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ORIGINAL ARTICLE

Enhanced frequency of micronuclei in lymphocytes from current as opposed to former uranium miners

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Summary
Micronuclei can be used as markers of past radiation exposure, but few pertinent studies have dealt with alpha radiation. Here we report on micronuclei in lymphocytes from uranium miners, comparing some that are currently active and others that retired 15–20 years ago. Their radiation exposure is assumed to come mainly from radon and its decay products in the air breathed at the work place.

Current miners showed a greater micronucleus frequency than former miners. This can be attributed to their recent radiation exposure, while the lower frequency in the former miners probably results from the disappearance of potentially micronucleus containing lymphocytes from the peripheral blood, which is known to occur with a half-life of about one year.

For current miners there is a significant correlation between micronucleus frequency and effective dose received over the last 12 months. The dose at which a doubling of the micronucleus frequency is observed is around 10 mSv. This is a much smaller dose than would usually be expected to be detectable with this test, and raises a number of questions about the induction of micronuclei by alpha radiation from radon and its decay products.

Key words: biodosimetry; micronucleus-centromere assay; alpha radiation; radon; uranium mining

INTRODUCTION

Micronuclei are structures in the cytoplasm which stain similarly to the main nucleus but are typically an order of magnitude smaller in diameter. They are surrounded by double membranes, and contain chromosomes or fragments of chromosomes which have not been incorporated into one of the daughter nuclei during cell division. Micronuclei occur spontaneously at a frequency of a few per mil in human lymphocytes and other cells, but the frequency varies from individual to individual, depending on age and other factors. Micronuclei can be induced by DNA breaking agents, so-called clastogens, such as radiation, chemicals like methyl methanesulphonate (MMS) or certain components of cigarette smoke (for review, see Müller and Streffer 1994), and are therefore used as markers of past exposure to such agents.

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Clastogen induced micronuclei tend to be different from those which occur spontaneously in that they contain acentric fragments rather than whole chromosomes with centromeres. This has been exploited to enhance the sensitivity of the micronucleus test by selectively analyzing micronuclei without centromeric signals (Norppa et al. 1993, Vral et al. 1997). The most wide-spread method for the identification of centromeres is *in situ* hybridization with centromere specific DNA probes (for review, see Norppa and Falck 2003). Monoclonal antibodies against specific centromere proteins, such as CENP-B (Earnshaw et al. 1987, Bejarano et al. 1993) can be used instead, to discriminate micronuclei without and with centromeres, i.e. those containing acentric fragments and those containing whole chromosomes. Here we employed such a technique to analyse micronuclei in uranium miners.

The world's biggest producers of uranium are Kazakhstan and Canada, which alone account for 50% of the world wide production of 50,000 t per year. Other countries producing in excess of 1000 t per year are Australia, Namibia, Russia, Niger, Uzbekistan, and the USA (World Nuclear Association 2010). The last functioning uranium mine in the European Union is Rožná in the municipality of Dolní Rožínka, Vysočina region, Czech Republic. It has an annual production of around 120,000 t of ore, with an average content of 2.11 kg of uranium per ton, which means an annual production of around 300 t of uranium. Less than 100 miners are currently active in the mine, the overall number of employees being around 550 (Sejkora et al. 2008, Kříbek et al. 2009). Thousands of former mine workers, however, are living in other regions of the country, where mines were closed at the beginning of the 1990s. By that time, the Czech Republic (or earlier, Czechoslovakia) had produced about 110,000 t of uranium from 64 uranium deposits. The largest deposit – at Příbram, Central Bohemia – alone produced half of that amount (Michálek 2007, Tomek 2008).

We decided to investigate genetic changes in the lymphocytes of miners from Rožná and Příbram with the help of the above-mentioned modified micronucleus test, focusing on differences between current and former miners. It is well known that micronuclei, or rather potentially micronucleus containing lymphocytes, disappear from the peripheral blood with a half-life of about one year (for review, see Müller and Streffer 1994, Thierens and Vral 2009). However, there is also evidence for enhanced genetic damage in uranium miners more than 10 years after they stopped working, which has been interpreted as evidence of persisting genomic instability induced by alpha radiation (Kryscio et al. 2001). The current project addresses these questions, comparing groups of miners from Rožná and Příbram as well as unexposed individuals. A preliminary analysis of current and former miners is presented here.

