Cytogenetic damage related to low levels of methyl mercury contamination in the Brazilian Amazon

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ABSTRACT

The mercury rejected in the water system, from mining operations and lixiviation of soils after deforestation, is considered to be the main contributors to the contamination of the ecosystem in the Amazon Basin. The objectives of the present study were to examine cytogenetic functions in peripheral lymphocytes within a population living on the banks of the Tapajós River with respect to methylmercury (MeHg) contamination, using hair mercury as a biological indicator of exposure. Our investigation shows a clear relation between methylmercury contamination and cytogenetic damage in lymphocytes at levels well below 50 micrograms/gram, the level at which initial clinical signs and symptoms of mercury poisoning occur. The first apparent biological effect with increasing MeHg hair level was the impairment of lymphocyte proliferation measured as mitotic index (MI). The relation between mercury concentration in hair and MI suggests that this parameter, an indicator of changes in lymphocytes and their ability to respond to culture conditions, may be an early marker of cytotoxicity and genotoxicity in humans and should be taken into account in the preliminary evaluation of the risks to populations exposed *in vivo*. This is the first report showing clear cytotoxic effects of long-term exposure to MeHg. Although the results strongly suggest that, under the conditions examined here, MeHg is both a spindle poison and a clastogen, the biological significance of these observations are as yet unknown. A long-term follow-up of these subjects should be undertaken.

Key words: Brazilian Amazon, Mercury, Mitotic Index, Cytogenetics.

INTRODUCTION

Since the late seventies extensive gold mining operations using techniques based on amalgation with mercury have developed in the Amazon Basin. In close association with these mining activities, there has been important deforestation for population settlements and development of agriculture. The mercury rejected in the water system from mining operations and lixiviation of soils after deforestation is considered to be the main contributor to the contamination of the ecosystem (Roulet et al. 1998a).

Studies conducted in the region of the

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Tapajós River, a major affluent of the Amazon River, have shown that mercury is present in all environmental compartments (water, soil, plant) (Roulet et al. 1988b, c; Castilhos et al. 1998, Malm 1998). The biotransformation of inorganic mercury into methylmercury in aquatic environments is a well known process that makes human exposure possible through consumption of contaminated fish (WHO 1990, 1991). In the region of the Tapajós River, where fish is the dietary mainstay, methylmercury exposure levels in humans, measured in hair ranges from a few μg/g up to 150 μg/g; the median values reported are in the order of 10 to 20 μg/g (Nakanishi 1992, Pfeiffer et al. 1993, Grandjean et al. 1993, Lebel et al. 1998).

In vitro studies have shown that methylmercury is a cytotoxic agent; reduced mitotic index and chromosomal aberrations are induced in human lymphocytes treated *in vitro* with methylmercury (Bahia et al. 1999, Nakatsuru et al. 1985, Verschaeve et al. 1985, Betti et al. 1992, 1993a, b). Few studies have been performed on possible cytotoxic effects in human populations with methylmercury contamination and authors, reviewing these studies, have pointed out that the findings are borderline or inconclusive (Nickle 1999, De Flora et al. 1994). Although there are methodological problems with each of the studies (small sample size, no control group, confounding factors not taken into account), the overall picture suggests that there may be an association between methylmercury exposure and cytogenetic deficiencies.

In the early seventies, two studies from Sweden, which included 9 and 23 exposed persons respectively, suggested correlations between blood mercury levels and lymphocyte structural chromosomal aberrations (Skerving et al. 1970, 1974). In 17 patients with Minamata Disease, Kato and Nakamura (1976) reported a 18.7% frequency of chromosome breakage and a 2.6% frequency of chromosome reunions and rearrangements, however, there was no control group. A study of 16 fish-eating subjects from a polluted area in Colombia found no differences in the frequency of sister-chromatid exchanges compared to controls; the frequency of chromosomal aberrations was higher in the exposed group only when achromatic lesions were included (Monsalve & Chiape 1987). In a larger scale study, Wulf et al. (1986) analyzed sister-chromatid exchanges in lymphocytes taken from 147 persons whose diet included seal meat; they showed mean frequencies 1.7 times higher in the heavy seal-meat eaters as compared to those who ate less; moreover, the frequency of sister-chromatid exchanges increased with increasing blood mercury levels. They did not report hair mercury levels, which may be a better indicator of longterm exposure.

