(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

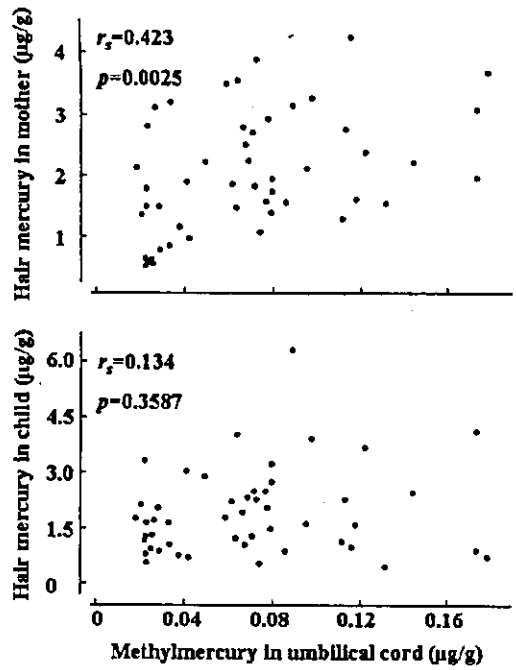

Fig. 1 Relationships between the methylmercury concentration in the cord tissue and hair mercury concentration in 49 mothers and children in Japan

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

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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

into four groups. Each bottle contained 50-100 g tissue. The representative samples were finally prepared by homogenization (Iyengar and Rapp 2001). The entire cord was stored in a clean glass tube without any preparation.

The mothers were finally asked to provide a sample of breast milk (more than 50 ml) one month after the delivery. A clean glass bottle was used for the shipping of breast milk.

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 (Date et al. 1996) 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. The testers had been trained in the training center at Nagasaki University School of Medicine, Japan.

Cognitive functions of the infants at 7 months old were evaluated using the Bayley Scale of Infant Development (BSID), second edition, the Kyoto Scale of Psychological Development (KSPD), and the Fagan Test of Infant Intelligence (FTII). BSID, an established psychodevelopmental test tool, consists of three major scales: the Mental Scale, the Psychomotor Scale, and the Behavior Rating Scale; only the first two scales are used. The mental scale assesses the infant's level of cognitive function (memory, learning, and problem solving), language development (expressive/receptive language, and vocalization), and personal/social development. The motor scale assesses fine and gross motor functions. Since there is no Japanese version of the standardized protocol of BSID, we translated the original manual into Japanese. To examine its reliability, the evaluation of testers were examined on the basis of the Gold Standard developed at the University of Rochester School of Medicine (Davidson et al. 1995). In addition, raw scores were used in the analysis because of the lack of Japanese age norms. KSPD is a Japanese standard developmental test (Maehara et al. 2002); therefore, the developmental performance of the infants is expressed as the developmental age (DA) for each behavior area and for all areas. The developmental quotient (DQ) is obtained by dividing the estimated DA by the chronological age and then multiplying the quotient by 100. FTII is a noninvasive test of information processing that may be applied to infants up to one year of age (Fagan and Detterman 1992).

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, but the battery of neurobehavioral tests is as yet undetermined.

Chemical determinations. Total mercury analysis was carried out by cold vapor atomic absorption spectrometry (Akagi and Nishimura 1991) with minor modifications. Briefly, without washing the hair samples, each sample, weighing approximately 20 mg, was acid digested with 0.5

ml of HNO_3, 0.5 ml of $HClO_4$ and 2 ml of H_2SO_4 at 200°C for 30 minutes. The resultant ionic mercury was then reduced to mercury vapor by adding 0.5 ml of 10% 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. Indeed, a few samples were analyzed to know the exact MeHg concentration by the method of Akagi and Nishimura (1991). MeHg in hair first extracted with hydrochloric acid and then with benzene. The organic layer was subjected to electron-capture detection gas chromatography (ECD-GC) at the National Institute for Minamata Diseases. The concentration of MeHg was confirmed to be more than 95% of the total mercury content. Total mercury analysis was also applied to other samples similarly.

