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Chromosome damage in underground coal miners: detection by conventional cytogenetic techniques and by submitting lymphocytes of unexposed individuals to plasma from at-risk groups

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ABSTRACT

Chromosome abnormalities and the mitotic index in lymphocyte cultures and micronuclei in buccal mucosa cells were investigated in a sample of underground mineral coal miners from Southern Brazil. A decreased mitotic index, an excess of micronuclei and a higher frequency of chromosome abnormalities (fragments, polyploidy and overall chromosome alterations) were observed in the miners when compared to age-paired normal controls from the same area. An alternative assay for clastogenesis in occupational exposition was tested by submitting lymphocytes from non-exposed individuals to a pool of plasmas from the exposed population. This assay proved to be very convenient, as the lymphocytes obtained from the same individuals can be used as target as well as control cells. Also, it yielded a larger number of metaphases and of successful cultures than with common lymphocyte cultures from miners. A significantly higher frequency of chromatid gaps, fragments and overall alterations were observed when lymphocytes from control subjects were exposed to miner plasma pools. Control plasma pools did not significantly induce any type of chromosome alterations in the cultures of normal subjects, thus indicating that the results are not due to the effect of the addition of plasma pools *per se*.

INTRODUCTION

Interest in occupational exposure and disease has greatly increased in the last 20 years and is related to growing interest in environmental quality control. Concern about the possibility that industrial wastes and products may cause illness in the general population and that workers are likely to be the first to show the effects caused by hazardous exposure has motivated the development of various research methods aiming at the detection and the identification of environmental exposure to genotoxic conditions (review in Stewart Houk, 1992).

Analysis of chromosome alterations is widely used for evaluating hazardous substances, since the relationship between clastogenesis, mutagenesis and carcinogenesis is a well-established fact. Cytogenetic analysis can provide a useful tool for monitoring at-risk populations and individuals (reviews in Ashby and Richardson, 1985 and Sorsa and Yager, 1987).

Natarajan *et al.* (1983) presented a bioassay for screening cytostatic drug activity in plasma by incubating Chinese hamster ovary (CHO) cells with

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human or rat plasma containing active metabolites of cyclophosphamide, and evaluating its genotoxic effect by determining the frequency of induced sister chromatid exchanges (SCE). Darroudi and Natarajan (1985), using the same system, extended these studies by evaluating the mutagenic action of 20 different directly and indirectly acting chemicals. Their data show that this method could be useful for the demonstration of genotoxicity. Arnsdorff-Roubicek and Targa (1990) performed a similar assay by using plasmas of rats treated with cyclophosphamide, and evaluating the genotoxic effects by determining the frequency of SCE in human lymphocytes, which they found to be increased.

MATERIAL AND METHODS

The coal miners sample studied in the present investigation consisted of 51 males, 20-40 years old, working for at least one year in underground coal mining in Criciúma, State of Santa Catarina, Brazil. The controls were 51 males, in the same age range as the miners, recruited among students and professional staff from the Federal University of Santa Catarina, Florianópolis, State of Santa Catarina, 200 km from Criciúma.

All participants answered a questionnaire on their health status, smoking habits, X-ray exposure and medications. The selected donors were all in healthy condition and were not using any medication for at least 60 days prior to sample collection. Miners and controls were matched by age and smoking habits.

Cell and blood collection

A sample of oral mucosa cells and 20 ml of venous blood were collected from each participant. The oral mucosa cells were collected by scraping both sides of the oral cavity with the edge of a microscope slide. The material thus obtained was immediately transferred to individual small tubes containing an isotonic saline solution. Peripheral blood collected for performing lymphocyte cultures was transported to the laboratory in the syringes used for drawing the individual samples. All material was transported in an ice box to the lab and kept in a refrigerator overnight.