The objectives of the present study were to examine cytogenetic functions in peripheral lymphocytes (mitotic index, polyploidal aberrations and chromatid breaks) among a population living on the banks of the Tapajós River with respect to methylmercury contamination, using hair mercury as a biological indicator of exposure.

MATERIAL AND METHODS

Population

Cytogenetic study of the analysed population was aproved by the Ethics Committees from the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) and the *Universidade Federal do Pará*, Belém – Pará, Brasil. The persons who participated in this study live in a small village, Brasília Legal, situated on the bank of the Tapajos River, an effluent of the Amazon (3°59′00″S, 55°30′00″W). The village is approximately 250 km downstream from gold-mining operations and the villagers are not exposed to mercury vapours. Brasília Legal is accessible only by water, a 12 hour boat trip from Santarém, a city of several hundred thousand. There are minimal health facilities in Brasília Legal and electricity for only 3 hours every evening. The adult population ($\geq$ 15 years) is made up of approximately 250 persons.

Recruitment of study participants was carried out from house to house with the help of the local community health nursing aides. The study objec-

tives were explained and villagers were invited to come to the Health Center where blood and hair samples were taken, and a questionnaire including socio-demographic information, smoking and drinking habits, medical and work history was administered by interview.

A total of 98 adults, ranging in age from 15-81 years, participated in the study. Their age distribution, which is similar to the overall population, is presented in Table I with other relevant characteristics: sex, smoking and drinking habits, a history of malaria and previous work in the gold mining region. For the current smokers, the number of cigarettes per day ranged from 1 to 4; 4% (3 persons) of the current drinkers reported drinking more than once or twice a week and only 2 persons reported previously using marijuana. One third (33.3%) of the men and two of the women had worked in the gold mining region, where they would have been exposed to mercury vapours. It is in this area that most of the cases of malaria were contracted; of the 24 persons with a history of malaria, 15 (79%) had worked in the gold mining region.

TABLE I

Characteristics of the study participants.

	n	%
age category		
$\geq 15 < 25$	35	35.7
$\geq 25 < 35$	24	24.5
$\geq 35 < 45$	17	17.3
$\geq 45 < 55$	12	12.3
≥ 55	10	10.2
sex		
women	46	46.9
men	52	53.1
smoking habits		
non-smoker	51	52.0
ex-smoker	29	20.4
smoker	27	27.6
alcoholic beverages		
non-drinker	37	37.8
drinker	61	62.2
malaria		
no	72	74.2
yes	25	25.8
gold mining region		
never	78	80.4
yes	19	19.6

Cytogenetic Techniques

Blood was obtained in heparinized vacutainers by venupuncture. In order to avoid potential loss of viability associated with transport and delay, cultures were prepared on the boat, using electricity provided by a generator. The blood was stored immediately in a refrigerator and within a maximum of 4 hours, two independent cultures were set up by adding 12 drops of whole blood in 5ml of RPMI 1640 (GIBCO), containing 20% fetal calf serun (Cultilab) with antibiotics (100 IU penicilin/ml and 100 μg streptomycin/ml, GIBCO) and 4% phytohemoagglutinin (Cultilab). Cultures were incubated in a controlled water-bath at 37°C for 48h. Colchicine (0.8mM, Sigma) was added to the cultures 2h before harvest to obtain a maximum of cells at metaphase.

Cells were harvested by centrifugation (1000 rpm/min) and treated during 10 minutes with KCl (0,075 M, MERCK). They were fixed with Carnoy fixative 1:3 (glacial acetic acid:absolute methanol). Slides were prepared, air-dried and stained for 10 minutes with 3% Giemsa stain (MERCK) diluted in buffer solution (pH = 6.8).

