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Effects of occupational exposure to mercury vapor on lymphocyte micronuclei

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BARREGÅRD L, HÖGSTEDT B, SCHÜTZ A, KARLSSON A, SÄLLSTEN G, THIRINGER G. Effects of occupational exposure to mercury vapor on lymphocyte micronuclei. *Scand J Work Environ Health* 1991;17:263—8. For 26 chloralkali workers exposed to inorganic mercury and 26 age-matched, occupationally unexposed referents, the frequency and size distribution of micronuclei were determined in peripheral lymphocytes stimulated with either phytohemagglutinin or pokeweed mitogen. For the exposed workers the mean concentrations of mercury in urine, plasma, and erythrocytes were 16 nmol/mmol of creatinine, 48 nmol/l, and 78 nmol/l, respectively, and their mean exposure time was 10 years. Neither the frequency nor the size of micronuclei was significantly different in the two groups; nor were there any correlations to current mercury levels. However, in the exposed group, and with phytohemagglutinin as the mitogen, a statistically significant correlation between previous exposure to mercury (cumulative exposure or number of blood mercury peaks) and the frequency of micronuclei was found. This association was also present when the effects of age and smoking were allowed for, and it may indicate an accumulation of cytogenetic effects in T-lymphocytes.

Key terms: chloralkali, cytogenetic, phytohemagglutinin, pokeweed mitogen.

High levels of mercury vapor (Hg^0) may be found in certain work environments (eg, plants for the production of chlorine, fluorescent tubes, or thermometers). Low-level exposure is common among dental personnel and is seen in the general population as a result of release from dental amalgam surfaces. Mercury vapor is readily absorbed through the lungs into the blood stream and is then distributed to various parts of the body. The critical organs are the central nervous system and the kidneys (1, 2).

The most evident genotoxic effect of inorganic mercury compounds is the induction of C-mitosis with inactivation of the mitotic spindle. This phenomenon results in aneuploidy and/or polyploidy. The effect on the spindle mechanism is probably due to the high affinity of mercury for sulfhydryl groups in the spindle apparatus (3—5). The ability of inorganic mercury to induce gene mutations seems, however, to be low (3, 4). Cytogenetic in vivo studies have, in most cases, shown no effects on human chromosomes (6—9). Only few studies of cancer in animals after exposure to mercury have been reported, and none used the inhalation of mercury vapor as the method of exposure (10, 11). In two epidemiologic studies of cancer in groups with occupational exposure to mercury vapor, an ex-

cess of lung cancer was reported. This excess was, however, probably related to factors other than the exposure to mercury (12, 13). In a study of dental personnel, the incidence of brain tumors was increased, and exposure to mercury vapor was discussed as a possible causative factor (14).

The micronucleus method has been developed into a rapid and sensitive cytogenetic procedure for the study of lymphocytes from peripheral blood (15). The micronucleus size depends on whether it originates from whole chromosomes or from chromosome fragments (16), as in aneuploidy and chromosome breakage. The aim of the present study was to examine whether the frequency and size distribution of micronuclei in peripheral lymphocytes were affected by exposure to mercury vapor in chloralkali workers.

Subjects and methods

We examined 26 male chloralkali workers exposed to mercury vapor and 26 occupationally unexposed referents from the same company. The exposed group included all workers exposed to mercury vapor for at least one year (except one diabetic) and available on the days of sampling. The average levels of mercury in the air had been 25—50 $\mu\text{g}/\text{m}^3$ for the past decade, and the mean duration of exposure to mercury was 10 years. The referents were individually matched to the exposed subjects for age (± 7 years) and smoking habits (table 1). They had no present or historical occupational exposure to mercury. Fish consumption was higher among the referents, however (table 1). The number of smokers was 14 in both groups. Two of the

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Table 1. Age and previous mercury exposure of the 26 mercury-exposed chloralkali workers and 26 occupationally unexposed referents. The cumulative exposure index denotes integrated whole blood mercury over employment time.

Group ^a	Age (years)			Fish consumption (meals per week)			Exposure time (years)			Cumulative exposure index (nmol/l · years)		
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
Exposed	37	33	21–65	0.9	1	0–2	10	10	1–31	910	710	85–4300
Reference	36	33	18–61	1.3	1	0–3	.	.	.	.	.	.

