogenetic studies performed on peripheral lymphocytes of workers exposed to chromic acid in the chromium plating industry have shown an increase in chromosome damage (Sarto *et al.*, 1982; Stella *et al.*, 1982; Lai *et al.*, 1998; Vaglenov *et al.*, 1999; Benova *et al.*, 2002), with the exception of one study on SCEs (Nagaya *et al.*, 1989). In general, the majority of cytogenetic surveillance studies on occupational chromium exposure have been negative, which has been explained by the expected low Cr^{6+} exposure of peripheral lymphocytes, as compared with the lungs (Kortenkamp, 1997). In the furnace sector of a ferrochromium plant 'a qualitatively limited increase' in aberrant cell frequencies was reported by Sbrana *et al.* (1990).

MN in epithelial cells of subjects occupationally exposed to chromium have earlier been examined in only two studies on electroplating workers (Sarto *et al.*, 1990; Benova *et al.*, 2002). Sarto *et al.* (1990) observed no increase in MN in buccal or nasal mucosa. Chromic acid is expected to reduce cell division strongly in nasal epithelium and, since MN are formed only in dividing cells, their production might not be detected. On the other hand, Benova *et al.* (2002) found twice as high frequencies of buccal MN in chromium platers than control persons; micronucleated cells were also very frequent in the controls (mean 11.48 per 1000 cells), allegedly due to air pollution. The effect concerned both C^- and C^+ MN, which appeared to agree with an *in vitro* study that showed induction of both kinetochore-positive and kinetochore-negative MN by chromium chloride (Cr^{3+}) and potassium dichromate (Cr^{6+}) (Seoane and Dulout, 2001).

In our present study no increase in C^- or C^+ MN could be found in the nasal cell samples of the workers. Again, this result is likely to reflect the relatively low exposure of the workers in the modern production line, where the average levels of Cr^{6+} in the air (0.4–0.5 μg/m^3) (Huvinen *et al.,* 1993, 2002) appear to be clearly lower than in the chromium plating industry (e.g. 11.8 μg/m^3) (Health and Safety Executive, 1990). In the study of Benova *et al.* (2002) the chromium platers who showed a doubling of micronucleated buccal cells had been exposed to ambient air Cr^{6+} levels of 4.2–47.2 μg/m^3.

The reductive capacity of the mucosa and reduction and trapping of Cr^{3+} and reactive oxygen species inside the target cells probably adequately protect the cells from the genotoxic effects of Cr^{6+}. These defence mechanisms may be overcome only at high exposures, so that a threshold has been proposed for Cr^{6+} carcinogenicity (De Flora *et al.*, 1997; De Flora, 2000). Cr^{6+} is taken up by cells much more efficiently than is Cr^{3+} and this difference appears to explain the greater genotoxicity of Cr^{6+} than Cr^{3+} in experimental settings. The mechanism of Cr^{6+} (or Cr^{3+}) genotoxicity is not known in detail. The process was suggested to involve the generation of reactive oxygen species (Mattagajasingh and Misra, 1995) and the DNA reactive forms were considered to result from the intracellular reduction of Cr^{6+} (Snow and Xu, 1991; Bridgewater *et al.*, 1994).

In conclusion, we have studied workers employed for an average of 23 years in modern ferrochromium or stainless steel production with low exposure to dusts or fumes containing Cr^{6+} or Cr^{3+}. The subjects did not show clear clinical or cytological changes in their nasal mucosa or an increase in symptoms of nasal diseases, except for changes in nasal mucosa and secretion that were possibly related to dust exposure. No genotoxic effects attributable to occupational chromium exposure were observed in the MN analysis of exfoliated nasal cells.

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