MATERIAL AND METHODS

Samples

Samples of heparinized blood were obtained by venipuncture from 30 miners each from Rožná (current miners) and Příbram (former miners). Transport to the laboratory took about 2.5 hours from Rožná and 0.5 hours from Příbram. Information about age, kind of work, duration of work under exposure conditions, smoking habits etc. was gathered in interviews. Informed consent was obtained in written form from each participant in the study. Personal dosimetry data were provided by the employer (DIAMO s. p., 100% owned by the Czech Ministry of Industry and Trade).

Cultivation of blood cells

Each sample was divided into 2.5 ml aliquots and topped up to 5 ml each with cultivation medium (RPMI 1640 with 20% fetal calf serum, both Gibco – Invitrogen, Paisley, Scotland). In order to stimulate lymphocyte proliferation, 15 µl/ml phyto-haemagglutinin (Gibco – Invitrogen, Paisley, Scotland) was added. After 44 h incubation at 37 °C and 5% CO_2, 1.5 µl/ml Cytochalasin B (Sigma, Deisenhofen, Germany) was added (final concentration of 5.6 µg/ml). Cultivation was continued for another 24 h under the same conditions. This procedure was first described by Fenech and Morley (1985).

Preparation of slides

Cell suspensions were centrifuged at 1000 rpm for 10 min. The supernatant was carefully sucked off and the sediment taken up in 5 ml lysis buffer (10 mM EDTA, 155 mM NH_4Cl, 10 mM $KHCO_3$) following Zeni et al. (2003). After whirling up the pellet, the cell suspension was immediately centrifuged at 1000 rpm for 6 min. This sequence (sucking off the supernatant – adding lysis buffer – whirling up the pellet – centrifugation) was repeated once, so that the overall time in lysis buffer was about 15 min. The cells were re-suspended in (serum free) medium, centrifuged again at 1000 rpm for 10 min and taken up in hypotonic solution (medium/distilled water 1:3) for 3 min following Thomson and Perry (1988). Cells were then cytospun onto uncoated slides at 1200 rpm

for 7 min using Rotofix32 (Hettich, Tuttlingen, Germany). Fixation was carried out on the slides with 1% paraformaldehyde for 5 min at room temperature.

Immunostaining

Slides were dipped in 0.1% Triton X-100 for 5 min for permeabilization, and incubated with rabbit H65 antibodies against CENP-B (Santa Cruz Biotechnology, Santa Cruz, California) 1:50 diluted in 0.1% Triton X-100 in PBS for 1 hour at room temperature. After being rinsed 3 times with PBS they were incubated with Alexa Fluor 488 goat anti-rabbit (Molecular Probes – Invitrogen, Paisley, Scotland) 1:500 in 0.1% Triton X-100 in PBS for 1 hour at room temperature. Cell nuclei were counterstained with DAPI. Finally, the slides were rinsed 3 times with distilled water and left to dry for 10 min following Abend et al. (1995). Cover slips were mounted with the help of ProLong Gold anti-fading medium.

Scoring and statistical analysis

The slides were scored under a fluorescence microscope OLYMPUS 1X71 with a 40× objective and 400× total magnification. 500 binuclear cells were scored per slide and two slides per donor. The scoring was done "blind", i.e. without the scorer knowing to which subgroup the donor belonged. Statistical differences were assessed using the unpaired Student's t-test as available in the Microsoft Excel 2003 software. For the linear regression analysis, the LAB Fit Curve Fitting Software (V 7.2.39, Wilton and Cleide P. Silva) was used.

RESULTS AND DISCUSSION

We present here results from the analysis of blood samples from 30 current and 30 former uranium miners. These numbers are to be more than doubled by the end of the project. Even now, however, we feel that some interesting features are apparent and should be reported.

