Table4.Odds ratios for endometrial cancer according to quartile of intake of foods

characteristics	Cases(n=231) Number(%)[b]	Control(n=234) Number(%)[b]	Odds ratios††	(95%CI)
Meat consumption, %				
Few	23 (6.9)	29 (12.4)	1	
1-2 times/weeks	89 (44.6)	86 (36.7)	1.36	(0.7-2.65)
3-4 times/ weeks	103 (38.6)	95 (40.6)	1.71	(0.88-3.32)
Almost every day	16 (9.9)	24 (10.3)	1.09	(0.44-2.71)
				p=0.62
Fish consumption, %				
Few	8 (3.4)	13 (5.6)	1	
1-2 times/weeks	46 (19.9)	60 (25.6)	1.29	(0.45-3.71)
3-4 times/ weeks	126 (54.6)	111 (47.4)	2.14	(0.78-5.86)
Almost every day	51 (22.1)	50 (21.4)	1.7	(0.59-4.89)
				p=0.51
Stirr fried foods consumption, %				
Few	37 (16.0)	47 (20.1)	1	
1-2 times/weeks	95 (41.1)	100 (42.7)	1.52	(0.99-2.32)
3-4 times/ weeks	64 (27.7)	59 (25.2)	1.71	(0.86-3.83)
Almost every day	35 (15.2)	28 (12.0)	1.22	(0.35-4.15)
				p=0.007
Deep fried foods consumption, %				
Few	108 (46.1)	126 (53.8)	1	
1-2 times/weeks	94 (40.7)	76 (33.3)	1.91	(0.47-3.01)
3-4 times/ weeks	23 (9.96)	24 (10.3)	1.54	(0.65-3.63)
Almost every day	6 (2.6)	6 (2.6)	1.96	(0.53-2.68)
				p=0.039

†† Adjusted for age, BMI, education, hypertension, diabetes, age at menarche, pregnancy,menopause status, Lactation ,oral contraceptives use and other cancer history

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分担研究報告書

高分解能GC/MSを用いた特定異性体によるPCB簡易分析への試み

分担研究者　岡村智佳子（東北大学大学院医学系研究科　婦人科学助手）
研究協力者　上瀧　智巳　（医科学分析センターＳＲＬ株式会社）

研究要旨

内分泌かく乱化学物質は、ヒトの健康および生態系に取り返しのつかない重大な影響を及ぼす危険性をはらんだ環境保全上の重要課題であり、早急な対応が求められている。中でもPCBは熱交換媒体や粘着剤、絶縁溶液などの工業用途から家庭生活まで広く長期に渡って使われてきた。今回われわれは内分泌かく乱物質のヒト健康影響を評価するために、ヒトを対象とする疫学研究を施行し、対象者の血液中のPCB分析を施行している。

一方、「ポリ塩化ビフェニル廃棄物の適正な処理の推進に関する特別措置法」（平成13年7月施行）により、近年全国規模でPCBs分解事業が行われつつある。これに伴い分解処理施設の周辺環境保全、周辺住民および作業従事者の暴露評価と安全衛生の観点からスクリーニングを目的としたPCBs分析が必要とされている。

現在のところ最も信頼の高いPCBs分析手法は、高分解能GC/MS（HRGC/HRMS）を用いた全異性体分析である。しかし、この分析手法は緻密な分析が可能である反面、全ての異性体について同定・定量を行う必要があるため、解析には多大な労力が必要とされスクリーニングを目的とした場合に必ずしも適した分析手法とは言えない。

そこで、今回我々はMuirとMoritaらがUNEP（国連環境計画）に提言[1]している7異性体（#28/31, 52, 101/90, 118, 138, 153, 180）および30異性体（#8/5, 18, 28, 31, 44, 49, 52, 95/66, 87, 99, 101, 105/132, 110, 118, 128, 146, 149, 151, 153, 138/163, 156, 183, 187, 201/157, 170, 180, 194, 195, 206, 209)を用いて、簡易分析の検証と暴露評価指標としてのこれら異性体の適用が可能かを試みたので報告する。

A. 研究目的

今回、HRGC/HRMSを用いたPCBs分析法により、MuirとMoritaらがUNEP（国連環境計画）に提言している7異生体および３０異生体を用いて、(1) 簡易分析の検証と (2) 暴露評価指標としてこれらの異性体の適用が可能かどうかを検討する。

B. 研究方法

<u>測定検体</u>

子宮体がん症例 32 例と、それに年齢や居住地をマッチングさせた対照 28 例の血液検体

<u>前処理操作</u>

試料を秤量し、クリーンアップスパイクを添加後、1mol/Lの水酸化カリウム/エタノールにて加水分解処理した。その後、ヘキサンにて3回抽出を行い、ヘキサン抽出液を蒸留水にて3回水洗し、無水硫酸ナトリウムにて脱水後、フロリジルカラムによる精製を行った。精製後のヘキサン溶出液を濃縮し、シリンジスパイクを添加、HRGC/HRMS測定用試料とした。前処理方法の概要フローを示す。

試 料
　| クリーンアップスパイク添加
アルカリ分解
　| 1mol/L KOH/EtOH
ヘキサン 液-液抽出
　| 蒸留水
　| ヘキサン×3
水 洗
　| 蒸留水×3
脱 水
　| 無水硫酸ナトリウム
フロリジルカラム精製
　| ヘキサン溶出
減圧濃縮
　| シリンジスパイク添加
HRGC/HRMS測定

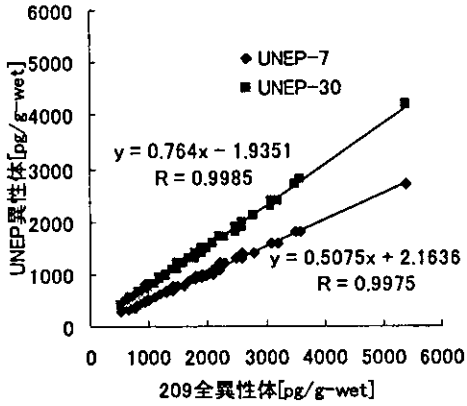

図1　209全異性体とUNEP異性体との相関

測定方法

HRGC/HRMS 測定条件は GC に Agilent 6890 Plus GC (Agilent Technologies)、MS には AutoSpec-Ultima (Micromass) を使用した。注入方法は PTV (Agilent Technologies) を用い、注入量は 5μL とした。分析カラムは HT8-PCB (内径 0.25mm、長さ 60m、関東化学) を使用し、昇温条件:60℃(2.5min)→20℃/min→180℃→2℃/min→260℃→5℃/min→300℃(4min)とした。

C. 研究結果と考察

UNEP-7 および UNEP-30 異性体による簡易分析の試み

血漿 60 例を用いて UNEP-7 異性体および UNEP-30 異性体のそれぞれの Total-PCBs 濃度と全異性体の Total-PCBs 濃度との相関を調べた。その結果、共に R = 0.997 以上の相関が認められた(図 1)。また、定量値としても UNEP-7 異性体の Total 値は全異性体 Total 値の約 5 割を占めており、UNEP-30 異性体の Total 値では約 8 割を占めていることが判明した。

このことから、UNEP-7 異性体または UNEP-30 異性体を用いて、相関式より得られたファクターより全異性体の Total-PCBs濃度が推測可能であることが確認され、UNEP-7 異性体または UNEP-30 異性体を用いる簡易分析の有用性が示唆された。