^a The number of smokers was 14 for both groups.

Table 2. Biological exposure indices for the 26 mercury-exposed chloralkali workers and 26 occupationally unexposed referents. (Hg = mercury, HgB = mercury in whole blood)

Group	Number of peaks (HgB > 150 nmol/l)			Plasma Hg (nmol/l)			Erythrocyte Hg (nmol/l)			Urinary Hg (nmol/l)			Urinary Hg (nmol/mmol creatinine)		
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
Exposed	2.2	0.5	0–29	48	31	14–200	78	52	24–323	252	178	25–930	16	13	4.2–37
Reference	.	.	.	7.5	7.7	4–13	33	30	12–101	19	15	1–48	1.3	1.2	0.2–2.9

exposed workers were on beta-blocker medication owing to hypertension, and another exposed worker used a bronchodilating spray to treat mild asthma, whereas none of the referents used any medical drugs regularly. Three workers and one referent took supplementary selenium. Two exposed workers and one referent had had a medical X-ray examination (other than dental) during the six months preceding our study.

Venous blood and morning urine samples were collected in metal-free heparinized tubes and bottles on the same occasions from the exposed subjects and referents. One whole blood sample was transported to the laboratory for lymphocyte cultivation on the day of the sampling. Plasma and erythrocytes were separated by centrifugation and then, with the urine samples, frozen until analysis for mercury content and creatinine.

Mercury was analyzed in urine, plasma, and erythrocytes with the use of the cold-vapor atomic absorption technique, as previously described (17). The samples were analyzed in duplicate. The coefficients of variation at mercury concentrations above and below 30 nmol/l were 7.6 % and 12 % for urine, 5.8 % and 12 % for plasma, and 5.6 % and 11 % for erythrocytes. Accuracy was initially tested by comparison with radiochemical neutron activation analysis (17). In addition, 18 of the samples in the concentration range 6–376 nmol/l were reanalyzed three years later (1990) together with five reference samples. The results for 17 of the samples averaged 96 (SD 14, range 70–114) % of the previous values. For the remaining sample, the result was 6 nmol/l, as compared with 13 nmol/l previously. The results obtained for the whole blood reference samples were 18, 46, and 500 nmol/l as compared with the recommended values of 20, 50, and 488 nmol/l, respectively (batches 904 and 905, Seronorm,[®] Nycomed, Oslo, Norway, and control blood for metals 2, lot number 620403, Behring

Institute, Behringwerke AG, Marburg, Germany). The results for lyophilized urine were 44 and 254 nmol/l, as compared with the assigned values of 48 and 255 nmol/l (Lanonorm[®] metals number 1 and Sero-norm[®] batch 108). For two exposed workers, the urinary mercury level had to be determined a few months before the present examination. For three exposed workers and three referents only the mercury content of the urine and whole blood was determined. For these subjects, the mercury content of the plasma and erythrocytes was estimated from the mercury concentration of the whole blood and ratios of mercury in blood components on other sampling occasions in the same year.

For the exposed group, we calculated a cumulative exposure index for each subject by integrating the previous yearly mean levels of mercury in blood over employment time.

In addition, the number of occasions when the blood mercury levels had been found to exceed 150 nmol/l was registered. This level is currently used at the factory as a biological exposure limit, and a higher level results in temporary removal from exposure.

For the micronucleus assay, buffy coat leukocytes from heparinized venous blood were incubated for 8 h in 1640 Roswell Park Memorial Institute (RPMI) medium with 15 % fetal calf serum. Parallel cultures were prepared with either phytohemagglutinin (PHA) (Gibco; 1 ml/100 ml) or pokeweed (PWM) (Gibco; 1 ml/100 ml) as the mitogen. It has been shown previously that the appropriate incubation time is the same for both mitogens (18). The cells were prepared according to the method described by Högstädt (15).