The slides were analyzed, using light microscopy, in the Cytogenetic Laboratory at the Federal University of Pará in Belém, Brazil. For lymphocyte analyses, one of the two cultures that were collected for each individual, was selected randomly. If the selected culture showed poor proliferative potential, the other one was used. At least one culture was adequate for every person. The mitotic index (MI) (frequency of metaphase nuclei in 1000 nuclei) and the number of polyploidal aberrations (PA) in 1000 cell was determined for each individual. For chromatid breaks, 100 clear metaphases were analyzed for each culture. The observers were unaware of subjects' exposure levels.

Hair Sampling and Analyses

Hair strands from the root were taken from the occipital region and then placed in plastic bags, with the root end stapled. Analyses for mercury determination were conducted in the laboratories of the Environmental Research Chair of the University of Québec in Montréal, using Cold Vapor Atomic Fluorescence spectrophotometry (CVAF).

Hair strands were cut in one cm segments and each segment was analyzed for total mercury, according to the procedure described by Bloom and Fitzgerald (1988) and adapted for hair. Inorganic mercury determination was done on the first segment using the methods described by Farant et al. (1981) and adapted for CVAF. Analytical quality was ensured by including a Health Canada sample of powdered hair in the series. In the present analysis, the mean value for total mercury of the 2 first centimeters was used.

RESULTS

Hair Mercury Levels

Total hair mercury levels for this population was log normally distributed and ranged from 0.57 μg/g to 153.8 μg/g. The latter however was an outlier and is treated separately in the analyses, the next highest value was 71.85μg/g. The median level was 13.50μg/g, with 7.95μg/g and 22.19μg/g, the 25th and 75th quartile values, respectively. Women have significantly lower levels (median: 10.8μg/g) as compared to men (median: 17.08μg/g; Mann-Whitney $U = 823.5$; $p < 0.05$). There was no relation between hair mercury levels and smoking status, alcohol consumption, age, having worked in the garimpos or having suffered from malaria.

Mitotic Index

For the participants in this study, the mitotic index ranged from 8 to 36 per 1000 cells, with a mean of 25.20 ± 7.8. Table II presents the mean mitotic indices with respect to the relevant characteristics of the group. Women had a significantly higher index as compared to men. Although smokers presented a significantly lower mitotic index as compared to ex-smokers and non-smokers, when sex was entered into the model, this difference was no longer significant (2 factor ANOVA: sex: F = 5.05; p = 0.03; smoking: F = 0.70; ns). The same pattern was observed for alcohol consumption, with drinkers having a lower mitotic index as compared to non-drinkers, however, when sex was included in the model, neither variable was significant (2 factor ANOVA: sex: F = 1.85; ns; alcohol consumption: F = 1.85; ns). No significant differences were observed with age, malaria and having lived in the gold mining area.

The relation between mitotic index for women

TABLE II

Mean mitotic index per 1000 cells with respect to the characteristics of the study participants.

	Mitotic Index	
	mean	st. dev.
age category		
$\geq 15 < 25$	26.5	7.8
$\geq 25 < 35$	24.2	7.4
$\geq 35 < 45$	22.8	8.7
$\geq 45 < 55$	23.7	8.3
≥ 55	27.4	7.4
sex		
women	27.1	6.8
men	23.2	8.4**
smoking habits		
non-smoker	26.0	7.7
ex-smoker	27.3	6.6
smoker	21.5*	8.2*
alcoholic beverages		
non-drinker	27.4	6.9
drinker	23.7*	8.2
malaria		
no	25.7	7.5
yes	22.8	8.5
gold mining region		
never	26.0	7.5
yes	22.1	8.6

ANOVA. **p<0.01; *p<0.05.

and men with respect to total hair mercury is presented in Figure 1. There appears to be an initial plateau where mitotic indices vary between 30 and 36; the relation is best described by a second degree polynomial ($r^2 = 0.64$; $F = 84.5$; $p < 0.001$) The outlier (not in the graph), whose HHg is 153.8 μg/g has a mitotic index of 12.

