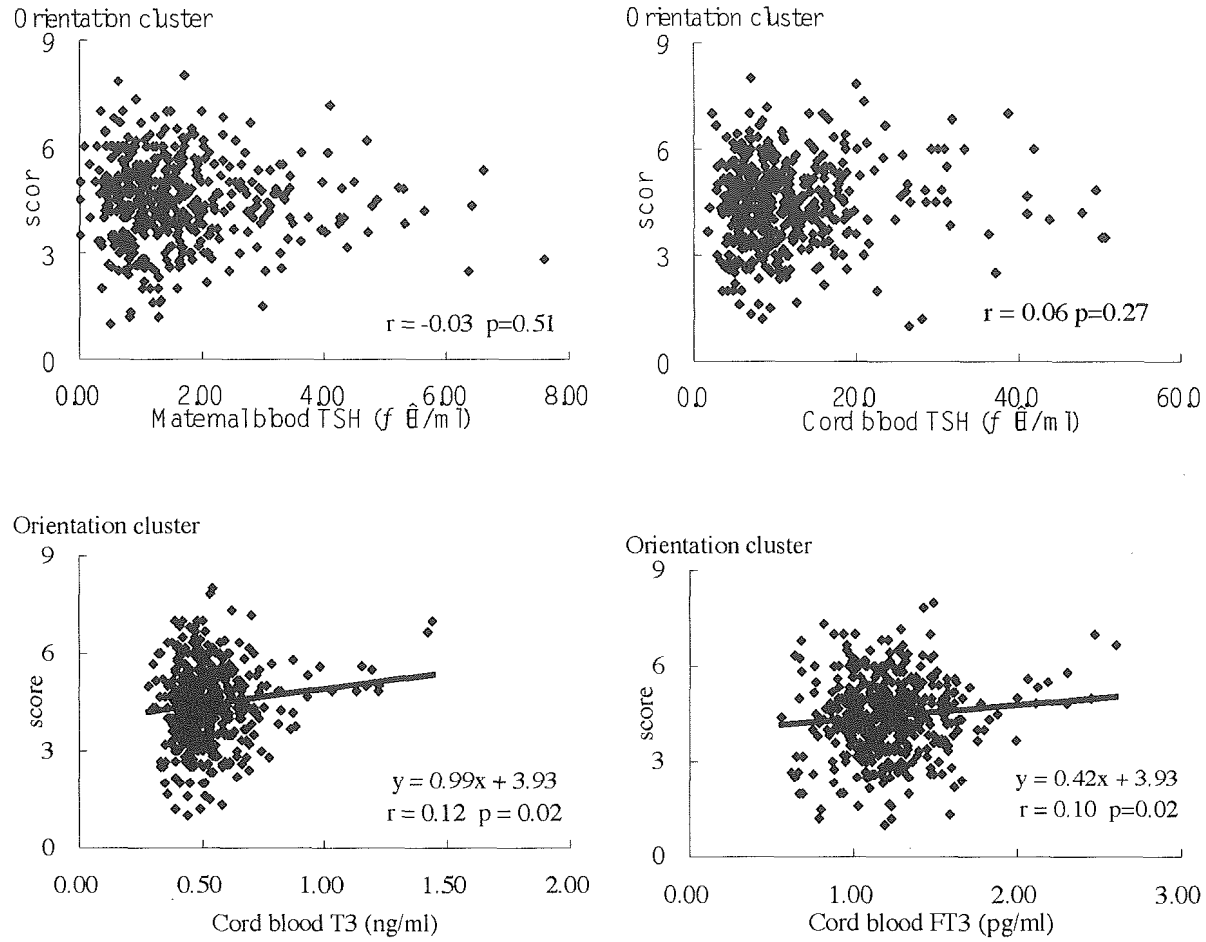

Fig 3. Associations of maternal/cord thyroid hormones with the Orientation cluster score of NBAS

Acknowledgments

We thank all the families who participated in this study. The study protocol was approved by the Medical Ethics Committee of the Tohoku University Graduate School of Medicine. This research was funded by Risk Analysis Research on Food and Pharmaceuticals from Japanese Ministry of Health, Labour and Welfare.