In the first step, 3000 lymphocytes from each individual were analyzed “blindly” by the same observer for the presence of intracellular micronuclei. In the second step, the number of cells corresponding to 10 micronuclei were scored, and the sizes of the cell nuclei

and the micronuclei were measured according to the method previously described (16). In no case were more than 3000 lymphocytes scored in the second step. The size of the micronucleus was expressed as the ratio between the surface of the micronucleus and the corresponding main nucleus of the cell. This procedure was necessary since the different phases of the cell cycle gave different sizes of cell nuclei and micronuclei. The method error in the determination of the frequency of micronuclei was calculated from repeated scoring by the same observer. The coefficient of variation was 17 % when the frequency of micronuclei was based on the examination of 3000 cells.

The frequencies of micronuclei were approximately normally distributed, while the differences in frequencies between matched pairs, the mean size ratios, the mercury concentrations, the numbers of mercury peaks, and the cumulative exposure indices were not. For comparisons of micronuclei frequencies and size ratios between groups, Wilcoxon's matched-pairs signed rank test was used. The proportions of "large micronuclei" were compared with the use of Fisher's exact test. Correlations between exposure variables and cytogenetic effects were calculated with Spearman's rank correlation coefficient (r_s). Associations between more than two variables were analyzed with the multiple linear regression technique. P-values of <0.05 (two-tailed) were considered statistically significant.

Results

The mercury levels in plasma, erythrocytes, and urine were higher in the exposed workers, as could be expected (table 2). All these levels were highly intercorrelated in the exposed group, and to some extent also associated in the reference group (correlations of neither group shown in table). The exposure times, the cumulative dose indices, and the numbers of peaks were highly intercorrelated in the exposed group. There was also, as expected, a positive correlation between fish consumption and the erythrocyte mercury concentration in the referents.

For a few subjects, the cultivation of lymphocytes was unsuccessful.

For lymphocytes stimulated with either PHA or PWM, the frequencies of micronuclei in the exposed

and reference groups were similar (table 3), and the differences were not statistically significant. Nor were there any statistically significant differences between the exposed group and the referents with respect to the individual mean size ratios of micronuclei in lymphocytes stimulated by PHA or PWM (table 4). The same held true when the numbers of subjects with "large" micronuclei were compared in the two groups (table 4).

There were no statistically significant differences between the smokers and nonsmokers in the combined group of exposed subjects and referents, with respect to either frequencies or sizes of micronuclei stimulated with either PHA (mean frequency 5.1 ‰ and 4.7 ‰ for the smokers and nonsmokers, respectively) or PWM (mean frequency 5.3 ‰ and 4.7 ‰ for the smokers and nonsmokers, respectively). There was no statistically significant correlation between the frequencies of micronuclei induced by PHA and PWM ($r_s = 0.24$, $P = 0.11$). The micronucleus frequencies showed, however, a significant positive association with age ($r_s = 0.38$, $P = 0.007$ and $r_s = 0.55$, $P < 0.0001$ for PHA and PWM, respectively). For lymphocytes stimulated with PHA, there was also a positive association between age and the individual mean size ratio of the micronuclei ($r_s = 0.37$, $P = 0.01$). No such effect was seen for the PWM-stimulated cultures. The effect of age on frequency and size (only PHA) remained present when smoking habits were taken into account.

Within the exposed group, there was a positive correlation between the frequency of micronuclei induced by PHA on one hand and the exposure time, cumulative exposure index, and number of mercury peaks in the blood on the other ($r_s = 0.46$, $P = 0.02$; $r_s = 0.60$,

Table 3. Frequency (‰) of micronuclei in lymphocytes stimulated by phytohemagglutinin (PHA) or pokeweed mitogen (PWM) in mercury-exposed chloralkali workers and unexposed referents. Frequencies are based on 3000 examined lymphocytes. (95 % CI = 95 % confidence interval)

Group	PHA stimulation			PWM stimulation		
	Number of subjects	Mean	95 % CI	Number of subjects	Mean	95 % CI
Exposed	24	5.2	4.3—6.1	24	5.1	4.2—6.0
Reference	26	4.7	3.8—5.5	25	5.0	4.5—5.5

Table 4. Individual mean size ratios (size of micronucleus/size of main nucleus) of micronuclei in lymphocytes stimulated by phytohemagglutinin (PHA) or pokeweed mitogen (PWM) in mercury-exposed workers and in unexposed referents. The mean size ratios were calculated only for subjects with ≥ 3 micronuclei. Furthermore, the number of subjects with at least one large (size ratio >0.1) micronucleus is shown. (GM = geometric mean, 95 % CI = 95 % confidence interval)