PCBs 暴露指標としての UNEP-7 および UNEP-30 異性体の活用

PCBs 暴露を想定し、血漿中 PCBs 濃度の約 4 ～ 30 w/w %程度量になるよう、血漿に PCB 標準溶液としてカネクロール 300 (以下 KC300) およびカネクロール 400 (以下 KC400) を 50pg、100pg、200pg、400pg 添加し、KC 無添加血漿と PCBs 濃度の比較を行った。その結果、全異性体の Total-PCBs 濃度において、KC 添加量に対し増加の傾向が見られたものの、KC 添加量 0～100pgの少量添加域では良好な直線性が認められなかった。(図2)。そこで、UNEP-7 および UNEP-30 異性体における KC 添加量に対する 3 塩素化同族体(T3)および 4 塩素化同族体(T4) の濃度変化を確認したところ、KC 少量添加域においても直線性が認められた(図3)。更に、個々の異性体に着目した場合は KC300 の添加に対して#8、＃18、＃28、＃31、＃44、＃49、＃52 で直線性のある濃度変化が確認され、また KC400 では＃18、＃28、＃31、＃44、＃49、＃52、＃87、＃95、＃101、＃110 において同様の濃度変化が確認された。KC300 および KC400 の添加に対する各異性体の濃度変化の一例を図4および図5に示す。

以上のような結果から、PCBsの暴露に対して UNEP-7 異性体や UNEP-30 異性体が、暴露評価の指標となり得る可能性が示唆され

た。

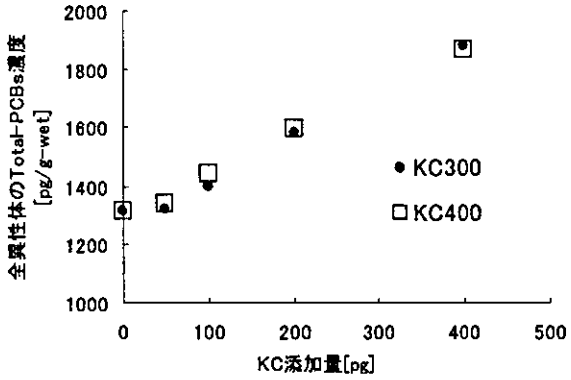

図 2 KC 添加量に対する全異性体 Total-PCBs濃度

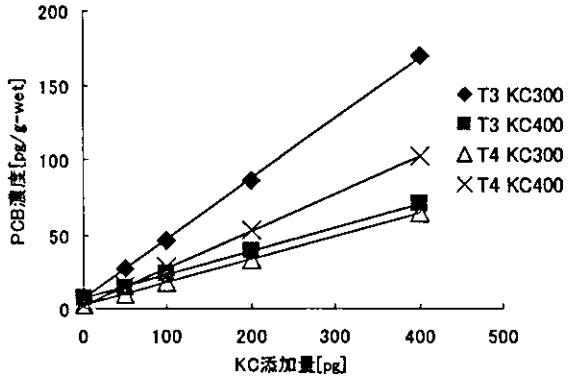

図 3 KC 添加量とUNEP-30 異性体における 3 塩素化および 4 塩素化同族体 Total 値との相関

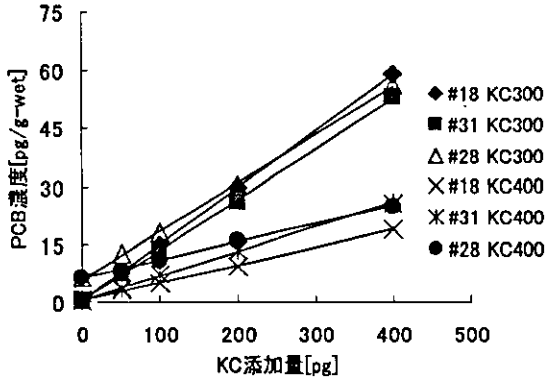

図4 KC 添加量と 3 塩素化異性体との相関

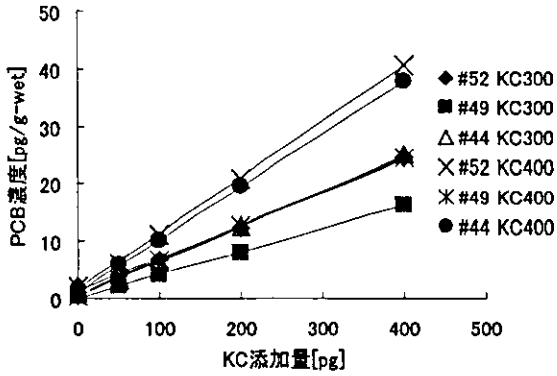

図 5 KC 添加量と4塩素化異性体との相関

D. 結論

PCB の７異生体および３０異生体を用いて、(1) 簡易分析の検証と (2) 暴露評価指標としてこれらの異性体の適用が可能であることが示唆された。暴露評価と安全衛生の観点からもスクリーニングをしていくことにより環境政策の一助になると思われた。

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IV. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Nakai, K, Satoh H. et al.	The Tohoku Study of Child Development: A Cohort Study of Effects of Perinatal Exposures to Methylmercury and Environmentally Persistent Organic Pollutants on Neurobehavioral Development in Japanese Children.	Tohoku J. Exp. Med.	202	227-237.	2004
Sakamoto M, Satoh H.et al.	Maternal and fetal mercury and n-3 polyunsaturated fatty acids as a consumption to fetus.	Environmental Science & Technology	38	3860-3863	2004
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V. 研究成果の刊行物・別刷

Int Arch Occup Environ Health (2004) 77: 571–579
DOI 10.1007/s00420-004-0542-1

ORIGINAL ARTICLE

Katsuyuki Murata · Mineshi Sakamoto
Kunihiko Nakai · Pal Weihe · Miwako Dakeishi
Toyoto Iwata · Xiao-Jie Liu · Tomoko Ohno
Tomoko Kurosawa · Kazuko Kamiya · Hiroshi Satoh

Effects of methylmercury on neurodevelopment in Japanese children in relation to the Madeiran study

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Abstract ***Objectives***: A cross-sectional study was carried out to assess the effects of methylmercury exposure on neurodevelopment in Japanese children, in relation to the Madeiran cross-sectional study, and to estimate benchmark dose (BMD) levels using the data of two studies. ***Methods***: Mercury levels in hair samples obtained from 327 Japanese mothers and their 7-year-old children, and methylmercury levels in the umbilical cord, were determined. Neurodevelopmental examinations, including the brainstem auditory evoked potential (BAEP), were performed on the children. ***Results***: The medians of hair mercury were 1.63 (0.11–6.86) μg/g for mothers and 1.65 (0.35–6.32) μg/g for children, and a significant correlation was seen between the hair mercury levels in mothers and children. The maternal hair mercury was significantly correlated with the methylmercury in the umbilical cords obtained from 49 children. In 210 children whose mothers had not changed their dietary habits since pregnancy, most of the neurodevelopmental variables were not significantly related to hair mercury levels. The BAEP latencies were significantly shorter in the Japanese children than in the 113 Madeiran 7-year-old children, whose mothers had hair mercury of 1.12–54.5 (median 10.9) μg/g. Significant relationships between the maternal hair mercury level and BAEP latencies (peaks III and V, and interpeak I–III) were found only in the merged data of Japanese and Madeiran children. When the lower 95% confidence limit of BMD (BMDL) was calculated, the BMDLs of mercury exposure for BAEP latencies in the merged data were between 6.9 and 10.5 μg/g, and lower than those in the Madeiran children. ***Conclusions***: It is suggested that Japanese children may ingest similar doses per body weight of methylmercury to their mothers. If maternal hair mercury was used as a proxy for mercury exposure at birth, no significant dose–effect associations with the BAEP latencies were observed in Japanese children with exposure levels below 6.9 μg/g of hair mercury, but only when higher-level exposures from Madeiran children were included. The BMDL was lower for the merged data than for Madeiran children alone.

