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Indicators of Genetic Toxicity in Leucocytes and Granulocytic Precursors After Chronic Methylmercury Ingestion by Cats

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Cytogenetic studies of leucocyte chromosomes from fish-eaters (SKERFVING et al. 1974) or subjects occupationally exposed to organic mercurials (VERSCHAEVE et al. 1976) revealed association of aberrations with blood mercury levels. However, neither the mechanism of induction of genetic damage, nor the lower limit of exposure required, has been established.

Expressions of the genetic toxicity of mercurials at the molecular level include inhibition of DNA repair in algae (DIMITRIEV et al. 1975, WHILIDAL et al. 1976), DNA strand breaks in a slime mould and in cultured mammalian cells (YATSCOFF and CUMMINS 1975, NAKAZAWA et al. 1975), inhibition of a variety of mammalian enzymes including adenyl cyclase (STORM and GUNSALUS 1974), and changes in macromolecular synthesis suggesting disturbances at the level of gene translation (BRUBAKER et al. 1973). The work reported here was undertaken to assess inhibition of capacity for DNA repair in leucocytes, and nuclear damage in bone marrow of cats after chronic exposure to low levels of dietary methylmercury.

MATERIALS AND METHODS

Details of animal care, feeding procedures, clinical, and pathological findings for the present study have already been published (CHARBONNEAU et al. 1976). Briefly, cats received mercury-contaminated fish (*Esox lucius*, northern pike), diluted with northern pike containing background levels of mercury (0.14 ppm) and incorporated at 35% by weight into nutritionally balanced diets. Of the total mercury in the diets (Table 1), 95% was methylmercury. Additional cats received the control fish diet supplemented with methylmercuric chloride. The study lasted 39 mo.

Blood taken just prior to necropsy was assayed for total mercury by flameless atomic absorption spectrophotometry (Table 1). After thiopental anaesthesia, 50 ml of blood was withdrawn from the abdominal aorta.

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Leucocytes were tested for (a) level of unscheduled DNA synthesis, as an indicator of direct damage to DNA and (b) capacity to repair damage due to methylmethanesulphonate (MMS).

Leucocytes separated by gel filtration (COULSON and CHALMERS 1964) were suspended at about 2 x 10^7 cells/ml in culture medium HB597 (Connaught Labs) supplemented with 1% fetal calf serum. Semi-conservative DNA synthesis was inhibited with hydroxyurea (10^{-2}M) for 1 hr. Each leucocyte culture was divided into four samples: two samples received MMS (10^{-3}M) and all samples received 10 µCi/ml tritiated thymidine. Cultures were incubated for 3 hr. DNA in each sample was extracted from trichloroacetic acid-precipitable material after digestion of RNA in 0.3N KOH (TROSKO et al. 1975). Three samples of each DNA extract (0.5 ml) were dissolved in Aquasol (New England Nuclear) and assayed in a Beckman Scintillation Counter, tritium isoset, with external standard. Quench was low and consistent between samples. Counting efficiency was 29%. DNA content was estimated in two 0.5 ml aliquots of extract (BURTON 1956). Each cat was characterized by mean dpm/mean µg DNA.

Bone marrow samples were obtained within minutes after cardiac arrest. Touch and smear preparations were made from femoral marrow and were stained with modified Leishman's stain. A preparation was scored positive if any abnormalities were observed in a sample of 500 cells.

RESULTS

Animals receiving 46 µg Hg/kg/day showed a slight neurological impairment accompanied by a minimal degree of CNS pathology (CHARBONNEAU et al. 1976). Groups receiving 20 or 8.4 µg Hg/kg/day showed no overt clinical signs, nor any pathological damage except for slight reduction in size and fibrosis of the atrio-ventricular node (MOODIE 1976). There was no evidence of toxicity towards circulating leucocytes in any dose group (CHARBONNEAU 1976).

Levels of hydroxyurea-resistant DNA synthesis in cultured leucocytes without challenge by the exogenous alkylating agent are shown in Table 2A. For cats receiving methylmercury from the fish source, mean ^{3}HTdR incorporation decreased regularly with increasing dose of methylmercury. No such regular trend was observed for the methylmercuric chloride source. Mean incorporation was lower for animals receiving methylmercury-contaminated fish than for animals receiving