Preparation of oral mucosa cells for micronuclei

The tubes containing the scraped material in isotonic saline were centrifuged at 1,500 rpm for 10 min and resuspended in fresh 3:1 (methanol:acetic acid) fixative. Slides were prepared in the same way as for lymphocyte cultures. They were Feulgen-stained and counter-stained with an ethanol solution of 1% Fast Green for one minute. A total of 2000 cells were scored for micronuclei per individual using the criteria proposed by Stich and Rosin (1983) and Sarto *et al.* (1987). All the analyses were performed by the same observer in a blind test.

Lymphocyte cultures

The blood samples were centrifuged at 1,200 rpm for 10 min and the plasmas were assembled into two pools: miner plasma pool (MP) and control plasma pool (CP). Each pool consisted of at least five plasma samples from different individuals collected on the same occasion. The buffy coat in each tube was removed and lymphocyte cultures were established by modification of the technique proposed by Moorhead *et al.* (1960). Individual duplicate cultures were established in 5 ml RPMI medium (15% fetal calf serum, phytohemagglutinin and 1% antibiotics):

(a) Miner lymphocyte cultures (M)
(b) Control lymphocyte cultures (C)
(c) Control lymphocyte cultures + 10% miner plasma pool (C + MP)
(d) Control lymphocyte cultures + 10% control plasma pool (C + CP)

For each miner one duplicate culture was established (M), and for each control, duplicate cultures were established without addition of plasma (C), with addition of miner plasma pool (C + MP) and with addition of control plasma pool (C + CP). The cultures were incubated for 48 h and processed in the conventional way. All slides were analyzed by the same observer in a blind test. Each individual culture was analyzed for evaluating the mitotic index (2,000 nuclei) and chromosome analysis (100 metaphases). Some of the cultures failed to yield metaphases suitable for analysis. The data presented in this paper include the results of at least 35 individuals in each group. Among the 35 successful cultures obtained from miners, 17 were from cigarette smokers and among the 40 successful cultures from control individuals 19 belonged to cigarette smokers, the remaining cultures being from non-smokers.

Plasma chemical analysis

Two pooled miner and one pooled control plasma samples were analyzed for metals, fluorides and polynuclear aromatic hydrocarbons at the Midwest Research Institute, Kansas City, Missouri. As the

shipping conditions were not ideal, the results on polynuclear aromatic hydrocarbons were not reliable.

Statistical analyses

The data were analyzed by means of standard parametric tests, after the original variables were submitted individually to suitable transformations to ensure normality, such as the Freeman-Tukey arc-sine transformation for proportions and the square root transformation of Anscombe for counts (Zar, 1984). We used standard normal tests for comparing means (paired *t*-tests for the case of plasma pools and non-paired *t*-tests for the remaining variables).

The size of the different samples differed according to limitations that were dependent on the kind of cytogenetic analysis performed: thus for chromosome and chromatid alterations the sample size was determined by the number of cultures that yielded at least 100 metaphases suitable for analysis. The data on mitotic index, estimated directly from lymphocyte cultures, were independent on the quantity of cells in metaphase, and the presence of micronuclei was determined in interphase cells scraped from the oral mucosa.

The *t*-tests used for analyzing our data are labelled as follows: 1) $\mathbf{t_1}$: *t*-tests for paired samples, with n-1 degrees of freedom, where n is the number of available data pairs; 2) $\mathbf{t_2}$: unpaired *t*-tests for two samples, where a pooled variance $s^2 = [s_1^2(n_1 - 1) + s_2^2(n_2 - 1)]/(n_1 + n_2 - 2)$ is used, averaged from the homogeneous variances of the two groups, and where the number of degrees of freedom is calculated after d.f. = $n_1 + n_2 - 1$; 3) $\mathbf{t_3}$: unpaired *t*-tests for two samples, where the variances s_1^2 and s_2^2 in the two samples of sizes n_1 and n_2 to be compared are not homogeneous; the *t*-test formula is then given by $t = |mean_1 - mean_2| / \sqrt{(s_1^2/n_1 + s_2^2/n_2)}$ and the number of degrees of freedom is adjusted according to the degree of variance heterogeneity, being calculated by d.f. = $1/[u^2/(n_1 - 1)+(1 - u)^2/(n_2 - 1)]$, where $u = n_2 s_1^2/(n_2 s_1^2 + n_1 s_2^2)$ (Diem and Lentner, 1968).