Keywords Methylmercury · Child neurodevelopment · Dose–effect relationship · Benchmark dose · Brainstem auditory evoked potential

K. Murata (✉) · M. Dakeishi
T. Iwata · T. Ohno · T. Kurosawa
Division of Environmental Health Sciences,
Department of Social Medicine,
Akita University School of Medicine,
1-1-1 Hondo, Akita 010-8543, Japan
E-mail: winestem@med.akita-u.ac.jp
Tel.: +81-188-846082
Fax: +81-188-362608

M. Sakamoto · X.-J. Liu
Department of Epidemiology,
National Institute for Minamata Disease,
Minamata, Japan

K. Nakai · H. Satoh
Environmental Health Sciences,
Tohoku University Graduate School of Medicine,
Sendai, Japan

P. Weihe
Department of Occupational and Public Health,
Faroese Hospital Systems, Faroe Islands

K. Kamiya
Environmental Health Department,
Ministry of the Environment, Tokyo, Japan

Introduction

Methylmercury is a worldwide contaminant of seafood and freshwater fish. Its toxicity can produce widespread adverse effects within the nervous system, especially when exposure occurs during brain development (Igata 1993; International Programme on Chemical Safety 1990; National Research Council 2000). Early adverse effects have been characterized by administering neurobehavioral tests to children exposed in utero from maternal seafood diets (Grandjean et al. 1997; Kjellström et al. 1989). The National Research Council

(National Research Council 2000) concluded that prenatal exposure was the most critical and emphasized the findings from a birth cohort study carried out in the Faroe Islands (Grandjean et al. 1997). Nevertheless, neurodevelopmental risks related to such low-level exposures of methylmercury (i.e., at approximately 10 μg/g hair) from contaminated seafood remain disputable.

As neurodevelopmental parameters, various neurobehavioral tests such as the Wechsler Intelligence Scale for Children, Child Behavior Checklist, McCarthy General Cognitive Test, Language Development Test, California Verbal Learning Test, Bender Copying Test, Boston Naming Test, McCarthy Motor Test, reaction time and finger tapping, have been used by many researchers addressing the risk assessment of methylmercury (Davidson et al. 1998; Grandjean et al. 1997; Kjellström et al. 1989). Some of the tests have been reported to be associated with exposure biomarkers at birth, but common tests to three prospective studies in the Faroe Islands (Grandjean et al. 1997), New Zealand (Kjellström et al. 1989), and Seychelles (Davidson et al. 1998) hardly existed (National Research Council 2000). Accordingly, a comparable study with common tests, as well as the test specific to the exposure, would be required. Also, neurophysiological tests such as the brainstem auditory evoked potential (BAEP) and electrocardiographic (ECG) R-R interval variability, may be useful for the assessment because such measurements have been reported to be sensitive to occupational and environmental hazardous substances (Araki et al. 1997; Counter 2003; Grandjean et al. 2004; Murata and Araki 1996; Murata et al. 1999a, c, 2002, 2004) and independent of the subjects themselves (e.g., mood, language or education) and socioeconomic factors (Chiappa 1997).

Apart from the above prospective studies, a cross-sectional study was conducted in 1995 to clarify the effects of methylmercury on child neurodevelopment (Murata et al. 1999a). One hundred and forty-nine children in first grade at two elementary schools near the fishing harbor of Câmara de Lobos, Madeira, Portugal, were invited to participate in the study; the mercury in the hair of the mothers who had not changed their dietary habits after pregnancy was used as a proxy for mercury exposure at birth. Since exposure levels in the Madeiran mothers seem to have been considerably higher than those in Japanese mothers (Sakamoto et al. 1993; Yasutake et al. 2003), it may be valuable to compare outcomes from two separate countries.

In Japan, a large-scale study on the developmental effect of methylmercury exposure from contaminated seafood, except for the Tohoku Study of Child Development that is now ongoing (Nakai et al. 2004), has never been conducted. We carried out a cross-sectional study with similar tests to the Faroese cohort study (Grandjean et al. 1997, 2004), to clarify whether Japanese children have any neurodevelopmental impairment due to prenatal methylmercury exposure, in relation to the Madeiran study (Murata et al. 1999a, 2002). Also, we determined benchmark dose (BMD) levels, using the results obtained from the two studies, to compare the current levels with previous ones (Budtz-Jørgensen et al. 2000; Cox et al. 1989; Crump et al. 1995, 1998, 2000).

Material and methods

Subjects

The study protocol was approved by the ethical review committee at the Akita University School of Medicine. The nature of the procedures used in the present study was explained to 926 parents in Akita and Tottori prefectures of Japan, and the mothers and their 7-year-old children were invited to take part in our study during the period of July–September in 2002 and 2003. The children, who were born between 2nd April 1995 and 1st April 1997, were chosen in accordance with the preceding study on the risk assessment of methylmercury exposure (Murata et al. 1999a). The children were in the first grade of 28 elementary schools, 14 of which were located near the fishing harbor. In Japan, there were many mines and smelters 30 years ago, and it was probable that soil or water has been contaminated by lead, copper, cadmium, etc; for this reason, the study population did not include those who came from such areas.

To make an international comparison, we merged into this study the data obtained from 149 Madeiran 7-year-old children (Murata et al. 1999a, 2002), because the Madeiran cross-sectional study was comparable to ours with regard to the exposure biomarker and outcome variables, such as the maternal mercury level in scalp hair, BAEPs and age of the study population; also, the BAEP was measured in the same manner by the same examiner. Detailed information on these subjects has been reported in another paper (Murata et al. 1999a). However, since there was a racial difference between the Japanese and Madeiran children, race was considered as a confounder in the merged data.

Exposure biomarkers

Samples of hair, cut close to the scalp, were collected from the occipital area in all mothers and children. The hair length was generally about 10 cm and ranged from 1 to 30 cm. Total mercury in aliquots of dried hair samples (15–20 mg), rinsed with acetone, was determined by the cold vapor atomic absorption spectrophotometry method at the National Institute for Minamata Disease (Akagi and Nishimura 1991). In addition, samples of dried umbilical cord from the children were obtained from parents who consented voluntarily to our proposal; according to an old tradition, most Japanese families used to preserve a small piece of the cord of the child as a birth memento. Methylmercury in the cord tissue, after the blood cells had been removed, was determined at the same institute

by ECD-gas chromatography after extraction by dithizone (Akagi and Nishimura 1991), because the umbilical cord may have been contaminated by inorganic mercury compounds (e.g., mercuric bromide of disinfectant). Total mercury concentrations in the children's and mothers' hair were used as the current mercury exposure and as a proxy for mercury exposure at birth, respectively. Methylmercury concentrations in the cord tissue were used to check the validity of the proxy for mercury exposure.

A detailed survey of medical records during pregnancy and delivery, including smoking and drinking habits, gestation period and birth weight, past and present history of illness in the child, and dietary habits in the mother, was conducted by a medical doctor at the schools or civic centers where examinations on child neurodevelopment were done. Also, a questionnaire on artificially waved hair was collected from the mothers to clarify the effects on hair mercury levels.