Polyploides

The frequency of polyploides per 1000 lymphocyte cells ranged from 0 to 16. The majority of participants (63.9%) did not present this aberration. The distribution of persons with polyploidal aberrations (PA) with respect to the characteristics of the group are presented in Table III. Although proportionally more men than women presented PA, the difference did not attain statistical significance. Polyploidal aberrations were observed significantly more frequently among alcohol drinkers as compared to non-drinkers and among those who had lived in the gold mining region as compared to those who had not. When stratified according to sex, these differences are no longer present.

Figure 2 shows the relation for women and men for PA frequency and hair mercury level. The lowest level at which PAs are observed is 7.25 μg/g HHg. Above this level there is an increase in both the number of aberrations observed as well as the relative frequency of persons presenting them. Using 7.25 as a cut-off point, the simple regression line between the frequency of polyploidal aberrations and total hair mercury level is highly significant ($r^2 = 0.41$; $F = 51.7$; $p < 0.001$). At HHg $\geq$ 20 μg/g, the prevalence of persons with polyploidal aberrations is 86.7% as compared to 18.8% for those with HHg $\geq$ 10 μg/g <20 μg/g and 8.8% for those with levels below 10. The differences are highly significant (Chi square: 48.9, df=2; $p < 0.001$). A total of 16 polyploides per 1000 cells were observed for the outlier.

Chromatid Breaks

Between 1-3 breaks were observed in lymphocytes for 14 persons (14.6%); for 11 there was only one break, for 2 there were 2 breaks and 1 person presented 3 breaks. The only characteristic that distinguishes those with breaks from those without is the level of HHg; those with chromatid breaks have significantly higher levels of HHg as compared to those without (30.46 μg/g $\pm$ 10.7 vs 14.5 μg/g $\pm$ 11.6; ANOVA F=23.3; $p<0.001$). Among those with HHg $\geq$ 20 μg/g, the prevalence of persons with breaks is 37.9%, as compared to 9.4% for those with HHg $\geq$ 10 μg/g $<$ 20 μg/g; none of the persons with HHg levels below 10 presented breaks. The differences are highly significant (Chi square: 19.4, df=2; $p<0.001$).

TABLE III

Distribution of persons with polypoidal aberrations with respect to the characteristics of the study participants.

	Polyploidal aberrations	
	n	%
age category		
$\geq 15 < 25$	9	25.7
$\geq 25 < 35$	13	54.2
$\geq 35 < 45$	7	43.8
$\geq 45 < 55$	4	33.3
≥ 55	2	20.0
sex		
women	12	26.1
men	23	45.1
smoking habits		
non-smoker	16	45.7
ex-smoker	6	17.1
smoker	13	37.1
alcoholic beverages		
non-drinker	8	21.6
drinker	27	45.0*
malaria		
no	22	31.0
yes	13	52.0
gold mining region		
never	23	29.9
yes	11	57.9*

Chi square: *p<0.05.

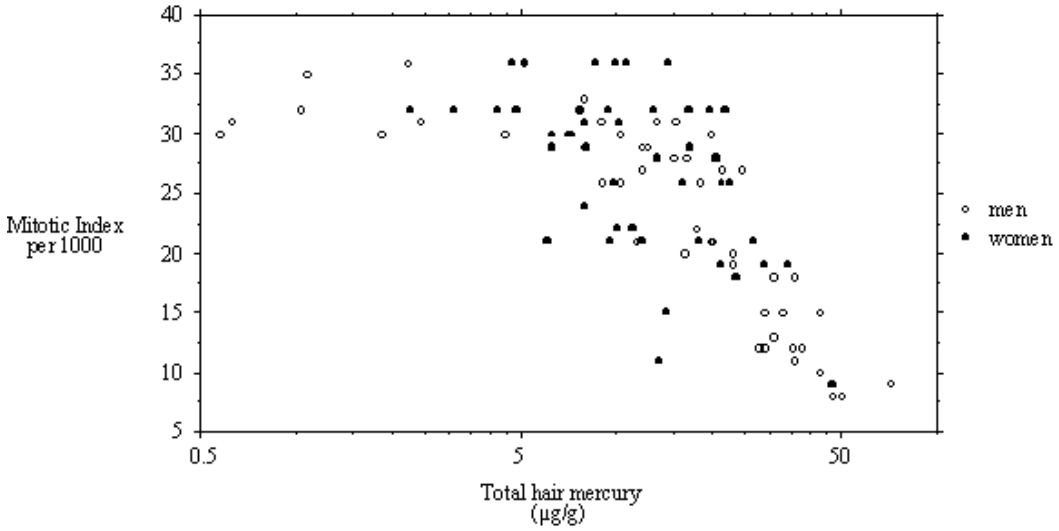

Fig. 1 – Relation between mitotic index for men and woman with respect to total hair mercury.