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. The analytical method was as follows: after biological samples were spiked with the ^{13}C-labeled standard mixture of PCBs, lipids in a sample were extracted and weighed. The extract redissolved by an organic solvent was purified in a multi-layer silica gel column. The purified solution was concentrated and analyzed for PCB after the addition of ^{13}C-labeled syringe spike. Four nonplanar PCB congeners (International Union for Pure and Applied Chemistry (IUPAC) nos. 118, 138, 153, and 180) are the predominant congeners found in human tissues and typically account for approximately 50-60% of total PCB (data not shown). Some earlier epidemiological studies attempted to assess PCB exposure using the sum of the above four major PCB congeners. For comparison with those earlier studies, the sum is also calculated in the present study.

A reporter gene assay of the toxic potency of dioxins and related chemicals was used for the assessment of dioxins. The Chemically Activated LUciferase gene eXpression (CALUX) assay was developed by Xenobiotic Detection Systems (XDS, Durham NC, USA) using a patented recombinant mouse cell line that contains the luciferase reporter gene under the control of dioxin-responsive elements (Denison et al. 1998). This analytical process consisted of the first extraction process as in PCB analysis and then column purification using sulfuric acid-impregnated silica gel and activated carbon column. The last purified extracts were given to the cells to produce luciferase, and the amount of light generated by the luciferase was directly related to dioxin toxic equivalent (TEQ) value. This assay has several advantages including its high sensitivity, easy pretreatment, and rapid determination, in comparison with HRGC/HRMS. This assay also requires only a smaller sample volume, which is another important advantage for epidemiological studies.

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. The standard reference material for analysis was NIST 1577b (bovine liver). Other major biochemical analyses of maternal and cord blood samples included those of plasma selenium and thyroid hormones including TSH, and total/free T4 and T3. Selenium was determined fluorometrically (Watkinson 1966). The assay of thyroid hormones 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) (Anme et al. 1998), which has been established in Japan modified after the Home Observation for Measurement of the Environment (HOME) score (Caldwell and Bradley 2001).

HOME is a validated instrument for the assessment of the home environment, but there is no Japanese version that matches the Japanese cultural context. The EES is a questionnaire that directly evaluates the interaction between the child and the caregiver. It was shown that the results of EES highly correlated with those of HOME (Anme et al. 1998).

The parental socioeconomic status (SES) was rated using the Hollingshead Four Factor Index of Social Status (Hollingshead 1975) with several modifications to make the category and prestige of occupation match the Japanese economical context.

Maternal intelligence quotient was measured using the Raven standard progressive matrices. Only the Raven colored progressive matrices have already been introduced in Japan only for people older than 40 years old. We therefore used the original Raven standard version and analyzed results using the raw data.

Other major potential confounders included were as follows: age at examination (days), gestational age (weeks), and alcohol consumption/smoking habits during pregnancy for the mothers, and the Apgar score, neonatal illness/jaundice, spontaneous delivery, parity, chronic diseases, and duration of breastfeeding (months) for the infants.

TABLE 2. *Variables measured at the Tohoku study*

Measurement	Description
Exposure assessment	
PCBs	Cord blood, placenta, breast milk, and maternal blood
Dioxins	Cord blood, placenta, and breast milk, expressed by CALUX-TEQ
Pesticides	Breast milk and placenta, but the exact assay method has not been decided.
MeHg	Maternal hair at delivery, maternal blood, cord blood, and placenta
Heavy metals	Other heavy metals including Pb and Cd, in cord blood, maternal blood, and placenta
Other biochemical measurements	
Selenium	Cord blood and maternal blood
TSH, T4/T3	Cord blood and maternal blood
Neurodevelopment assessments	
NBAS	Infants at 3 days old
BSID	Infants at 7 and 18 months old
KSPD	Infants at 7 and 18 months old
FTII	Infants at 7 months old
K-ABC	Children at 42 months old
Confounders/covariates	
EES	A questionnaire regarding the home environment
SES	Hollingshead four factor index with modifications for application in Japan
Maternal IQ	Raven standard progressive matrices
FFQ	An interview method, with 122 single foods and recipes, and some additional seafood items
Questionnaires	Alcohol consumption/smoking during pregnancy, educational background, hair cosmetic treatments, dental amalgam, and duration of breastfeeding (months),
Other factors	**Mother**: age at delivery, spontaneous delivery/cesarean section, and chronic diseases **Infant**: Apgar score, body weight, body height, head circumference at birth, gestational age (weeks), neonatal illness/jaundice, parity, and age at examination (days)

Results and Discussion

The present report describes the study design and protocol for the prospective cohort study on the effects of perinatal exposures to MeHg and other environmentally POPs on neurobehavioral development in Japanese children. All variables measured are summarized in Table 2. To our knowledge, this is the first cohort study that examines these hazardous risks to children in Japan.