Outcome variables

Three trained examiners examined tremor, postural sway, ear–hand coordination, and auditory reaction time (at station A); corrected Q–T interval (QTc) on ECG, ECG R-R interval variability, and eye–hand coordination (at station B); and BAEP latencies (at station C) for a total of 1 h per child, using the Neurobehavioral Test System (CATSYS 2000, Danish Product Development Ltd, Denmark), the ECG-Amplifier 1271SP (NEC-Sanei Co., Japan), the ECG-9202 electrocardiography and Neuropack μ electromyography (Nihon Kohden Co., Japan).

Hand tremor was measured successively for each hand for 16.4 s: the subjects were asked to hold a light stylus as they would hold an ordinary pen, with their elbows bent at a right angle and free of body contact or any obstacles (Despres et al. 2000). The stylus was held horizontally, parallel to the abdomen at approximately 10 cm in front of the navel, and the index finger was positioned about 1 cm from the tip of the stylus. Ear–hand coordination was examined and was composed of a drum that recorded hand pronation–supination movements (Despres et al. 2000). This test was performed with each hand separately under the following standard condition: hand pronation–supination at a constant slow (1 Hz) and a constant fast (2.5 Hz) metronome beat. Eye–hand coordination was examined by operation of the mouse in front of the portable computer, and the subjects were asked to move the arrow of the mouse, not onto a blue square but onto a red one, and to click the left switch as soon as the movable square appeared on the display. Reaction time to a sound stimulus was measured with each hand separately (Despres et al. 2000). Postural sway was measured on a flat floor (Despres et al. 2000). Subjects were asked to stand quietly on a platform without foam under eyes-open and eyes-closed conditions; again, they were asked to stand on a platform with foam in the same manner; the transversal and sagittal sway distances, area and velocity were measured for eyes open and eyes closed.

After the subject had lain quietly supine for at least 5 min, 300 R-R intervals on ECG were measured, and consecutive 100 R-R intervals with the minimal standard deviation (SD) were automatically extracted from the data obtained to avoid non-stationarities. The CV_{RR} (%) was defined as the ratio of the standard deviation of the R-R intervals to the average value (RR_{mean} ms). The power spectrum of R-R intervals was computed by autoregressive spectral analysis (Grandjean et al. 2004; Murata et al. 1992, 1997). The spectrum of each of two components, i.e., the high frequency (HF) component at the center frequency of 0.15–0.4 Hz and low frequency (LF) component at 0.01–0.15 Hz, was separated by component analysis. Each component coefficient of variation (i.e., CCV_{HF} and CCV_{LF}) was defined as the ratio of the square root of each component power spectral density (PSD_k, ms^2) to the RR_{mean}: CCV_k (%) $= 100 \times (PSD_k)^{1/2}/RR_{mean}$, where k = HF or LF. As parasympathetic blockade with atropine abolishes the HF component but beta-sympathetic blockade has no effect on it, the CCV_{HF} reflects the parasympathetic activity, and the LF component is considered to be derived from the fluctuation in the vasomotor activity through the baroreflex mechanism and to show a beta-adrenergically mediated increase in the standing posture (Ewing 1992; Pagani et al. 1986). With regard to the assessment of the cardiovascular function, the electrocardiograph automatically calculated the QTc from the R-R and Q-T intervals on ECG according to Bazett's formula; QTc = (Q-T interval)/(R-R interval)$^{1/2}$ (Murata et al. 1999b).

The BAEP was recorded in subjects lying comfortably. Click signals with an intensity of 65 dB HL were presented to the right ear through electromagnetically shielded earphones at 20 Hz and 40 Hz, independently (Grandjean et al. 1997; Murata et al. 1999a, 2002); the other ear was masked with white noise of intensity of 45 dB HL. Evoked potentials were recorded by three standard EEG electrodes placed on the vertex, the right mastoid ipsilateral to stimulation and the left mastoid (ground). The responses were averaged 2,000 times after amplification and filtration (bandpass 200–2,000 Hz), with one replication for each rate. The peaks I, III and V are thought to reflect the volume-conducted electrical activity from the acoustic nerve, pons and midbrain, respectively (Stockard et al. 1986). The coefficients of variation in the BAEP latencies at 20 Hz and 40 Hz, in a 20-year-old student, for 14 days were 3.0% and 3.4% for peak I latencies; 1.4% and 1.6% for peak III latencies; 0.9% and 1.6% for peak V latencies, respectively. Although the device for the BAEP measurement in the Japanese children differed from that in the Madeiran children, despite the same setting conditions, we did not find any obvious differences between pairs of three peak latencies measured with the two devices in eight volunteers (data not shown).

Data analyses

The relationships among exposure biomarkers were assessed by the Spearman rank correlation coefficient (r_s). The differences in outcome variables both between boys and girls and between Japanese and Madeiran children were analyzed by the analysis of covariance to control for age (and height and gender). The partial correlation coefficient (r) was calculated to examine the dose–effect relations of neurobehavioral and neurophysiological variables to mercury exposure after adjustment for age and gender (and height and race).

The BMD was defined as the mercury concentration in maternal hair that resulted in an increased probability of abnormal test performance by a benchmark response (BMR), i.e., from P_0 to $P_0+\mathrm{BMR}$ at the BMD (National Research Council 2000), when the P_0 and BMR represented an abnormal probability in an unexposed population and an excess risk in an exposed population, respectively. The BMD and cutoff value (C) were calculated from a statistical dose–effect model based on power functions for the dependence (μ) of the outcome variable on the mercury concentration ($g(d)=d^K$) and confounders (age, gender and race) as follows (Budtz-Jørgensen et al. 2001): (1) $\mu(d)=\beta_0+\beta_1\times g(d)+\beta_2\times(\mathrm{age})+\beta_3\times(\mathrm{gender})+\beta_4\times(\mathrm{race})$, (2) $P_0=1-\Phi[(C-\beta_0)/\sigma]$, and (3) $\mathrm{BMD}=g^{-1}\{[\Phi^{-1}(1-P_0)-\Phi^{-1}(1-P_0-\mathrm{BMR})]\sigma/\beta_1\}$ (the Φ and σ indicated the normal cumulative distribution function and SD, respectively, of the outcome variable in an unexposed population). The normalized value for each confounder was employed in the above regression model. A lower confidence limit for BMD (BMDL) was then calculated as the statistical 95% lower bound of the BMD (Budtz-Jørgensen et al. 2001), which has been applied as an alternative to the no-observed-adverse-effect level (NOAEL) to provide a point of departure for low-dose extrapolation (National Research Council 2000). The power parameter K has been restricted to values equal to or above 1, thus allowing the dose–effect curve to be nonlinear. Since previous applications of this method have used a P_0 of 5% and a BMR of 5% (Budtz-Jørgensen et al. 2001; Murata et al. 2002), we applied the linear and K-power dose–effect curves, set at the same P_0 and BMR. All analyses were performed with the Statistical Package for the Biosciences (Murata and Yano 2002).