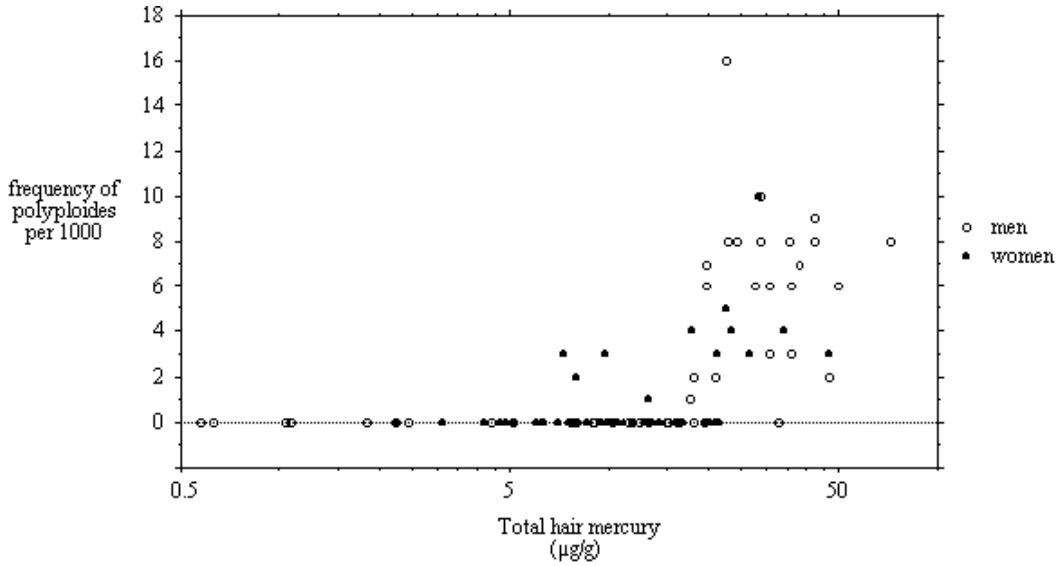

Fig. 2 – Relation for men and woman for polyploidal aberrations frequency and hair mercury level.

DISCUSSION

The findings of this study show a clear relation between methylmercury contamination and cytogenetic damage in lymphocytes at levels well below 50μg/g, the level at which initial clinical signs and symptoms of mercury poisoning occur (IPCS 1990). The first apparent biological effect with increasing MeHg hair level was the impairment of lymphocyte proliferation measured as mitotic index, the proportion of cells in M-phase of the cell cycle (Rojas et al. 1993). Decreased MI reflects inhibition

of cell-cycle progression and/or loss of proliferative capacity. During *in vitro* experiments on chromosomal aberrations, variation in MI is used to monitor induced cellular toxicity. This information on the degree of cytotoxicity is essential to adequately select harvest time and test concentration, and is especially important when the results are used in risk assessment of compounds to which humans may be exposed. Mitotic index suppression has been suggested for use in dose selection for cytogenetic testing for regulatory purposes (UKEMS 1990). However, this parameter is rarely considered for *in vivo* studies.

The relation between mercury concentration in hair and MI suggest that this parameter, an indicator of changes in lymphocytes and their ability to respond to culture conditions, may be an early marker of cytotoxicity and genotoxicity in humans and should be taken into account in the preliminary evaluation of the risks to populations exposed *in vivo*. In a study of chronic exposure to another metal, arsenic (As), impairment of lymphocyte proliferation was observed in exposed individuals as compared to controls (Ostrosky-Wegman et al. 1991, Gonsebatt et al. 1994).