References

1. Nakai K, Satoh H. (2002) Tohoku J Exp Med 196: 89-98
2. Nakai K, Suzuki K, Oka T, Murata K, Sakamoto M, Okamura K, Hosokawa T, Sakai T, Nakamura T, Saito Y, Kurokawa N, Kameo S, Satoh H. (2004) Tohoku J Exp Med 202: 227-237
3. Porterfield S. (2000) Environ Health Perspect. 108: 433-438
4. Brouwer A, Longnecker MP, Brinbaum LS, Cogliano J, Kostyniak P, Moore J, Schantz S, Winneke G. (1999) Environ Health Perspect 107: 639-649.
5. Koopman-Esseboom C, Morse DC, Weisglas-Kuperus N, Lutkeschiplolt IJ, Van der Paauw CG, Tuinstra LG, Brouwer A, Sauer PJ. (1994) Pediatr Res 36(4): 468-473.
6. Longnecker MP, Gladen BC, Patterson DG Jr, Rogan WJ. (2000) Epidemiology 11(3): 239-241.
7. Hagmar L. (2003) Thyroid 13(11): 1021-1028.

Organochlorine Pesticide Residues in Human Breast Milk and Placenta in Tohoku, Japan

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Introduction

Organochlorine pesticides are compounds widespread in the environment due to their persistence and highly lipophilic nature, and they accumulate in biological systems. Newborns are exposed to these organochlorine compounds across the placenta and through breastfeeding. Perinatal exposure to these compounds may induce several adverse effects such as lower birth weight [1], neurodevelopmental delay [2], and disturbance of thyroid hormone status [3]. DDT, especially, has been suggested to be a neuroendocrine disruptor as well as a functional teratogen in humans [4 5]. Other pesticides such as dieldrin and endosulfan were also recognized to have estrogenic hormonal activity in animal studies.

Recently, we have started a birth cohort study to examine the effects of exposure to persistent organochemical pollutants and heavy metals on neurodevelopment in Japanese children, The Tohoku Study of Child Development [6]. In this cohort study, biological samples, including maternal peripheral blood, cord blood, placenta, cord tissue, and breast milk have been collected from more than six hundred mother-infant pairs for chemical determinations. The growth of infants has been monitored using neurodevelopmental tests, including the Brazelton Neonatal Behavioral Assessment Scale, the Bayley Scale of Infant Development, the Kyoto Scale of Psychological Development, and others. Exposures to dioxin and related compounds, polychlorinated biphenyls, methylmercury, and several heavy metals were assessed. Additionally, since perinatal exposure to organochlorine pesticides may affect the neurodevelopment of children, we examined the effects of those pesticides in the cohort study.

In the present study, several organochlorine pesticides were analyzed in human breast milk and placenta from 20 mothers to identify the major pesticide compounds found in the cohort subjects. The relationship between pesticides in breast milk and the placenta was analyzed to examine the utilization of the placenta as the material for exposure assessment. Some information regarding the factors affecting the contamination of breast milk and the placenta with organochlorine pesticides

are also discussed.

Methods and Materials

This study was performed as part of our prospective cohort study [6]. Healthy pregnant women were recruited with their informed consent at obstetrical wards of two hospitals in Tohoku between January 2001 and September 2003. Twenty subjects were randomly selected from the registered subjects of the cohort study, and pairs of breast milk samples and placenta samples were used. The ages of mothers ranged from 21 to 39. The placenta was taken immediately after the delivery, and divided into 20-30 pieces that were randomly separated into 4 groups. Each bottle contained 50-100 g of tissue. The representative samples were finally prepared by homogenization. The mothers were asked to provide breast milk one month after the delivery. The breast milk sample was taken directly into a clean glass bottle. These samples were frozen at –80°C until analysis. Each mother completed a questionnaire to provide personal information such as the number of births, smoking, alcohol consumption during pregnancy, occupation, educational background, food intake, and place of residence. The study protocol was approved by the Medical Ethics Committee of the Tohoku University Graduate School of Medicine.