Results

Exposure biomarkers

The participating subjects, from whom informed consent was obtained, were 327 mothers aged 35.8±4.5 (range 24–49) years and the same number of children at 6.9±0.3 (6.3–7.5) years (participation rate 35.3%). The summary of exposure biomarkers in these subjects is shown in Table 1. Medians of mercury in hair were 1.63 μg/g for the mothers and 1.65 μg/g for the children, and the maximum was 6.86 μg/g for the mothers and 6.32 μg/g for the children; there was no significant difference in the hair mercury between the mother and child (Wilcoxon signed rank test, $P>0.5$). No significant differences in hair mercury levels were found either between subjects residing in cities and towns or between those in non-fishing and fishing areas (two-way analysis of variance with repeated measurements, $P>0.05$). In addition, the hair mercury level was significantly lower in the 108 mothers (0.11–6.86, median 1.31 μg/g) with artificially waved hair than in the 219 mothers (0.39–5.83, median 1.81 μg/g) without (Mann–Whitney U test, $P<0.0001$).

There was a significant relationship between hair mercury levels in the mothers and children ($r_s=0.249$, $P<0.0001$). As shown in Fig. 1, the hair mercury level in 49 mothers was significantly correlated with the methylmercury level in umbilical cord (0.018–0.178, median 0.067 μg/g), but its association was not significant in the 49 children.

Possible confounders

Results of the body weight at birth, gestation period, smoking and drinking habits during pregnancy obtained by interview and questionnaire are shown in Table 2. Of 327 children, 21 had a low birth weight of less than 2,500 g. There was no child with phenylketonuria, maple

Table 1 Summary of hair mercury concentrations in 327 participating subjects in Japan

Locality	Participating subjects	Prefecture (number)	Hair mercury concentrations (mean[a], range)	
			Mother (μg/g)	Child (μg/g)
Urban areas (cities)	181	Akita 135[b]	1.87, 0.11–6.86	1.85, 0.35–5.32
		Tottori 46[c]	1.66, 0.44–5.62	2.20, 0.43–5.83
Rural areas (towns and villages)	146	Akita 108[d]	2.06, 0.53–5.38	1.79, 0.56–6.32
		Tottori 38[e]	1.85, 0.42–4.79	1.90, 0.67–4.39

[a]Arithmetic mean
[b]Sixty-four boys and 71 girls
[c]Thirty-one boys and 15 girls
[d]Fifty-five boys and 53 girls
[e]Seventeen boys and 21 girls

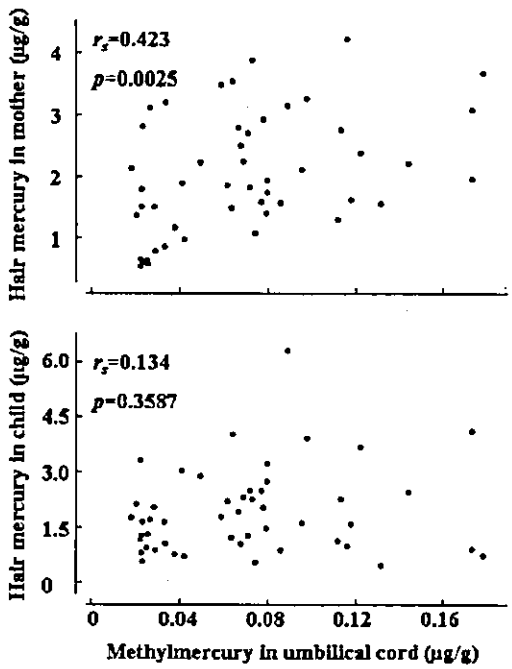

Fig. 1 Relationships between the methylmercury concentration in the cord tissue and hair mercury concentration in 49 mothers and children in Japan

Table 2 Basal characteristics of 327 mothers and their children in Japan

Characteristic	Mean (or number, %)	SD	Range
Body weight at birth (g)	3,142	436	1,568–4,568
Gestation period (weeks)	39.0	1.5	33–42
Smoking during pregnancy	25 (7.6%)		
Drinking during pregnancy	43 (13.1%)		
Natural delivery	290 (88.7%)		
Gestosis (edema, anemia, etc)	130 (39.8%)		
Past history of illness in child			
Febrile convulsion	30 (9.2%)		
Otitis media	132 (40.4%)		

syrup urine disease, homocystinemia, galactosemia, congenital hypothyroidism, neuroblastoma, or adrenal hyperplasia. According to present and past history of illness, there were one child with spinal progressive muscular atrophy, one with cleft palate, and one with epilepsy. In addition, to consider the current mercury level in maternal hair as a proxy for the exposure level at birth, we had to exclude 102 children whose mothers had changed their dietary habits with regard to fish consumption. Accordingly, a total of 210 Japanese children without the above diseases or low birth weight was employed in the analysis of dose–effect relationships. With regard to the postural sway test and BAEP latencies, all parameters in the 113 boys were significantly larger than those in the 97 girls (Table 3).

Effects of mercury exposure on child neurodevelopment in Japan

In calculating the partial correlation coefficients to control for age and gender (plus height only in the postural sway test), we found that there were significant relationships between the maternal hair mercury level and both sagittal sway distance in eyes open and right mean difference in slow rhythm of ear–hand coordination (Table 3), and between the hair mercury level in children and the SD of the eye–hand coordination ($r=0.175$, $P=0.0119$), but significant associations with the other neurobehavioral or neurophysiological variables were not found ($P>0.05$, data not shown).

Comparison between data in Japan and Madeira and benchmark dose

Of 149 mothers and their children participating in the Madeiran cross-sectional study (Murata et al. 1999a), 36 children were excluded because their mothers had changed their dietary habits after pregnancy (Murata et al. 2002). The age (mean ± SD, 6.92 ± 0.30 years) of the 113 Madeiran children was similar to that (6.90 ± 0.30 years) of the 210 Japanese children. Medians of maternal hair mercury were 10.9 (range 1.12–54.4) μg/g in Madeira and 1.67 (0.11–5.83) μg/g in Japan; similarly, those in children were 4.09 (0.38–25.95) μg/g in Madeira and 1.64 (0.45–6.32) μg/g in Japan. The mercury exposures were significantly higher in Madeira than in Japan ($P<0.0001$). The BAEP latencies, except the interpeak I–III latency, were significantly longer in the Madeiran children than in the Japanese children (Table 4). Additionally, significant relationships between the mercury exposure level in maternal hair and BAEP latencies, except the interpeak III–V latency, were found in the combined data (Murata et al. 2002) of Japanese and Madeiran children (these partial correlation coefficients, after age, gender and race had been controlled for, were between 0.139 and 0.230; $P<0.05$), but the exposure level in the children's hair was not significantly related to any BAEP latencies ($P>0.05$, data not shown).

Since no significant relationships between hair mercury and BAEP latencies were found in the Japanese children alone (Table 3), the BMD/BMDL calculation was meaningless. Therefore, the BMDs and BMDLs in the Madeiran children alone and in the Madeiran and Japanese children were calculated after adjustment for age, gender and race (Table 5). The BMDLs (mean 8.65 μg/g) in the combined data became lower than those (mean 9.36 μg/g) in the Madeiran data alone (paired sample t-test, $P=0.0220$).