Recruitment. We recruited 687 healthy pregnant women between January 2001 and September 2003 at the obstetrical wards of two hospitals in Sendai, but 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 from Sendai to other places. Sample size is essential for the statistical power, and this is especially important to test whether exposures to low levels of chemicals have the hazardous effects. In addition to the theoretical approach to decide the appropriate sample size, recent epidemiological studies that assessed neurobehavioral consequences of perinatal exposure to PCBs are useful in considering this issue. The Dutch cohort study was started with 418 healthy infants and 395 children were examined at 42 months of age (94% of the original cohort) (Patandin et al. 1999). The German cohort study consisted of 171 mother-infant pairs; 126 mothers provided milk samples and 91 mothers remained in the final examination of children at 42 months of age (approximately 70% of the mothers participating in the postnatal follow-up cohort) (Winneke et al. 1998). In the Faroe cohort study, PCBs could be analyzed in cord tissues from 435 of 1022 children who underwent neurodevelopment examination at 7 years old (Grandjean et al. 2001). These cohort studies showed a negative correlation between prenatal/postnatal PCB exposure and neurobehavioral development in children. Considering that the exposure level of Japanese women was similar to that of European women, and that the potential risk is almost identical, our sample size is probably sufficient.

Neurodevelopment assessment. There are six sets of cohort studies on health hazardous effects of perinatal PCB exposure in children, and all these studies approached this issue by the method of neurodevelopment assessment. Four sets of studies employed BSID to measure the development of infants, and three of them found a significant correlation between the outcomes of BSID and PCB exposure (Schantz et al. 2003). Based on these findings, BSID is expected to be a useful tool for evaluating the risks and the results can be easily compared among the studies. This was the reason why we employed BSID as one of the major components in our tests. On the other hand, BSID is a developmental test based on the developmental milestone concept, and there are no standardized data in Japan. Thus, BSID does not provide us information on MPI and PDI, the two standard indexes of the relative status of development in a population. We therefore used KSPD, the most commonly used neurodevelopmental test in Japan, to calculate DQ. Both BSID and KSPD were originally developed based on the work of Gessell (Ikuzawa et al. 1985; Black and Matula 1999). We also applied FTII and K-ABC to assess children at the ages of 7 and 42 months, respectively. The present study was the first trial to use FTII in Japan. FTII is a novelty preference task designed to predict the later development and intelligence of children (Fagan and Detterman 1992). These two intelligence tests were shown to be sensitive in detecting the adverse effects of low levels of perinatal PCB exposure (Jacobson et al. 1985; Patandin et al. 1999; Darvill et al. 2000; Walkowiak et al. 2001).

Chemical determinations. In a review (Schantz et al. 2003) of epidemiological studies on the possible adverse effects of perinatal expo-

sure to PCBs, it was concluded that a more complete information regarding the neurotoxicity of individual congeners or congener groups may be helpful for risk assessment. There are 209 PCB congeners, and a large number of these congeners were indeed found to be present in human tissues. Since their relative potency to produce nerve system effects is entirely unknown, a congener-specific analytical technique is essential for risk assessment. Despite the fact that several recent studies have used sophisticated congener-specific analytical techniques, there have been no attempts to analyze individual PCB congeners probably present in cord blood, mainly due to the lack of assay sensitivity. The delay of cognitive development may be more related to prenatal PCB exposure, as measured by the sum of concentrations of three or four major PCB congeners in either cord or maternal blood, but not with the postnatal PCB exposure, as measured by the sum of concentrations of PCBs in breast milk samples (Schantz et al. 2003). These findings suggest the importance of PCB congener-specific analysis in cord blood. In the present study, the detailed assessment of individual PCB congeners in cord blood and other samples was designed using a very sensitive HRGC/HRMS.