The average frequency of micronuclei (number of cells containing micronuclei per 100 binucleate cells) is slightly higher in the group of current miners from Rožná than in the group of former miners from Příbram, but the difference is not statistically significant (Table 1). One might expect a difference because the half-life of micronuclei, or perhaps rather of potentially micronucleus-containing lymphocytes in the peripheral blood, is usually assumed to be about one year (for review, see Müller and Streffer 1994, Thierens and Vral 2009). Therefore, miners who have been exposed recently should show more micronuclei than those exposed 20 years ago, even if their accumulated doses from work in the mines were similar. One might also expect the difference, if any, to be rather small, because the average doses received by the Rožná miners over the last 12 months were in the order of 5 to 20 mSv (see below), which is usually considered to be insufficient to show up at all in the micronucleus test. Another factor to be considered, however, is the well-known age dependence of the background micronucleus frequency. Literature data suggest that it rises by a few percent per year of age (for review, see Müller and Streffer 1994). Our comparative analysis of miners below and above median age within each of the two groups separately shows the same trend (Table 1), although the difference is significant only for the Rožná miners. As the Příbram miners are on average 25 years older, their average micronucleus frequency should therefore be considerably higher in comparison, and the fact that their frequency instead is somewhat lower indicates that we are really dealing with radiation induced micronuclei in the case of the Rožná miners.

The same differences and trends are seen when the fraction of micronuclei with centromeres is considered (Table 1). Because micronuclei with centromeres are supposedly spontaneous and not radiation induced (see Introduction), one would expect their fraction to be lower in the more recently exposed workers from Rožná, which is indeed the case. The difference is not statistically significant, but again the trend is opposite to what would be assumed on the basis of age dependence. In both the Rožná and Příbram groups, the fraction of centromere containing micronuclei decreases with age (although again not significantly). This is somewhat surprising, because other authors have shown that most of the age dependent increase in the overall micronucleus frequency is due to centromere-containing micronuclei (Thierens et al. 1999, 2000). It is not clear why it seems to be opposite in our case, but the age dependence which we find is such that it supports the assumption of a real difference in the fraction of centromere-containing micronuclei between the miners from Rožná and Příbram.

That the micronuclei found in current miners are at least partly the direct result of exposure to alpha radiation, while those observed in former miners are not, is confirmed by an analysis of the dispersion index. This quantity is defined as the quotient of the variance and the mean of a statistical distribution. In our case, it reflects the relative numbers of cells with 0, 1, 2, or more micronuclei. The dispersion index turned out to be 1.25 for current miners and 1.07 for

Table 1. **Micronucleus numbers and content for different groups of miners.**

Miners from	Age	Number of cells with micronuclei among 100 binucleate cells	Fraction of micronuclei with centromeres (%)
Rožná	34.5±6.1	0.78±0.62	57±43
– age below median	29.8±2.8	0.59±0.48	70±45
– age above median	39.2±4.7	0.97±0.69	44±40
Příbram	59.9±9.5	0.62±0.53	79±28
– age below median	52.6±5.8	0.47±0.44	83±45
– age above median	67.3±6.1	0.68±0.64	67±40

former miners. This difference is not very large, but seems reasonable assuming that a considerable part of the micronuclei even in recently exposed miners would not be radiation induced. Lymphocytes exposed to alpha radiation *in vitro* showed a dispersion index of about 1.5 at the highest dose applied, in contrast to lymphocytes exposed to X-rays, for which values around 1.1 were found (Johannes et al. 2010).

Finally for the Rožná miners, we looked at the correlation between the results of personal dosimetry and those of the micronucleus test. When the number of micronuclei containing cells per 100 binucleate cells was plotted against the dose received during the last 12 months (gamma and alpha combined), a very clear correlation was seen (Fig. 1). With a linear regression model, a background frequency of 0.4% could be estimated, and an increase of 0.05% per mSv. The overall numbers of micronuclei containing cells per 100 binucleate cells were thus somewhat smaller than in other studies, but comparable (Thierens et al. 1999, Kryscio et al. 2001). What was unexpected, however, was the statistically significant (p=0.024) increase with doses between 1 and 20 mSv. As mentioned above, such doses are usually considered to be too small to be picked up by the micronucleus test (Vral et al. 1997, Thierens et al. 1999, Wojcik et al. 2000). Enhanced frequencies of micronuclei have, however, been found in the lymphocytes of industrial radiographers (Sari-Minodier et al. 2002) and nuclear power plant workers (Hadjidekova et al. 2003) whose annual doses, albeit of γ radiation, were apparently in the same order of magnitude as for our miners.