The pesticides examined were hexachlorobenzene (HCB), α-hexachlorocyclohexane (HCH), β-HCH, γ-HCH, δ-HCH, cis-chlordane, trans-chlordane, oxy-chlordane, cis-nonachlor, trans-nonachlor, p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, p,p'-DDD, o,p'-DDD, aldrin, endrin, dieldrin, α-endosulfun, β-endosulfun, heptachlor, heptachlorepoxide, and methoxychlor. Gas chromatographic determination of these organochlorine pesticides was performed with the collaboration of SRL, Inc. (Tokyo, Japan) for sample extraction and Toray Research Center (Tokyo, Japan) for gas chromatography. Briefly, after the samples were spiked with $^{13}C_6$-HCB, $^{13}C_6$-β-HCH, $^{13}C_{12}$-p,p'-DDT, $^{13}C_{12}$-endosulfun, and $^{13}C_{10}$-chlordane, they were extracted with ethanol/hexane. The organic extracts were finally purified with the use of a Florisil column, and the eluates were concentrated and spiked with $^{13}C_{12}$-pentaPCB(#118). A mass spectrometer (AutoSpec, Micromass) coupled to a Hewlett-Packard model HP6800 capillary gas chromatograph equipped with a capillary column (BPX-35, 0.25 mm ID x 25 m, film thickness 0.33 μm, SGE) was used for determination of pesticides. Residue levels were expressed as ng/g extracted fat.

Results and Discussion

HCB, β-HCH, oxy-chlordane, cis-nonachlor, trans-nonachlor, p,p'-DDT, p,p'-DDE, dieldrin, and heptachlorepoxide were found from all breast milk samples and placenta samples as shown in Table 1, whereas levels of α-HCH, γ-HCH, δ-HCH, cis-chlordane, trans-chlordane, o,p'-DDT, o,p'-DDE, p,p'-DDD, o,p'-DDD, aldrin, endrin, α-endosulfun, β-endosulfun, heptachlor, and methoxychlor were very low or below the detection limit (data not shown). Since using of these organochlorine compounds had been prohibited in the field in the 1970-1980s in Japan, these results reconfirmed their environmentally persistent nature. In Japan, the concentrations of PCBs, β-HCH, and DDTs in breast milk declined gradually from the peak levels observed at the mid-1970s and almost reached equilibrium states [7]. However, it remains to be elucidated whether the current low levels of organochlorine pesticides affect the neurodevelopment of children.

The concentration of organochlorine pesticides in breast milk mainly depends on their accumulation in the maternal fatty tissue and their subsequent mobilization. Indeed, numerous studies around the world have used human breast milk samples to determine maternal body burden and lactational transfer of pesticides to infants. Since there were excellent correlations of all major pesticides between breast milk samples and placenta samples (Table 1, and the two typical relationships in Fig. 1), placenta is also suggested to be the useful material to estimate the maternal body burden. In addition, the concentrations of some organochlorine pesticides such as HCB, oxy-chlordane, and trans-nonachlor, in the placenta samples had significant negative correlations with parity (Table 2). This finding clearly shows that the mothers eliminate these pesticides during pregnancy and by breastfeeding them into their children. Considering that the concentration of pesticides in breast milk samples had no significant correlation with parity, monitoring of the placental pesticide concentration may contribute to determining the prenatal exposure of infants to organochlorine pesticides. The placenta is a relatively large organ, and is usually discarded after delivery. Utilization of the placenta is possibly suggested for the purpose of assessment of exposure to chemicals.

Table 1: Organochlorine pesticide concentrations in the human milk samples and placenta samples, and the relationship between the 2 samples.