TABLE 1

Source, Dietary Level, and Equilibrium Blood Level of Mercury

Diet	Fish Diet (ppm Hg)	CH_3HgCl µg Hg/ kg/day	Dose* µg/Hg/ kg/day		Terminal Blood Hg (ppm Hg ± SE)
Control	0.05	0	3.0	M	0.28±0.02
				F	0.24±0.03
"Fish" Source	0.14	0	8.4	M	0.99±0.13
				F	0.51±0.04
	0.33	0	20.0	M	3.14±0.43
				F	1.99±0.19
	0.76	0	46.0	M	7.43±0.81
				F	4.15±0.35
"Chloride" Source	0.05	5.4	8.4	M	1.00±0.09
				F	0.79±0.04
	0.05	17.0	20.0	M	3.44±0.57
				F	3.45±0.41
	0.05	43.0	46.0	M	13.53±2.60
				F	5.51±0.10

* All cats received 60g diet per kg bodyweight per day.

methylmercuric chloride ($F=3.78$), $p=0.06$). Males receiving the lowest fish-source dose (3 µg Hg/kg/day) displayed higher ^{3}HTdR incorporation than corresponding females ($F=4.94$, $p=0.03$).

Levels of unscheduled DNA synthesis (UDS) following challenge by the alkylating agent MMS are displayed in Table 2B. Leucocytes from all groups responded with elevated UDS. However, for the fish source, the magnitude of the response decreased regularly with increasing dose of methylmercury. For treated animals (but not controls) male response to MMS was lower than female response ($F=3.75$, $p=0.06$). This sex difference may be due either to the greater blood mercury levels attained in males (Table 1), or to inherent differences in susceptibility (EL-BEGEARMI et al. 1976).

Least squares regression analysis indicated a negative correlation between blood mercury and UDS ($\alpha<0.05$). It is of interest that both regression lines, relating UDS ±MMS to blood mercury level, anticipated complete inhibition at just over 10 ppm

TABLE 2

Hydroxyurea-Resistant DNA Synthesis in Cat Leucocytes After Chronic Ingestion of Methylmercury

μg Hg/kg/day		Control	Treated		
		3	8.4	20	46
		A. WITHOUT EXOGENOUS ALKYLATION			
Fish Source	M	60, 82	14, 14	39, 39	34, 27
	M	66, 57	49, 44	46, 47	*
	M	93, 92	73, 70	*	25, 27
	F	36, 31	56, 64	34, 43	50, 36
	F	60, 62	79, 78	53, 54	43, 45
	F	34, 33	32, 32	45, 46	37, 39
	Mean	59 ± 9	51 ± 10	44 ± 3	36 ± 4
CH_3HgCl	M		33, 33	52, *	33, 33
	M		128, 81	54, 59	56, 115
	M		26, 38	39, 37	28, 39
	F		44, 42	52, 55	50, 50
	F		49, 49	71, 68	68, 60
	F		49, 79	79, 86	*
	Mean		54 ± 9	59 ± 6	53 ± 10
		B. RESPONSE TO METHYLMETHANESULPHONATE (10^{-3}M)			
Fish Source	M	167, 123	34, 28	112, 153	94, 87
	M	120, 111	63, 78	57, 77	102, 113
	M	179, 211	211, 216	75, 77	57, 64
	F	149, 110	216, 183	93, 112	34, 47
	F	190, 182	230, 202	107, 102	79, 98
	F	80, 71	86, 80	116, 98	77, 68
	Mean	141 ± 18	136 ± 34	98 ± 10	77 ± 10
CH_3HgCl	M		67, 68	150, 197	67, 98
	M		117, 92	117, 135	107, 234
	M		72, 67	90, 83	32, 33
	F		167, 160	161, 192	127, 125
	F		101, 96	123, 132	168, 178
	F		142, 116	90, 99	168, 178
	Mean		106 ± 15	131 ± 16	117 ± 27

Each table entry gives mean dpm/μg DNA, for replicate cultures from each cat, incubated 3 hr with ^{3}HTdR and HU. M±S.E. *Data Missing.

mercury in blood. Clinical assessment of the cats indicated that signs of neurotoxicity began to appear when blood and brain mercury levels reached 10 ppm (CHARBONNEAU et al. 1976). However, the confidence intervals associated with the regression lines were wide.

Adjustment of estimates of UDS for the proportions of neutrophils and lymphocytes in each culture did not alter the observed trends. Further, effects of methylmercury on the relative amounts of radioactive and endogenous thymidinetriphosphate cannot explain the results, since the ratio of radioactivity incorporated into DNA +MMS/-MMS was not constant for the four dose groups. Finally, the hypothesis that methylmercury reduced permeability to MMS and caused an artefactual decrease in UDS response, cannot explain the decreased ^{3}HTdR incorporation in the unchallenged cell cultures. The possibility that low chronic exposure to mercury-contaminated fish directly inhibited unscheduled DNA synthesis must be entertained.