Discussion

None of the Japanese 7-year-old children participating in our study had the neurological signs or symptoms

Table 3 Outcome variables of neurobehavioral and neurophysiological tests in 113 boys and 97 girls in Japan: results of analysis of covariance after controlling for age (and height)

Outcome variable	Boys (mean ± SD)	Girls (mean ± SD)	Difference (*P*)	Correlation[a]
Postural sway test without foam				
Transversal sway distance (mm), EO[b]	5.42 ± 1.85	4.49 ± 1.11	< 0.0001	0.084
Sagittal sway distance (mm), EO	5.60 ± 1.86	4.81 ± 1.53	0.0011	0.160*
Sway area (mm^2), EO	827 ± 486	575 ± 273	< 0.0001	0.120
Sway velocity (mm/s), EO	16.0 ± 4.5	14.2 ± 4.0	0.0025	0.055
Transversal sway distance (mm), EC[c]	6.10 ± 2.00	5.04 ± 1.51	< 0.0001	0.123
Sagittal sway distance (mm), EC	6.16 ± 1.82	5.31 ± 1.47	0.0003	0.104
Sway area (mm^2), EC	1,240 ± 797	806 ± 473	< 0.0001	0.126
Sway velocity (mm/s), EC	22.7 ± 7.4	19.1 ± 5.9	0.0002	0.124
Postural sway test with foam				
Transversal sway distance (mm), EO	6.19 ± 1.63	5.07 ± 1.15	< 0.0001	−0.014
Sagittal sway distance (mm), EO	6.89 ± 2.08	6.24 ± 2.31	0.0364	−0.035
Sway area (mm^2), EO	1,297 ± 671	935 ± 480	< 0.0001	−0.054
Sway velocity (mm/s), EO	23.6 ± 6.5	19.8 ± 5.7	< 0.0001	−0.051
Transversal sway distance (mm), EC	7.65 ± 2.45	6.23 ± 1.62	< 0.0001	−0.011
Sagittal sway distance (mm), EC	7.66 ± 2.44	6.83 ± 1.98	0.0090	0.027
Sway area (mm^2), EC	2,058 ± 1,591	1,493 ± 920	0.0025	0.014
Sway velocity (mm/s), EC	33.3 ± 11.4	28.4 ± 9.21	0.0010	0.057
Tremor test				
Intensity (m/s^2), right	0.189 ± 0.073	0.167 ± 0.046	0.0106	−0.041
Center frequency (Hz), right	5.52 ± 0.93	5.48 ± 0.87	0.7594	0.011
Intensity (m/s^2), left	0.219 ± 0.091	0.205 ± 0.067	0.2133	−0.000
Center frequency (Hz), left	5.07 ± 0.91	5.059 ± 0.73	0.9269	0.042
Ear-hand coordination test				
Mean difference in slow rhythm (s), right	−0.073 ± 0.059	−0.080 ± 0.057	0.3725	0.147*
Mean difference in slow rhythm (s), left	−0.076 ± 0.054	−0.068 ± 0.056	0.2794	0.017
Mean difference in fast rhythm (s), right	−0.085 ± 0.051	−0.068 ± 0.056	0.0253	0.092
Mean difference in fast rhythm (s), left	−0.086 ± 0.050	−0.068 ± 0.054	0.0141	0.080
Reaction time				
Mean time (s), right	0.353 ± 0.061	0.357 ± 0.050	0.6136	0.085
Mean time (s), left	0.373 ± 0.067	0.383 ± 0.058	0.2609	0.114
Eye-hand coordination test				
Mean time (ms)	655 ± 76	679 ± 72	0.0268	0.123
Variance (SD, ms)	167 ± 37	160 ± 40	0.1798	0.132
Error number	6.40 ± 4.75	3.81 ± 3.53	< 0.0001	−0.020
Brainstem auditory evoked potentials				
Peak III latency (ms), 20 Hz	3.94 ± 0.17	3.85 ± 0.18	0.0002	0.023
Peak V latency (ms), 20 Hz	5.76 ± 0.20	5.65 ± 0.23	0.0001	−0.035
Peak III latency (ms), 40 Hz	4.04 ± 0.19	3.93 ± 0.19	< 0.0001	0.024
Peak V latency (ms), 40 Hz	5.91 ± 0.20	5.77 ± 0.24	< 0.0001	−0.033
Electrocardiogram				
Heart rate (/s)	84.1 ± 9.0	88.1 ± 9.3	0.0018	−0.004
Corrected QT interval (ms)	391 ± 15	391 ± 15	0.9567	−0.030
Electrocardiographic R-R interval variability				
CV_{RR} (%)	6.35 ± 2.25	6.44 ± 2.35	0.7682	−0.064
CCV_{HF} (%)	4.04 ± 2.14	4.22 ± 2.31	0.5587	0.005
CCV_{LF} (%)	4.21 ± 1.70	4.54 ± 2.01	0.1960	−0.023
%LF	52.4 ± 11.7	52.6 ± 12.9	0.9005	−0.061

*$P < 0.05$
[a]Partial correlation with maternal hair mercury levels in 210 children after adjustment for age and gender (and height)
[b]Eyes open
[c]Eyes closed

that had been reported in the literature for Minamata disease (methylmercury poisoning) (Igata 1993; Kurland et al. 1959), such as paresthesia, constriction of visual field, intention tremor, impairment of hearing/speech, mental disturbances, or unsteady gait. This would be due to the fact that exposure levels for the Japanese children or mothers did not exceed the safe limit (10 μg/g) of the International Programme on Chemical Safety (1990) or the BMDL and NOAEL of methylmercury, which have been reported to be 12 μg/g calculated from the Faroese birth cohort study by the US Environmental Protection Agency (2001) and 15.3 μg/g from the Seychelles Child Development Study by the Agency for Toxic Substances and Disease Registry (1999), respectively. Additionally, hair mercury levels in the Japanese children were slightly associated with those in their mothers, and there was no difference in current hair mercury levels between the mothers and children. By contrast, hair mercury levels in the Faroe Islands and Madeira were considerably higher in mothers than in

Table 4 Latencies of brainstem auditory evoked potential (mean ± SD) in Japanese and Madeiran children

Parameter	Japan (n=210)	Madeira (n=113)	Difference[a] (P)
20 Hz			
Peak III	3.90±0.17	4.10±0.29	<0.0001
Peak V	5.71±0.22	5.95±0.31	<0.0001
Interpeak I–III	2.12±0.13	2.12±0.22	0.9828
Interpeak III–V	1.81±0.15	1.86±0.17	0.0126
40 Hz			
Peak III	3.99±0.19	4.23±0.35	<0.0001
Peak V	5.84±0.22	6.20±0.34	<0.0001
Interpeak I–III	2.17±0.14	2.16±0.26	0.5598
Interpeak III-V	1.85±0.14	1.97±0.21	<0.0001

[a]Analysis of covariance was used to control for age and gender

children (Murata et al. 1999c, 2004). The latter findings suggest that Japanese children may ingest similar doses per body weight of methylmercury to their mothers, different from the two Western countries consuming much seafood.

In the present study, methylmercury levels in umbilical cord had a close relation to maternal hair mercury levels, although we could not observe such a relation in the children's hair. In the Madeiran cross-sectional study, the regression (i.e., gradient) of the peak III latency of the BAEP on maternal hair mercury was similar to that on maternal hair mercury at birth in the Faroese birth cohort study, and the BMDs and BMDLs, calculated from the former alone, were almost similar to those from the combined data of both children (Murata et al. 2002). Cernichiari et al. (1995) have also come to a similar conclusion in the Seychelles study. Thus, qualitative evidence has been provided that maternal hair mercury levels can be used as a proxy for mercury exposure levels at birth.