RESULTS

Micronuclei

The average number of cells with micronuclei from the buccal mucosa was significantly higher (t_3 = 10.17; d.f. = 88; P < 0.001) among miners (mean ± 1 SD = 0.006 ± 0.003) than among controls (0.002 ± 0.001). The distribution of individuals from both samples according to the number of cells with micronuclei is shown in Figure 1.

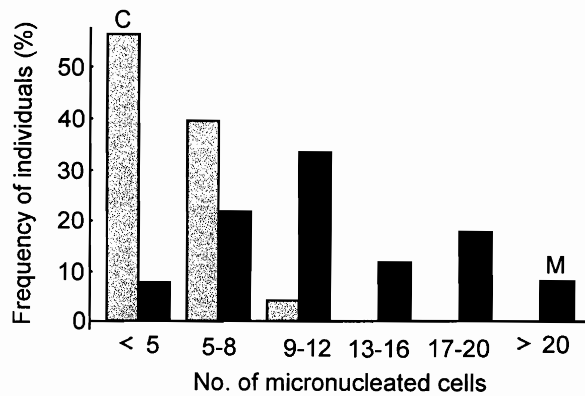

Figure 1 - Distribution of controls (C) and miners (M) according to the frequency of oral mucosa cells (out of 2,000 scored per individual) with micronuclei.

Lymphocyte cultures

a) Mitotic index: the mitotic index in lymphocyte cultures from controls (mean ± 1 SD = 0.047 ± 0.02) was significantly higher (t_2 = 6.10; d.f. = 85; P < 0.001) than that found in miners (0.027 ± 0.01) but did not differ from the value found in control cultures exposed to control plasmas (0.051 ± 0.02). The mitotic index of control cultures exposed to miner plasmas (0.034 ± 0.01), on the other hand, was significantly lower than that found among control cultures exposed to control plasmas (t_1 = 5.10; d.f. = 37; P < 0.001) and higher than that found among miners (t_2 = 2.78; d.f. = 85; P = 0.007).

b) Chromosome alterations: the frequencies of cells showing at least one type of chromosomal alteration (100 cells scored per individual) are shown in Table I.

There was a significantly higher frequency of cells with at least one fragment (t_3 = 7.14; d.f. = 58; P < 0.001), polyploid mitoses (t_3 = 4.76; d.f. = 39; P < 0.001) and cells with at least one type of chromosome alteration (t_2 = 5.04; d.f. = 73; P < 0.001) in miner cultures than in the control cultures. The comparisons between control lymphocytes exposed to miner plasma and to control plasma pools showed a significantly higher frequency of chromatid gaps (t_1 = 3.34; d.f. = 37; P < 0.002), fragments (t_1 = 3.65; d.f. = 37; P = 0.001) and overall alterations (t_1 = 6.38; d.f. = 37; P < 0.001) in the former group. We also observed an increased frequency of fragments in the miner cultures when they were compared to control cultures exposed to miner plasmas (t_2 = 3.08; d.f. = 85; P < 0.001). No significant difference was found when control cultures were compared to control cultures exposed to control plasmas, as expected.

The distribution of cultures according to the frequencies of cells with chromosome abnormalities in

Table I - Frequencies of cells with chromosome alterations (100 cells scored per individual): a) mean frequency ± 1 SD (original variables); b) mean frequency ± 1 SE (original variables); c) mean frequency ± 1 SD (transformed variables).