Pesticide	Milk (ng/g-fat)	Placenta (ng/g-fat)	Correlation Coefficient Milk x Placenta
Hexachlorobenzene	17.1±10.1	9.9±4.1	0.693**
β-HCH	83.4±55.1	21.5±12.6	0.919**
oxy-Chlordane	7.2±3.4	2.3±0.9	0.644**
cis-Nonachlor	3.7±1.7	0.8±0.4	0.589**
trans-Nonachlor	18.8±8.6	3.8±2.2	0.679**
p.p'-DDT	6.2±3.5	1.4±0.6	0.746**
p.p'-DDE	142.3±73.5	46.0±34.6	0.569**
Dieldrin	5.0±3.6	1.7±1.1	0.808**
Heptachlorepoxide	3.7±1.4	1.4±0.3	0.881**

Spearman's correlation analysis, ** $p<0.01$, * $p<0.05$

Table 2: Correlation coefficient values of organochlorine pesticides with fish intake, maternal age at delivery, and parity.

Pesticide	Fish consumption		Maternal age		Parity	
	Milk	Placenta	Milk	Placenta	Milk	Placenta
Hexachlorobenzene	0.023	-0.127	-0.421	-0.223	-0.429	-0.625**
β-HCH	0.064	0.034	0.244	0.375	0.025	-0.064
oxy-Chlordane	0.609**	0.515*	-0.208	0.033	-0.428	-0.521*
cis-Nonachlor	0.486*	0.356	-0.085	0.234	-0.093	-0.354
trans-Nonachlor	0.701**	0.475*	-0.133	0.155	-0.282	-0.471*
p.p'-DDT	0.341	0.267	-0.179	0.06	-0.053	0.089
p.p'-DDE	0.054	0.412	-0.165	0.169	-0.174	-0.131
Dieldrin	0.463*	0.518*	-0.109	0.004	0.12	0.033
Heptachlorepoxide	0.566**	0.711**	-0.185	-0.169	-0.054	-0.235

Spearman's correlation analysis, ** $p<0.01$, * $p<0.05$

Some organochlorine pesticides have been thought to be introduced to humans partly through the consumption of fish and related products [8]. The concentrations of oxy-chlordane, nonachlors, dieldrin, and heptachlorepoxide in breast milk samples and placenta samples were indeed correlated with fish consumption; however, HCB, HCH, and DDE had no association. These results indicated that the contribution of fish consumption to the intake of pesticides was dependent on the kind of pesticide. More information regarding risk analysis of pesticide intake is needed for risk management. Maternal age at the time of delivery and parity have been shown to be important factors affecting the concentration of pesticides in breast milk samples [8]. Although parity was a potent factor in our data (Table 2), maternal age had no significant relationship with the concentrations of pesticides in breast milk samples and placenta samples. However, since parity correlated significantly with maternal age (data not shown), multiple regression analysis should be performed to control for the effects of covariates. These issues, and identification of the factors affecting the contamination levels of organochlorine pesticides in breast milk and placenta will be readdressed when we increase the sample size.