The dose accumulated over the last 12 months, of course, is not necessarily what is "visible" in the

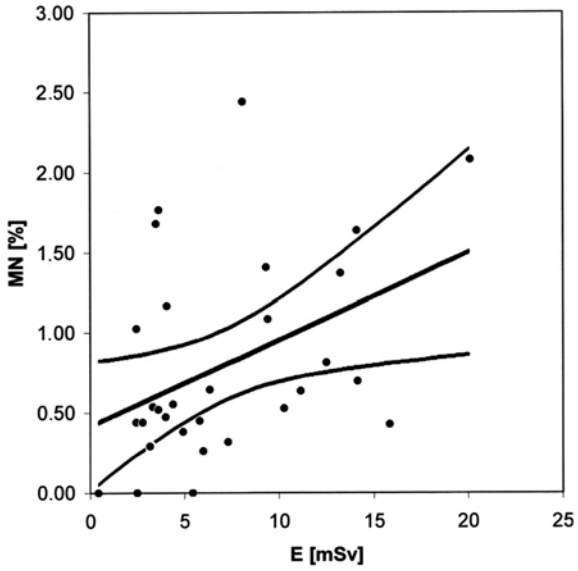

Fig. 1. **Correlation between effective dose accumulated over the last 12 months** before analysis and the number of micronucleus containing cells per 100 binucleate cells. Using linear regression analysis we found MN= (0.42±0.19)+(0.051±0.022)*E with r=0.398 (p=0.024)

micronucleus test. As mentioned above, the half-life of micronuclei is usually assumed to be about 1 year, so that micronuclei induced 12 months ago would still be found with 50% probability, those induced 24 months ago with 25% probability, and so on. Some simple calculations show that if the exposure rate is constant, 72% of the micronuclei induced over the

last 12 months will still be found at the time of investigation, or 14% of those induced over the last 10 years. If, however, the half-life of micronuclei is longer, say 3 years – as has been suggested on the basis of model calculations for people exposed to chronic low-dose γ- irradiation (Chang et al. 1999) – these values are 90% for the last 12 months, and 43% for the last 10 years. The complete results of personal dosimetry for the Rožná miners are available to us, but because certain methodological changes were introduced at the end of 2008, more effort has to be spent on a precise calculation of "micronucleus-relevant" doses from earlier years.

Also to be considered when assessing the reasonability of our results are the ways in which equivalent and effective doses are calculated. The quality factor for alpha radiation is assumed to be 20 in personal dosimetry, but the Relative Biological Effectiveness of alpha radiation with respect to micronucleus induction may be different. A number of studies suggest that it is in fact smaller, say around 4 (Bilbao et al. 1989, Yamada et al. 2002), which would mean our alpha doses could be a factor of 5 too high, and our finding of an increase of micronucleus frequency with dose would be even more surprising. On the other hand, the alpha dose to circulating lymphocytes, which should mainly stem from exposure in the tracheobronchial tract and the lungs, may be quite different from the effective dose calculated in personal dosimetry, but the exact relationship remains to be established (Harley and Robbins 2009, Little et al. 2009).

In conclusion, we would like to state that the micronucleus test, in particular in combination with the labelling of centromeres, is sensitive enough to demonstrate genetic damage in the lymphocytes of uranium miners who have been exposed to less than 20 mSv over the last 12 months. Further studies will have to include information about exposures more than 12 months back, and for comparison will not only rely on miners who retired 15 to 20 years ago, but will compare both current and former miners with people who have not been occupationally exposed to alpha radiation at all.

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