Group	Number of sampled individuals		Chromatid breaks	Chromosome breaks	Chromatid gaps	Chromosome gaps	Fragments	Rings	Total	Frequency of polyploid cells
Miners	35	a	0.007 ± 0.012	0.003 ± 0.011	0.006 ± 0.010	0.002 ± 0.006	0.012 ± 0.010	0.000 ± 0.000	0.029 ± 0.027	0.013 ± 0.017
		b	0.007 ± 0.002	0.003 ± 0.002	0.006 ± 0.002	0.002 ± 0.001	0.012 ± 0.002	0.000 ± 0.000	0.029 ± 0.005	0.013 ± 0.003
		c	0.051 ± 0.031	0.036 ± 0.023	0.046 ± 0.028	0.036 ± 0.019	0.070 ± 0.024	0.029± 0.000	0.097 ± 0.041	0.064 ± 0.039
Controls	40	a	0.003 ± 0.006	0.000 ± 0.002	0.003 ± 0.006	0.001 ± 0.003	0.002 ± 0.004	0.000 ± 0.000	0.008 ± 0.013	0.001 ± 0.003
		b	0.003 ± 0.002	0.000 ± 0.000	0.003 ± 0.001	0.001 ± 0.001	0.002 ± 0.001	0.000 ± 0.000	0.008 ± 0.002	0.001 ± 0.000
		c	0.051 ± 0.031	0.030 ± 0.006	0.038 ± 0.020	0.031 ± 0.012	0.035 ± 0.016	0.029 ± 0.000	0.054 ± 0.032	0.032 ± 0.011
Controls + pool of miner plasmas	40	a	0.009 ± 0.016	0.002 ± 0.009	0.005 ± 0.008	0.004 ± 0.009	0.007 ± 0.009	0.001 ± 0.004	0.026 ± 0.026	0.004 ± 0.008
		b	0.009 ± 0.003	0.002 ± 0.001	0.005 ± 0.001	0.004 ± 0.001	0.007 ± 0.001	0.001 ± 0.001	0.026 ± 0.004	0.004 ± 0.001
		c	0.052 ± 0.037	0.035 ± 0.021	0.046 ± 0.026	0.040 ± 0.025	0.051 ± 0.027	0.033 ± 0.014	0.092 ± 0.042	0.045 ± 0.024
Controls + pool of control plasmas	39	a	0.002 ± 0.005	0.000 ± 0.000	0.001 ± 0.003	0.001 ± 0.002	0.001 ± 0.004	0.000 ± 0.000	0.005 ± 0.009	0.003 ± 0.007
		b	0.002 ± 0.001	0.000 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.001	0.000 ± 0.000	0.005 ± 0.001	0.003 ± 0.001
		c	0.035 ± 0.016	0.029 ± 0.000	0.033 ± 0.012	0.031 ± 0.009	0.033 ± 0.014	0.029 ± 0.000	0.044 ± 0.025	0.039 ± 0.022

miners and controls is shown in Figure 2 and in control lymphocyte culture in the presence of control or miner plasma pools in Figure 3.

Comparison of smokers and non-smokers

Comparisons for micronuclei ($t_2 = 1.06$; d.f. = 97; P = 0.30), mitotic index ($t_2 = 1.1$; d.f. = 85; P = 0.27) or chromosome alterations ($t_2 = 1.82$; d.f. = 73; P = 0.07) between smokers and non-smokers showed no significant differences.

Correlation analysis

We found a statistically significant positive correlation between the frequency of cells with chromosome aberrations and the frequency of cells with micronuclei (r = 0.46; t = 4.47; d.f. = 73; P < 0.001) in the control + miner group and a negative correlation between the frequency of cells with chromosome aberrations and the mitotic index when all four samples were pooled (r = -0.34; t = 4.53; d.f. = 152; P < 0.001).