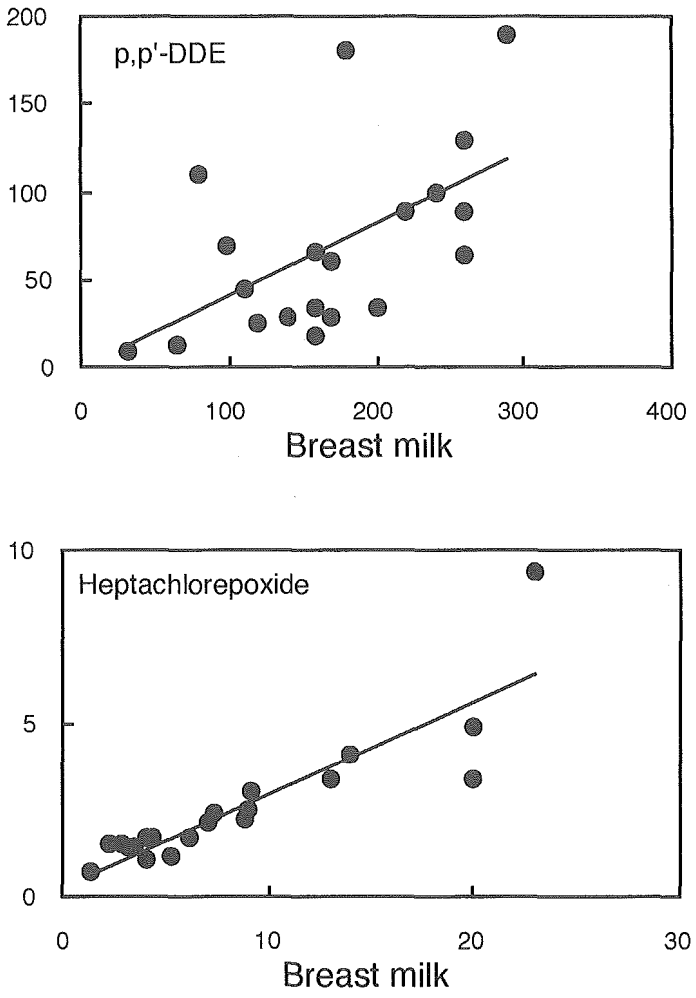

Fig. 1.: Relationship of p,p'-DDE (upper) and heptachlorepoxide (lower) between breast milk and placenta. ng/g-fat, N=20.

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References

1. Siddiqui M.K., Srivastava S., Srivastava S.P., Mehrotra P.K., Mathur N. and Tandon I. (2003) Int Arch Occupational Environ Health 76, 75-80.
2. Ribas-Fito N., Cardo E., Sala M., Eulalia de Muga M., Mazon C., Verdu A., Kogevinas M., Grimalt J.O. and Sunyer J. (2003) Pediatrics 111, e580-585.
3. Nagayama J., Okamura K., Iida T., Hirakawa H., Matsuda T., Tsuji H., Hasegawa M., Sato K., Yanagawa T., Igarashi H., Fukushige J. and Watabane T. (1998) Chemosphere 37, 1789-1793.
4. Dorner G. and Plagemann A. (2002) Neuroendocrinol Lett 23, 427-431.
5. Haradell L., Lindstrom G. and Van Bavel B. (2001) Environ Health Perspect 88, 141-144.
6. 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. (2004) Tohoku J Exp Med 202, 227-237.
7. Konishi Y., Kuwabara K. and Hori S. (2001) Arch Environ Contam Toxicol 40, 571-578.
8. Harris C.A., Woolridge M.W. and Hay A.W.M. (2001) Chemosphere 43, 243-256.

A Cohort Study of Effects of Perinatal Exposures to Methylmercury and Environmentally Persistent Organic Pollutants on Neurobehavioral Development in Japanese Children: Study design and status report

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Abstract: Adverse effects of perinatal exposures to methylmercury (MeHg) and environmentally persistent organic pollutants (POPs) have been apparent from several birth cohort studies, but little is known about the hazardous effects in Japanese, whose fish consumption is high. The present study was designed to examine the effects of perinatal exposures to MeHg, polychlorinated biphenyls (PCB), dioxins, pesticides, and other chemicals in Japanese children. Six 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 a battery of neurobehavioral tests. The children will be continuously followed up to ages 6-7. 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.

Key words: epidemiology, methylmercury, pregnant women

INTRODUCTION

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, 2002). It was shown that prenatal MeHg exposure causes the delay of development of cognitive functions in Faroe Islands (GRANDJEAN, 1997), although studies conducted in the Seychelles showed the absence of toxic effects of prenatal exposures to MeHg (DAVIDSON, 1998). Several epidemiological studies have also shown the evidence of the adverse effects of perinatal PCB exposure on neurodevelopment.

In this report we present a protocol of our cohort study, the Tohoku Study of Child Development, of the effects of perinatal exposures to MeHg and POPs on neurobehavioral development among Japanese children (NAKAI, 2004). We hypothesize that the prenatal/postnatal exposures to the above chemicals delay or disturb the normal growth and neurobehavioral development of children.