Group	PHA stimulation				PWM stimulation			
	Number of subjects	Size ratio		Number of subjects with >1 large micronucleus	Number of subjects	Size ratio		Number of subjects with >1 large micronucleus
		GM	95 % CI			GM	95 % CI	
Exposed	23	0.033	0.028—0.040	7	24	0.033	0.029—0.039	6
Reference	23	0.032	0.027—0.038	6	25	0.038	0.033—0.043	11

Figure 1. Frequency of micronuclei in 3000 lymphocytes (mitogen PHA) for workers exposed to mercury vapor, as a function of the cumulative exposure index calculated from the previous levels of mercury in whole blood (1A) and the number of recorded blood mercury (HgB) peaks > 150 nmol/l (1B). One subject (indicated with an arrow) had 29 recorded peaks.

Table 5. Linear and multiple linear regression with the frequency (%) of micronuclei in peripheral lymphocytes (phytohemagglutinin as the mitogen) as the dependent variable for 24 mercury-exposed subjects. The cumulative exposure index denotes the integrated whole blood level of mercury (HgB) over employment time. (R^2 = fraction of the total variance explained by the regression model)

Independent variables	Regression coefficient	P	R^2
Single variables			
Cumulative exposure index	0.0013	0.0035 ^a	0.33
Number of HgB peaks	0.18	0.014	0.24
Age	0.075	0.035	0.19
Smoking (yes/no)	1.2	0.18	0.08
Multiple regression model 1			
Cumulative exposure index	—	—	0.38
Age	0.0010	0.046 ^b	—
Smoking	0.040	0.25	—
Multiple regression model 2			
Number of HgB peaks	—	—	0.48
Age	0.18	0.0067	—
Smoking	0.077	0.01	—
	0.55	0.44	—

^a $P = 0.0022$ if the cumulative exposure index is log-transformed.

^b $P = 0.034$ if the cumulative exposure index is log-transformed.

$P = 0.002$; $r_s = 0.50$, $P = 0.01$, respectively). The frequency of micronuclei has been plotted against the cumulative exposure index and the number of blood mercury peaks in figures 1A and 1B. In a multiple linear regression model, also including age and smoking, the associations between micronucleus frequency on one

hand and the cumulative exposure index and the number of blood mercury peaks on the other were still present and statistically significant (table 5). A log-transformation of the cumulative exposure index increased the association with PHA-induced frequency of micronuclei somewhat more, as could be expected from figure 1A. As shown in figure 1B, there was one "outlier" with many blood mercury peaks. When that worker was excluded, the aforementioned correlation coefficients remained nearly unaffected, and the association between micronucleus frequency and blood mercury peaks in the multiple regression model was still statistically significant.

No associations to PHA-induced frequency were seen for the actual mercury levels or for the frequency of micronuclei induced by PWM or the size ratios with either PHA or PWM as the mitogen.

Discussion

The micronuclei frequencies were not significantly increased among the mercury-exposed workers as compared with the referents. The power of the present study permits a chance of 80 % to show a 25 % increase in the micronucleus frequencies ($\alpha = 0.05$, one-tailed) of lymphocytes stimulated by either PHA or PWM. Blood and urinary mercury levels reflect ex-

posure during the past weeks and months, respectively. As we found no associations between micronucleus frequencies on one hand and the concentrations of mercury in these biological fluids on the other, it seems unlikely that micronucleus frequencies are influenced by moderate recent exposure to mercury vapor.

With respect to long-term exposure, the interpretation of the data is more complex. There was, for lymphocytes stimulated by PHA, a statistically significant positive association between micronucleus frequency on one hand and the cumulative exposure index and the number of recorded blood mercury peaks on the other. If this is not merely a random finding, but a true causal relationship, it indicates that long-term and/or high exposure to mercury may damage the genetic material in lymphocytes, whereas the lack of an association with the actual mercury levels in biological fluids speaks against a temporary effect of the mercury present in the cultured lymphocytes at the moment of cell division.