Abnormalities in the bone marrows of the cats were scored, and each cat was characterized simply as with, or without, multinucleated or abnormal cells (Table 3). "Bi- or multinucleated" granulocytic precursors were characterized by the presence of two or more nuclei of even or various sizes (micronuclei). "Nuclear abnormalities" were of three recurring types: 1) nuclear bisection either partial, or total, as seen in nodular lymphosarcoma (MCKENNA et al. 1975); 2) nuclear pleomorphism with irregular nuclear contour, one to several indentations, or accordion-like structure; or 3) nuclear fragmentation with some portions of the nucleus attached together by filaments. The nuclear shape of these cells was reminiscent of Rieder cell nuclei or cells seen in rat acute mononuclear leukemias. However, the staining properties of nuclear chromatin, number and appearance of nucleoli, and cytoplasm were similar to other normal myeloid precursors. Statistically significant increases in myeloid (but not erythroid) anomalies were seen in the group receiving 20 µg Hg/kg/day as contaminated fish, and for all groups receiving methylmercuric chloride.

DISCUSSION

Capacity for unscheduled DNA synthesis (DNA repair) was inhbitied in leucocytes from cats fed 35% of calories as methylmercury-contaminated fish (0.14, 0.33, or 0.76 ppm Hg in the total diet). Further, frequency of abnormalities in the myeloid series of bone marrow was increased. Both effects occurred at blood Hg levels below those associated with clinical signs.

Table 3

Incidence of Abnormalities in Bone Marrow of Cats After Chronic Ingestion of Methylmercury

			Control	Treated		
		µg Hg/kg/day	3	8.4	20	46
Fish Source (M F)	Myeloid Series	Bi- or Multinucleated	0/10	0/7	0/5*	2/8
		Nuclear Abnormalities	0/10	0/7	6/8*	0/8
	Erythroid Series	Bi- or Multinucleated	4/10	2/7	5/8	4/8
		Nuclear Abnormalities	0/10	0/7	2/8	0/8
Chloride Source (M F)	Myeloid Series	Bi- or Multinucleated		0/5	0/8	0/5
		Nuclear Abnormalities		4/6*	7/8*	3/5*
	Erythroid Series	Bi- or Multinucleated		2/6	2/8	3/5
		Nuclear Abnormalities		0/6	0/8	0/5

Each table entry gives no. of cats scored positive/total no. of cats.
*Significantly different from control ($P < 0.05$).

The types of nuclear abnormalities observed were similar to those described following mercurial exposure of man and rabbit by DOWNEY et al. (1930). These nuclear configurations suggest that genetic effects of methylmercury may be mediated, at least in part, by altered tertiary structure of DNA. In DNA from mouse liver, methylmercury was found preferentially in euchromatic regions of the chromosome (BRYAN et al. 1976). It is conceivable that inhibition of DNA repair might result, and lead to strand breakage, as observed in cultured cells.

The absence of inhibitory effect on UDS after exposure to methylmercuric chloride was surprising, since higher doses of both fish and chloride sources of methylmercury gave rise to comparable neurological signs. All cats received the same amount (g/kg body weight/day) of fish of the same species, caught in geologically similar lakes, ruling out an effect due to the fish itself. Selenium concentrations in the mercury-contaminated and control diets were identical. However, the methylmercuric chloride was dissolved in corn oil prior to addition to the diet. Route of absorption, distribution amd metabolism of protein-CH_3Hg (fish source) and lipid-CH_3Hg (chloride source) might well differ.

Although the major clinical expression of organic mercury poisoning in man is neurological, human experience of intoxication was associated with chromosome anomalies in leucocytes (SKERFVING et al. 1974, VERSCHAEVE et al. 1975), diffusive inflammation of the digestive tract, partial or scant formation of bone marrow, atrophy of lymph glands, and an excess mortality (among females) due to bacterial infections (TAKEUCHI 1976). Effects on leucocyte DNA may be responsible for these immunological manifestations of mercury intoxication.

It may be germaine to point out some parallel features of methylmercury intoxication and *Ataxia telangiectasia*. While the former is chemically induced and the latter is inherited, both are associated with neurological dysfunction; with atrophy of Purkinje cells; with optic atrophy and reduction of periferal vision; with reduced immunocompetence (PETERSEN et al. 1966, PATERSON et al. 1976) and, now, with defective DNA repair. This parallel leads us to speculate, that derangement of DNA repair may underlie the neurotoxicity of methylmercury.

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