STUDY DESIGN

Healthy pregnant women were recruited with their informed consent at obstetrical wards of two hospitals in Tohoku, Japan. 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 study protocol was approved by the Medical Ethics Committee of the Tohoku University Graduate School of Medicine.

The hair samples were collected from the mothers after delivery. Maternal peripheral blood samples were collected at 28 weeks of pregnancy. They were centrifuged within 4 hours for 20 minutes at 3000 rpm; plasma and whole blood were stored at –80 °C until analysis. A blood sample 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. The mothers were finally asked to provide a sample of breast milk one month after the delivery.

Questionnaire. Several types of questionnaire were administered after the delivery. To assess the fish-intake and the general nutrition status of the mothers a food-intake frequent questionnaire (FFQ) for 122 individual foods and recipes, and some additional items regarding seafood was administered. This is a standardized FFQ that enables the assessment of the intake of not only major nutrients but also several essential nutrients including retinol and folic acid in the Japanese population. Other questionnaires were administered with the following items: educational background, occupation, income, smoking habit including passive smoking, alcohol consumption during pregnancy, hair treatments including bleaching, permanent wave and coloring, and dental amalgam treatment.

Neurodevelopment assessment. All testers who performed neurodevelopment assessments were not informed of exposure information including alcohol consumption/ smoking habit, FFQ data, and feeding method. The Brazelton Neonatal Behavioral Assessment Scale (NBAS) was administered when the infants were 3 days old. Cognitive functions of the infants at 7 months old were evaluated using the Bayley Scale of Infant Development, second edition (BSID), the Kyoto Scale of Psychological Development (KSPD), and the Fagan Test of Infant Intelligence (FTII). BSID and KSPD were also used for the assessment of neurobehavioral development when the children were 18 months old. The Japanese version of Kaufman Assessment Battery for Children (K-ABC) was employed to assess the development and intelligence of children when they are 42 months old. The growth and development of the children will be followed up until they are 6-7 years old.

Chemical determinations. Total mercury analysis was carried out by cold vapor atomic absorption spectrometry. Briefly, without washing the hair samples, each sample was acid digested with HNO_3, $HClO_4$ and H_2SO_4 at 200 °C for 30 minutes. The resultant ionic mercury was then reduced to mercury vapor by tin chloride to a flameless atomic absorption monitor (HG-201, Sanso Co., Ltd., Tokyo). Analytical accuracy was ensured by analyzing the Human Hair Reference Material NIES CRM No. 13 from the National Institute of Environmental Studies (Lot #650, Tsukuba). In fish-eating populations, total mercury in hair consists mostly of MeHg (more than 95 %).

Assessment of PCB exposure was performed by determining PCB levels in cord blood, placenta, breast milk, and maternal blood. All 209 PCB congeners were analyzed by high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) using the isotope dilution method.

A reporter gene assay of the toxic potency of dioxins and related chemicals was used for the assessment of dioxins. The CALUX (Chemically Activated LUciferase gene eXpression) assay was developed by Xenobiotic Detection Systems (XDS, NC, USA) using a patented recombinant mouse cell line that contains the luciferase reporter gene under the control of dioxin-responsive elements. This assay has several advantages including its high sensitivity, easy pretreatment, and rapid determination, in comparison with HRGC/HRMS.

Cadmium and lead were determined by graphite furnace atomic absorption spectrometry and inductively coupled plasma mass spectrometry, respectively, after samples were digested in a microwave oven with ultrapure nitric acid. Other major biochemical analyses of maternal and cord blood samples included those of plasma selenium and thyroid hormones. Selenium was determined fluorometrically. The assay of TSH, and total/free T4 and T3 were performed using a radioimmunoassay technique.