There was no gender difference in ECG-related variables except heart rate in the Japanese children, but significant differences in some tests, such as the BAEP and postural sway, were observed between both genders (Table 3); these findings are consistent with those in previous reports (Araki et al. 1994; Grandjean et al. 1997; Murata et al. 1992; Murata and Araki 1996). Nonetheless, the gender difference could not be explained by mercury exposure, birth weight or height (Araki et al. 1994). For that reason, it is crucial to control for the effects of gender, as well as age, in the data analysis.

We failed to find any dose–effect relationships in most of the outcome variables in Japanese children alone (Table 3), while a few of the postural sway and ear–hand coordination variables had subtle but significant associations with maternal hair mercury. Given the multiple significance test, we could conclude from these findings that Japanese children with mercury exposure levels of less than 6.9 μg/g at birth had no adverse effects on neurodevelopment. However, three notes of warning should be struck against the negative findings: (1) The Faroese birth cohort had an enormously wide range of mercury exposure (Grandjean et al. 1997), but the range of our exposure biomarker was extremely small. (2) The Faroese sample number was three times as much as ours. A larger population including higher-level exposures would increase the statistical power. (3) The effects of possible confounders other than age and gender, e.g., artificially waved hair (Iwasaki et al. 2003; Yamamoto and Suzuki 1978; Yasutake et al. 2003), may have been included in the present study. Certainly, the mothers with artificially waved hair in our study had approximately 72% of the hair mercury levels in the mothers without it. Such exposure misclassification may have underestimated the true effect in risk assessment (Grandjean et al. 2002).

In our study the BAEP latencies, except for the interpeak I–III latency in the Madeiran children, were prolonged when compared with those in the Japanese children; also, the peak III and V, and interpeak I–III latencies were associated with maternal hair mercury levels in the combined data after controlling for the

Table 5 Benchmark dose (BMD, μg/g) and its lower 95% confidence limit (BMDL, μg/g) at benchmark response level of 0.05 according to dose–effect models for latencies of brainstem auditory evoked potential at 20 Hz and 40 Hz in 113 Madeiran and 210 Japanese children (maternal hair mercury levels (μg/g) were used as a proxy for exposure biomarker at birth)

P_0=0.05	Data in Madeira alone				Combined data of Madeira and Japan			
	Linear model[a]		Power model[b]		Linear model[a]		Powermodel[b]	
	BMD	BMDL	BMD	BMDL	BMD	BMDL	BMD	BMDL
20 Hz								
Peak III latency	19.62	10.52	19.62	11.18	15.31	9.41	15.49	9.56
Peak V latency	18.44	10.15	18.60	10.32	16.75	9.99	16.89	10.15
Interpeak I–III latency	14.99	9.08	15.20	9.24	12.10	8.05	12.28	8.20
40 Hz								
Peak III latency	12.61	8.04	12.61	8.60	9.71	6.90	9.87	7.04
Peak V latency	19.23	10.42	19.41	10.60	17.48	10.49	17.64	10.66
Interpeak I–III latency	12.22	7.92	12.40	8.07	10.00	7.08	10.15	7.21

[a]Linear model: $[BAEP] = b_0 + b_1 \times [dose] + b_2 \times [age] + b_3 \times [gender]\ (+ b_4 \times [race])$
[b]Power model: $[BAEP] = b_0 + b_1 \times [dose]^k + b_2 \times [age] + b_3 \times [gender]\ (+ b_4 \times [race])$

effects of age, gender and race. In other reports, the interpeak I–III and I-V latencies of the BAEP were significantly prolonged in patients with fetal Minamata disease (Hamada et al. 1982), and there were significant differences in interpeak III–V and I–V latencies of the BAEP between the Ecuadorian children, exposed to methylmercury-contaminated food and elemental mercury vapors, with blood mercury levels of 20–89 μg/l and with levels below 20 μg/l (Counter 2003); the differential effects of prenatal and postnatal exposures may explain the difference between the interpeaks in the two studies (i.e., I–III and III–V latencies) (Murata et al. 2004). In addition, significant dose–effect associations of the BAEP latencies have been observed in the Faroese birth cohort and Madeiran children (Murata et al. 1999a, c, 2004). In many cases, neurotoxic effects of occupationally hazardous substances have shown prolonged latencies of cerebral evoked potentials (Araki et al. 1997). It is therefore suggested that these differences in the BAEP latencies between the Japanese and Madeiran children may have been due to mercury exposure and that the BAEP latencies, as well as the neuropsychological tests including the Boston Naming Test and California Verbal Learning Test employed in the Faroese birth cohort study (Grandjean et al. 1997), are one of the most sensitive endpoints to methylmercury exposure. Additional study is necessary to explain the difference in the interpeak III–V latency.

A mean BMDL of 8.65 μg/g in maternal hair for BAEP latencies in the combined data of Japanese and Madeiran children is somewhat low when compared with recently calculated BMDLs for other neurological outcome variables in the Faroese children (Budtz-Jørgensen et al. 2000) and in a New Zealand population (Crump et al. 1998). From several curve functions an average BMDL of approximately 10 μg/g was calculated for crude neurological abnormalities in children exposed in connection with the poisoning incident in Iraq (Cox et al. 1989, Crump et al. 1995). Higher BMDLs were also reported in a study in the Seychelles, where clear effects on psychological tests have not been detected so far (Crump et al. 2000). Judging from these reports, as the endpoint examined in each study shifted away from clinical to subclinical effects (or, from non-specific to domain-specific tests), the exposure level at which such an effect emerged appeared to become lower, like a declining threshold of harm for mercury (Schettler et al. 2000). Additionally, the lower BMDLs calculated from the combined data may have been due to the wide spectrum of mercury exposure, compared with the exposure in either the Japanese or Madeiran children.

According to the a priori hypothesis, the cord-blood mercury concentration is expected to be the best predictor for neurobehavioral decrements in children (Grandjean et al. 1992, 1999). Also, it has been demonstrated that the mercury concentration in the umbilical cord tissue was well associated with the mercury concentration in cord blood ($r_s = 0.85$), rather than that in maternal hair ($r_s = 0.77$) (Dalgård et al. 1994). On the other hand, Japanese maternal hair mercury levels in this study could explain only 18% of the variation of prenatal exposure ($r_s = 0.42$). Therefore, if we can obtain more umbilical cords from the same subject population, it will enable us to address the effects of prenatal methylmercury exposure on child neurodevelopment in the retrospective study.

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The Tohoku Study of Child Development: A Cohort Study of Effects of Perinatal Exposures to Methylmercury and Environmentally Persistent Organic Pollutants on Neurobehavioral Development in Japanese Children

KUNIHIKO NAKAI, KEITA SUZUKI, TOMOKO OKA, KATSUYUKI MURATA,[1] MINESHI SAKAMOTO,[2] KUNIHIRO OKAMURA,[3] TORU HOSOKAWA,[4] TAKEO SAKAI,[5] TOMOYUKI NAKAMURA,[6] YOSHINORI SAITO,[6] NAOYUKI KUROKAWA, SATOMI KAMEO and HIROSHI SATOH

Department of Environmental Health Sciences, Tohoku University Graduate School of Medicine, Sendai 980-8575,
[1]Division of Environmental Health Science, Akita University School of Medicine, Akita 010-8543,
[2]National Institute for Minamata Disease, Minamata 867-0008,
[3]Department of Obstetrics, Tohoku University Graduate School of Medicine, Sendai 980-8575,
[4]Department of Human Development, Faculty of Education, Tohoku University, Sendai 980-8576,
[5]Miyagi Children's Hospital, Sendai 989-3126, and
[6]Miyagi Prefectural Institute of Public Health and Environment, Sendai 983-8666