Plasma chemical analysis

Chemical analysis of the plasma pools revealed elevated lead and depressed zinc concentrations in miners when compared to controls as well as to normal tolerance limits. The respective values were: a) lead (control = 0.60 µg/dl; miner plasma pool 1 = 3.0 µg/dl, miner plasma pool 2 = 2.0 µg/dl; normal concentration (graphite fumace AAS) = 0.1-2.2; Iyengar and Woittiez, 1988; Versieck and Cornelis, 1989); b) zinc (control: 1.0 µ/ml; miner plasma pool 1 = 0.50 µg/ml; miner plasma pool 2 = 0.64 µg/ml; normal concentration (DCP) =

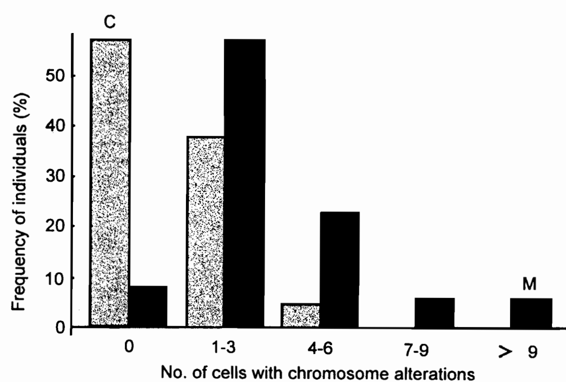

Figure 2 - Distribution of lymphocyte cultures from controls (C) and miners (M) according to the number of cells (out of 100 scored per culture) with chromosome abnormalities.

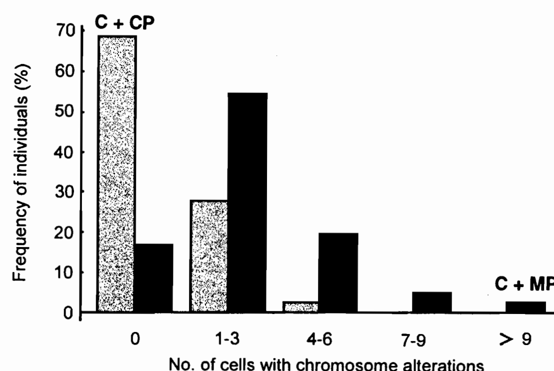

Figure 3 - Distribution of control lymphocyte cultures exposed to control plasma pool (C + CP) and to miner plasma pool (C + MP) according to the number of cells (out of 100 scored per culture) with chromosome abnormalities.

0.7-1.2; Baselt and Cravey, 1989; Iyengar and Woittiez, 1988).

An elevated phenantrene concentration (3.6 μg/ml) was detected in one of the two miner plasma pools. The reported limits are 0.5 μg/ml (Baselt and Cravey, 1989).

DISCUSSION

As far as we know, only two other reports in the literature deal with clastogenic effects due to professional coal mining. Sram *et al.* (1985) detected an increase of chromosomal damage in a sample of 27 soft coal open cast mining workers from Czechoslovakia and Al-Sabti *et al.* (1992) reported chromosome alterations in a sample of nine coal miners from Slovenia, in a study that included several professionally exposed groups of individuals, but they gave no information about the kind of mining (open cast or underground).

We showed that in the coal miners of Criciúma, Brazil, there is an increased risk of genetic injury. We observed an increase in the frequency of micronucleated cells of the oral mucosa and an increase of cells with fragments, chromatid gaps and chromosome alterations in lymphocyte cultures from miners when compared to controls. We did not detect any influence of cigarette smoking on the frequency of micronuclei or chromosome abnormalities. An increase of chromosome alterations was detected by Bueno *et al.* (1992) in rodents collected at sites that receive wastes from coal washing and tobacco culture in Criciúma.