Potential confounders/covariates. The quality of the home environment was assessed using a questionnaire, the Evaluation of Environmental Stimulation (EES), which has been established in Japan modified after the Home Observation for Measurement of the Environment (HOME) score. The parental socioeconomic status (SES) was rated using the Hollingshead Four Factor Index of Social. Other major potential confounders included were as follows: intelligence quotient by the Raven standard progressive matrices, age at examination (days), gestational age (weeks), and alcohol consumption/smoking habits during pregnancy for the mothers, and the Apgar score, neonatal illness/jaundice, delivery type, parity, chronic diseases, and duration of breastfeeding (months) for the infants.

RESULTS AND DISCUSSION

The present report describes the study design and protocol for the prospective cohort study of the effects of prenatal exposures to MeHg and other environmentally POPs on neurobehavioral development in Japanese children. To our knowledge, this is the first cohort study that examines these hazardous risks to children in Japan.

We recruited 687 healthy pregnant women between January 2000 and September 2003. Although the final number of babies registered in this study is not yet determined because the delivery of pregnant women registered in this study is ongoing, the percentage of babies fulfilling the criteria for inclusion with the mothers' consent to participate in the assessment using NBAS was 85 %. The percentage of babies participating in the next assessment at 7 months old was 86 % of those participating in the assessment using NBAS. This reduction was mainly due to family relocation to other places.

The results of this cohort study will allow us to evaluate associations between the neurobehavioral development of children and prenatal exposures to MeHg and environmentally POPs in Japan. A recent report from the cohort at Faroe Islands (MURATA ET AL., 2004) indicated that the adverse effects of prenatal exposure to MeHg were still observed in the children at age 14 years by neurophysiological tests, suggesting that some neurotoxic effects from prenatal exposures are irreversible. To clarify this issue, the subjects should be followed until their adolescent ages. The present report describes the study design for children aged 0 to 42 months. When any significant associations between child development and chemical exposures is observed in this study, the further follow-up is essential to know the persistency of adverse effects.

Acknowledgments

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REFERENCES

DAVIDSON, P. W., MYERS, G. J., COX, C., AXTELL, C., SHAMLAYE, C., SLOANE-REEVES, J., CERNICHIARI, E., NEEDHAM, L., CHOI, A., WANG, Y., BERLIN, M. & CLARKSON, T. W. (1998): Effects of prenatal and postnatal methylmercury exposure from fish consumption on neurodevelopment: outcomes at 66 months of age in the Seychelles Child Development Study; *JAMA* 280, pp. 701-707.

GRANDJEAN, P., WEIHE, P., WHITE, R. F., DEBES, F., ARAKI, S., YOKOYAMA, K., MURATA, K., SORENSEN, N. & DAHL, R. (1997): Cognitive deficit in 7-year old children with prenatal exposure to methylmercury; *Neurotoxicol. Teratol.* 19, pp. 417-428.

MURATA, K., WEIHE, P., BUDTZ-JORGENSEN, E., JORGENSEN, P. J., & GRANDJEAN, P. (2004): Delayed brainstem auditory evoked potential latencies in 14-year-old children exposed to methylmercury; *J. Pediatr.* 144, pp. 177-183.

NAKAI, K. & SATOH, H. (2002): Developmental neurotoxicity following prenatal exposures to methylmercury and PCBs in humans from epidemiological studies; *Tohoku J. Exp. Med.* 196, pp. 89-98.

NAKAI, K., SUZUKI, K., OKA, T., MURATA, K., SAKAMOTO, M., OKAMURA, K., HOSOKAWA, T., SAKAI, T., NAKAMURA, T., SAITO, Y., KUROKAWA, N., KAMEO, S. & SATOH, H. (2004): *Tohoku J. Exp. Med.* 202, pp. 227-237.