The rationale for using both PHA and PWM as mitogens is the fact that PWM, in contrast to PHA, not only stimulates T-lymphocytes, but also the B-lymphocytes, and that the effects of different environmental agents on these classes of lymphocytes may not be the same (19, 20). As certain fractions of lymphocytes have a very long life, a cytogenetic effect accumulated over many years is not theoretically illogical. Such an accumulation of cytogenetic effects is a more likely explanation for the association between the cumulative exposure and the micronucleus frequency than an accumulation of mercury, which has not been shown to occur in the hematopoietic system. Previous damage to lymphocyte chromosomes may increase the tendency to form micronuclei when these lymphocytes divide in culture.

The validity of our exposure indices and the cytogenetic analyses is, naturally, crucial for the ability to prove an adverse effect of the exposure. The cumulative exposure index and the number of blood mercury peaks suffer from substantial errors, being dependent only upon a few blood mercury measurements per year from the exposed subjects. Furthermore, as the age distribution of human lymphocytes is unknown, we cannot know a priori whether the cumulative exposure during one year, two years, or the like or the total exposure time is the optimum for detecting a potential adverse effect. A misclassification of exposure due to imprecise methods should, however, be nondifferential (21) and tend to weaken a possible dose-response relation.

The cumulative exposure index is, naturally, highly correlated with the total exposure time and also with the occurrence of high peaks of mercury exposure. In the multiple regression model, the association with micronucleus frequency was somewhat stronger for the blood mercury peaks than for cumulative exposure. However, with the limited size of the material and the frequency of mercury sampling, the distinction be-

tween the intensity and length of the previous mercury exposure remains uncertain.

As to the cytogenetic assays, the method errors are not negligible. Again, however, a nondifferential misclassification would blur a "true" difference between our two groups and also a possible dose-response relation. Therefore, it could not explain the significant association between previous exposure within the mercury-exposed group and the PHA-induced micronucleus frequency.

The hypothesis that exposure to inorganic mercury, a spindle poison, may change the micronucleus size distribution by increasing the number of "large" micronuclei was not supported by our data. If aneuploidy accounts for a low fraction of the total number of micronuclei, the power to detect an increase in such an effect may be too low in a study of micronuclei, and traditional methods of studying numerical chromosome aberrations may be preferable, as has been shown for exposure to methylmercury (22). It should, however, be pointed out that adverse effects on the mitotic spindle in lymphoid cells in bone marrow or lymph nodes may also result in "instability" and thus give rise to chromosome aberrations other than numerical ones in later cell generations (ie, in circulating lymphocytes or after cell division in cultures).

No in vitro or in vivo studies of micronuclei in human lymphocytes exposed to mercury have yet been reported. Popescu et al (7) found an increased number of chromosome aberrations, but no increased aneuploidy, in four workers with high exposure to mercury vapor (urinary mercury level 710–1930 nmol/l). Another study (6) reported increased aneuploidy in 17 workers with low mercury vapor exposure, but then no effect was found in a study of 28 chloralkali workers with moderate exposure to mercury vapor (8). Mabile et al (9) found no increased frequency of structural chromosome aberrations in 22 workers exposed moderately to mercury vapor. The presence of aneuploidy was not reported.

The increase in frequency and size of micronuclei with age (PHA as the mitogen) has been described previously (18). In the present study, the effect of age on micronucleus frequency (but not on size) was also seen in lymphocytes stimulated by PWM.

As most cytogenetic studies, the present investigation was made on peripheral lymphocytes. These cells are easy to study, but they are not necessarily representative of possible cytogenetic damage caused by mercury in other tissues or cell types, as such an effect is dependent, for example, on the concentration of mercury in the various cell types, and the presence of detoxifying and repair mechanisms.

When considered together, the limited studies conducted to date do not give any strong indications of adverse cytogenetic effects in lymphocytes at low or moderate exposure to mercury vapor. In view of the findings in the report by Popescu et al (7) and the results of the present study, future investigations of

subjects with high and/or prolonged exposure seem, however, to be justified. Furthermore, the general opinion that inorganic mercury is not carcinogenic in humans (1, 23, 24) should be substantiated by additional animal (preferably inhalation) studies and epidemiologic studies of cancer incidence among exposed workers.

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