NAKAI, K., SUZUKI, K., OKA, T., MURATA, K., SAKAMOTO, M., OKAMURA, K., HOSOKAWA, T., SAKAI, T., NAKAMURA, T., SAITO, Y., KUROKAWA, N., KAMEO, S. and SATOH, H. *The Tohoku Study of Child Development: A Cohort Study of Effects of Perinatal Exposures to Methylmercury and Environmentally Persistent Organic Pollutants on Neurobehavioral Development in Japanese Children.* Tohoku J. Exp. Med., 2004, **202** (3), 227-237 —— Several birth cohort studies have shown adverse effects of perinatal exposures to methylmercury (MeHg) and environmentally persistent organic pollutants (POPs). These chemicals are ingested mainly through fish consumption, but little is known about the hazardous effects in Japanese, whose fish consumption is high. The present study, the Tohoku Study of Child Development, was designed to examine the effects of perinatal exposures to MeHg, polychlorinated biphenyls (PCB), dioxins, pesticides, and other chemicals in Japanese children. Six

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Address for reprints : Kunihiko Nakai, V.M.D., Ph.D., Department of Environmental Health Sciences, Tohoku University Graduate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan.
e-mail: nakaik@mail.tains.tohoku.ac.jp

Some results from this study were presented at the NIMD Forum 2003 held at Niigata, Japan, on November 20, 2003.

hundred eighty-seven pregnant women were participated in this study with their written informed consent. Maternal peripheral blood, cord blood, cord tissue, placenta, and breast milk samples were collected for chemical analysis. Maternal hair was also taken for MeHg analysis. Infants born at full term were assessed by neurobehavioral tests: the Brazelton Neonatal Behavioral Assessment Scale at three days old, the Kyoto Scale of Psychological Development and the Bayley Scales of Infant Development at 7 and 18 months old, and the Fagan Test of Infant Intelligence at 7 months old. The children will be continuously followed up to ages 6-7 years. Maternal food intake frequency, maternal IQ, socioeconomic status, and home environment were assessed as covariates. The results of this cohort study will allow us to evaluate associations between the neurobehavioral development of children and perinatal exposures to MeHg and environmentally POPs in Japan. ——— cohort; development; dioxin; methylmercury; polychlorinated biphenyls

The neurobehavioral effects of prenatal exposures to methylmercury (MeHg) and environmentally persistent organic pollutants (POPs) including polychlorinated biphenyls (PCBs), dioxins, and pesticides are of great concern worldwide (Nakai and Satoh 2002). It was shown that prenatal MeHg exposure causes the delay of development of cognitive functions in Faroe Islands (Grandjean et al. 1997), Madeira Islands (Murata et al. 1999), and New Zealand (Kjellstorm et al. 1986), although studies conducted in the Seychelles showed the absence of toxic effects of prenatal exposures to MeHg (Davidson et al. 1998). Several epidemiological studies have also shown the evidence of the adverse effects of perinatal PCB exposure on neurodevelopment. Cohort studies in North Carolina (Rogan et al. 1986), Michigan (Jacobson et al. 1985, 1990), NewYork (Darvill et al. 2000; Stewart et al. 2000), The Netherlands (Patandin et al. 1999; Vreugdenhil et al. 2002), Germany (Winneke et al. 1998; Walkowiak et al. 2001), and Faroe Islands (Grandjean et al. 2001) demonstrated negative associations between perinatal PCB exposure and cognitive functions in children.

MeHg and POPs constitute a group of persistent environmental chemicals. Due to their hydrophobic nature and resistance towards metabolism, they are found in every level of the food chain. Consequently, these chemicals accumulate in humans mostly through the consumption of food, particularly that of fish and shellfish origins. Indeed, the consumption of fish and shellfish is the major route of dioxin exposure (>80% of all food sources) in Japan (Ministry of Health, Labour and Welfare 2002). From the nutritional perspective, fish is usually recommended for pregnant women because it is rich in some nutrients such as n-3 polyunsaturated fatty acids (PUFA) essential for the perinatal growth of the brain. Therefore, from the perspective of risk assessment, the above health hazard issues are particularly of importance in fish-eating populations.

In this report we present a protocol of our cohort study, the Tohoku Study of Child Development, on the effects of perinatal exposures to MeHg and POPs on neurobehavioral development among Japanese children. We hypothesize that the prenatal/postnatal exposures to the above chemicals delay or disturb the normal growth and neurobehavioral development of children. Exposure assessment includes measurements of multiple chemicals that may potentially affect the child development. Health risk of children was mainly evaluated by neurobehavioral tests. In studies designed to examine neurobehavioral development, multiple confounding factors including food intake habit, home environment,

TABLE 1. *Inclusion criteria for the Tohoku study*

Mother

1. Absence of thyroid dysfunction, mental and psychological diseases, hepatitis, immune deficiency, malignant tumor, diabetes mellitus requiring antidiabetic agents, and any other severe diseases that may affect the normal growth of fetus
2. No severe preeclampsia and severe gestational diabetes mellitus
3. No in vitro fertilization
4. Japanese as the mother tongue
5. Written consent

Infant

1. Absence of congenital anomalies or severe diseases
2. Singleton birth at term from 36 to 42 weeks of gestation
3. Body weight of more than 2400 g, and when the term was 36 weeks of gestation, body weight of more than 2500 g

socioeconomic status, and others must be considered. These issues that must be considered in a study design are reported.

Study design

Recruitment of cohort. Healthy pregnant women were recruited with their informed consent at obstetrical wards of two hospitals in Sendai. To establish an optimal study population, only infants born at term (36 to 42 weeks of gestation) without congenital anomalies or diseases are included. Pregnancy and delivery should have been completed without overt signs of serious illness or complications. The inclusion criteria are shown in Table 1. The study protocol was approved by the Medical Ethics Committee of the Tohoku University Graduate School of Medicine.

Sample collections. The hair samples were collected from the mothers after delivery. Most epidemiological studies on MeHg exposure have used mercury concentration in hair to estimate the body burden (WHO 1990). Since hair growth rate is independent of gender or racial differences (Cernichiari et al. 1995), by assuming a constant rate of hair growth equal to 1.1 cm per month (Cox et al. 1989), it is possible to generate a profile of MeHg exposure based on the mercury concentrations in serial segments of scalp hair. The hair samples were cut next to the scalp, in the nape area, with stainless steel scissors. The samples were placed in a plastic bag and kept in a desiccator until analysis.

Since most commercially available plastic and glass materials are possibly contaminated with a significant amount of chemicals such as POPs, all glassware used for sample collection and storage was treated by heating at 400°C in a chemically clean chamber to exclude the possible contamination with PCBs and dioxins. All other materials were confirmed to be clean before use.

Blood samples were collected from mothers at 28 weeks of pregnancy. For blood collection, a vacuum system heparin tube confirmed to be without contamination was used to collect peripheral blood (30 ml), and centrifuged within 4 hours for 20 minutes at 3000 rpm; plasma and whole blood were stored at –80°C until analysis.

A blood sample (more than 50 ml) from the umbilical cord was collected into a bottle using heparin as the anticoagulant after the delivery. Placenta and cord tissues were also collected after the delivery. Since the placenta is a large organ, which is a heterogeneous mixture of placental cells and decidual tissues containing maternal and fetal blood, representative samples of placenta were obtained as follows: the placenta was divided into 20-30 pieces that were randomly separated