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ORIGINAL ARTICLE

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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

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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

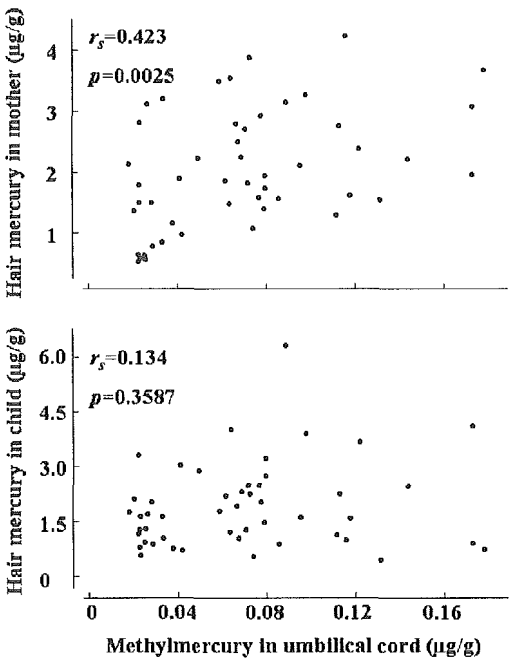

Fig. 1 Relationships between the methylmercury concentration in the cord tissue and hair mercury concentration in 49 mothers and children in Japan

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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References

Agency for Toxic Substances and Disease Registry (1999) Toxicological profile for mercury. US Department of Health and Human Services. http://www.atsdr.cdc.gov/toxprofiles/tp46.pdf. Cited 2 Jan 2004

Akagi H, Nishimura H (1991) Speciation of mercury in the environment. In: Suzuki T, Imura N, Clarkson TW (eds) Advances in mercury toxicology. Plenum, New York, pp 53–76

Araki S, Murata K, Yokoyama K, Okajima F, Grandjean P, Weihe P (1994) Neuroelectrophysiological study of children in low-level methylmercury exposure in Faroe Islands: methodology and preliminary findings. In: Proceedings of the international symposium on assessment of environmental pollution and health effects from methylmercury. National Institute for Minamata Disease, Minamata, pp 141–151

Araki S, Yokoyama K, Murata K (1997) Neurophysiological methods in occupational and environmental health: methodology and recent findings. Environ Res 73:42–51

Budtz-Jørgensen E, Grandjean P, Keiding N, White RF, Weihe P (2000) Benchmark dose calculations of methylmercury-associated neurobehavioural deficits. Toxicol Lett 112-113:193–199

Budtz-Jørgensen E, Keiding N, Grandjean P (2001) Benchmark dose calculation from epidemiological data. Biometrics 57:698–706

Cernichiari E, Brewer R, Myers GJ, Marsh DO, Lapham LW, Cox C, Shamlaye CF, Berlin M, Davidson PW, Clarkson TW (1995) Monitoring methylmercury during pregnancy: maternal hair predicts fetal brain exposure. Neurotoxicology 16:705–710

Chiappa KH (1997) Brainstem auditory evoked potentials methodology. In: Chiappa KH (ed) Evoked potentials in clinical medicine, 3rd edn. Lippincott-Raven, Philadelphia, pp 199–268

Counter SA (2003) Neurophysiological anomalies in brainstem responses of mercury-exposed children of Andean gold miners. J Occup Environ Med 45:87–95

Cox C, Clarkson TW, Marsh DO, Amin-Zaki L, Tikriti S, Myers GG (1989) Dose–response analysis of infants prenatally exposed to methyl mercury: an application of a single compartment model to single-strand hair analysis. Environ Res 49:318–332

Crump K, Viren J, Silvers A, Clewell H, Gearhart J, Shipp A (1995) Reanalysis of dose–response data from the Iraqi methylmercury poisoning episode. Risk Anal 15:523–532

Crump KS, Kjellström T, Shipp AM, Silvers A, Stewart A (1998) Influence of prenatal mercury exposure upon scholastic and psychological test performance: benchmark analysis of a New Zealand cohort. Risk Anal 18:701–713

Crump KS, Van Landingham C, Shamlaye C, Cox C, Davidson PW, Myers GJ, Clarkson TW (2000) Benchmark concentrations for methylmercury obtained from the Seychelles Child Development Study. Environ Health Perspect 108:257–263