In this Amazonian population, no specific effect of age on proliferative ability of lymphocytes was observed. These results are similar to other human studies which reported no variation in lymphocyte proliferative rate with age in a control group from the United Kingdom (Anderson et al. 1988). Similarly, the sex-related differences in mitogen-induced lymphocyte proliferation observed here are in conformity with others. In non-exposed individuals, Anderson et al. (1991), observed higher blastogenic transformation in PHA stimulated lymphocytes from women as compared to men. Although Gonsebatt et al. 1994 report a greater impairment of lymphocytes in women exposed to arsenic in drinking water as compared to men, they have no measure of dose, which may prove to be higher in women. Sager et al. (1984) reported that the number of cells in the developing cerebellar external layer in 2-day-old mice was significantly reduced after methylmercury treatment in males but remained unaltered in females.

The fact that exposure was associated with changes in the proliferative potential of lymphocytes raises the question of possible immunological implications. There is a large experimental data base on the immunosuppressive properties of metals, their inorganic salts and organometallic components (Koller 1980). Methylmercury, together with calcium, lead and As, have shown to cause immunological changes in laboratory animals (NRC 1992). The observed reduced lymphocyte proliferation associated with low levels of mercury may translate into reduced resistance to disease in this Amazonian population.

The positive results found for polyploidy and chromatid breaks with mercury exposure and their appearance following MI perturbations are in agreement with the molecular mechanism of action inferred from *in vitro* observations. The genotoxicity of mercury is generally attributed to its binding with tubulin-SH, causing the impairment of spindle function and chromosome segregational error during cell division (Onfelt 1983, Sager et al. 1983). This has been regarded as the cause of the increased frequency of polyploidy and aneuploidy in Allium and Drosophila (Ramel & Magnusson 1969, 1979, Ramel 1969, Fiskesjo 1988). Moreover, *in vitro* polymerization and depolymerization of microtubules is affected by MeHg (Vogel et al. 1985). This could explain some of the multiple effects of MeHg on the cell cycle, including a lengthened G1 and decreased transition probability after short term exposure of cycling cells, and a G2 accumulation after a longer term exposure (Vogel et al. 1986). Studies on the potentiating effects of organic and inorganic mercuries on clastogen-induced chromosome aberrations induced in CHO cells suggest that they inhibit some of the protein activities involved in the DNA repair process (Yamada et al. 1993).

The findings of the present study support the notion that methylmercury has a clastogenic effect, causing chromatid breaks. Mercury like other metals such as cadmium, chromium, zinc, magnesium

and manganese may be genotoxic through generation of free oxygen radicals (Ochi et al. 1983, Cantoni et al. 1984a, b, Snyder 1988). Methylmercury is known to generate free radicals through induction of lipid peroxidation in animal tissue (Ganther 1978, Shinada et al. 1990). Therefore the observed breaks could result from free radical damage.

In contrast to inorganic mercury, methylmercury easily passes the placenta barrier at least in some species. The occurrence of prenatal intoxication in children shows that this is the case also in humans. It remains to be investigated whether tissues from exposed fetuses have an increased frequency of cells with polyploids or chromatid breaks. It is not yet known whether it is associated with an increased frequency of spontaneous abortions. Theoretically, methylmercury-induced chromosome damage in germline cells could give rise to abnormal offspring.

This is the first report showing clear cytotoxic effects of long-term exposure to methylmercury. The group was sufficiently large with a wide range of mercury exposure, based on a well-known biological marker, hair mercury. In the situation of this riverbank population on the Tapajós River, it is estimated that exposure to mercury has been present over the past twenty years. Although the results strongly suggest that, under the conditions examined here, MeHg is both a spindle poison and a clastogen, the biological significance of these observations is as yet unknown. A long-term follow-up of these subjects should be undertaken. It is noteworthy that lower MIs preceded appearance of chromosomal endpoints such as polyploidy and chromatid breaks suggesting that MI may prove to be a valuable early indicator for this health hazard.

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