Besides different organic compounds, acids and toxic fumes due to mining and spontaneous combustion of pyrites, the following metals have been found to be in excess in Criciúma: iron, manganese, copper, lead, zinc, selenium, silver, mercury, arsenic, barium and cadmium (FATMA, 1990). The environmental conditions of Criciúma include, therefore, many substances that are genotoxic. Given the complexity of the biological and environmental interactions involved, it is not possible to assign our cytogenetic findings to the presence of a specific polluting agent. However, in the miner plasma samples investigated in the present study the only significant alterations were elevated lead level concentration and a depressed zinc concentration, besides an excess of phenantrene found in one of the miner plasma pools. A decrease in cell division rates due to zinc deficiency has been reported by Wu and Wu (1987). However, the decrease in the mitotic index we found in miners cannot be attributed to this deficiency, as the culture media with fetal calf serum certainly compensated any natural deficiency present *in vivo*. Because of temperature variation during transportation of the plasma samples for the chemical analysis performed in the USA, it is possible that their organic composition was altered.

By using the lymphocytes of non-exposed individuals as exposure target cells for plasmas of miners, we obtained a much higher mitotic index and a larger frequency of successful cultures than in cultures of miner lymphocytes. On the other hand, the frequency of overall chromosome alterations was not different from that found in lymphocyte cultures from miners. Nevertheless, an excess of fragments was detected in miner cultures.

Our data indicate that plasma pools *per se* did not significantly affect the frequency of chromosome alterations, the mitotic index, or the culture success when non-exposed lymphocytes were cultured with plasma pools of non-exposed individuals. Our results on control lymphocytes exposed to 10% miner plasmas for 48 h, which show the same amount of chromosome damage as chronic *in vivo* exposure for more than one year, might at first sight seem incongruent. However, the lower mitotic index, low efficiency of culture success, an excess of small nuclei typical of cells that were not properly stimulated by phytohemagglutinin and the presence of cells with chromosome pulverization in direct miner cultures are evidence for toxic or genotoxic effects with consequent impaired capacity to respond to stimulation and cell division. The negative correlation found between the frequency of chromosome alterations and the mitotic index indicates that genotoxic effects may be responsible for the lower mitotic indices found in exposed populations. Thus, if an excess of chromosome abnormalities is one of the events related to the low mitotic index found in direct cultures of miner lymphocytes, the damage detected by exposing unexposed control lymphocytes to plasmas of the exposed population is an underestimate of the actual amount of chromosome damage, but constitutes a very convenient methodology for detecting damage in situations when the conditions under study interfere with cell division capacity. This methodology also presents the following features:

a) The lymphocytes of the same unexposed individuals can be used both as exposure target cells and controls.

b) The probabilities of culture success and yield of metaphase cells seem to be higher, thus improving the conditions for chromosome analysis.

c) In the case of culture failure, there is no need for further collection of material in the exposed population, as the plasmas can be frozen and later tested

on different samples of lymphocytes from unexposed individuals.

d) The lymphocytes from exposed populations can be used for other bioassays, e.g. the detection of DNA adducts.

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RESUMO

Estudos citogenéticos foram realizados em mineiros de carvão mineral de Criciúma (SC) e em controles fumantes e não-fumantes selecionados por sexo e idade. Nas culturas de linfócitos da amostra de mineiros observamos uma diminuição do índice mitótico e um excesso de fragmentos cromossômicos e em células da mucosa oral um excesso de micronúcleos. Um ensaio alternativo para detecção de clastogênese em casos de exposição ocupacional foi testado: os linfócitos de indivíduos não-expostos foram submetidos a uma mistura de plasmas de mineiros e também a uma mistura de plasmas de indivíduos não expostos, com a finalidade de servirem como controles pareados no experimento realizado. Os resultados revelaram que o teste é muito conveniente, uma vez que linfócitos dos mesmos indivíduos podem ser utilizados tanto como alvo como controle. Neste experimento detectamos um aumento de falhas cromatídicas, fragmentos e alterações cromossômicas em geral nos linfócitos expostos à mistura de plasmas de mineiros. A mistura de plasmas dos controles não induziu nenhuma alteração significante, indicando que os resultados obtidos não foram devidos ao efeito de adição das misturas de plasma *per se*.

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