Only the Dutch cohort study (Patandin et al. 1999) examined the adverse effects of dioxin exposure on neurobehavioral development in children, in which the perinatal exposure, as measured by GC/MS in breast milk samples collected at 2 weeks postpartum, showed no noticeable correlation with cognitive functions measured later. However, the interpretation of these findings is complicated by the results that total PCB in breast milk samples showed no correlation with cognition functions, even though the same study showed negative correlation when total PCB in cord blood was used for analysis. These findings suggest that the characterization of prenatal exposure is more important to clarify the adverse effects of dioxins; the effects of prenatal dioxins exposure should be examined by analyzing levels of dioxins in cord blood. Because dioxins could not be measured by HRGC/HRMS in small volume of cord blood and maternal blood samples, the CALUX assay, a reporter gene assay to determine the all dioxin-like substances, is useful for this purpose. Previously, we already confirmed that data obtained by CALUX assay showed an extremely good correlation with TEQs obtained by HRGC/HRMS in environmental materials (Nakamura et al. 2002).

However, in practice, several problems in exposure assessment remain. First, the metabolites of PCBs are likely included in the adverse effects of PCB exposure. The main hypotheses are that PCB effects on neurodevelopment include the disruption of thyroid hormone homeostasis (Porterfield and Source 2000), and that candidate PCB congeners that may disturb the homeostasis may include several minor congeners and their OH-metabolites (Cheek et al. 1999; Chauhan et al. 2000). The measurement of all possible metabolites of PCBs is not realistic. Second, although there are limited available data describing the neurotoxicity of pesticides in humans, these chemicals may indeed affect the neurodevelopment of children (Schettler 2001). In the present study, however, the assay methods for pesticides including organochlorine and organophosphorus chemicals are not yet determined because the number of chemicals is too large. Third, the measurement of all chemicals including PCBs, dioxins, heavy metals and pesticides from cord blood is difficult because of the shortage of sample volume and the insufficient detection limit. Other biological samples such as placenta are promising for identifying the surrogate marker for exposure assessment. A recent report suggested a good correlation of total PCB in placenta with that in cord blood, maternal blood, and breast milk samples (Wang et al. 2004). Further studies are necessary in order to examine the importance and usefulness of placenta and cord tissues in the assessment of prenatal exposure effects.

Confounders. Despite the major source of MeHg and POPs is via fish intake, fish consumption itself is though to have several beneficial

aspects. Selenium is considered to play an essential role in protection against MeHg toxicity (Watanabe 2002). Fish is usually rich in selenium, and almost 70% of the daily total selenium is through the fish intake in Japan (Miyazaki et al. 2002). However, the bioavailability of fish-derived selenium is still controversial. Fish is also rich in PUFA which may be essential for the normal development of an infant brain (Horwood and Fergusson 1998). However, the beneficial effects of increased amount of PUFA in cord blood on the later developmental period are also still controversial (Bakker et al. 2003). In the present study, these confounding factors including selenium and PUFA were considered from nutritional perspectives in the risk assessment of eating fish.

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. 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 are observed in this study, the further follow-up is essential to know the persistency of adverse effects.

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Maternal and Fetal Mercury and *n*-3 Polyunsaturated Fatty Acids as a Risk and Benefit of Fish Consumption to Fetus

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Maternal fish consumption brings both risks and benefits to the fetus from the standpoint of methylmercury (MeHg) and *n*-3 PUFA (polyunsaturated fatty acids). MeHg is one of the most risky substances to come through fish consumption, and mercury concentrations in red blood cells (RBC-Hg) are the best biomarker of MeHg exposure. Docosahexaenoic acid (DHA, C22:6*n*-3), which is one of the most important fatty acids for normal brain development and function, is also derived from fish consumption. Our objective in this study was to examine the relationships between RBC-Hg and plasma fatty acid composition in mother and fetus at parturition. Venous blood samples were collected from 63 pairs of mothers and fetuses (umbilical cord blood) at delivery. In all cases, fetal RBC-Hg levels were higher than maternal RBC-Hg levels. The geometric mean of fetal RBC-Hg was 13.4 ng/g, which was significantly ($p < 0.01$) higher than that of maternal RBC-Hg (8.41 ng/g). While the average fetal/maternal RBC-Hg ratio was 1.6, the individual ratios varied from 1.08 to 2.19, suggesting considerable individual differences in MeHg concentrations between maternal and fetal circulations at delivery. A significant correlation was observed between maternal and fetal DHA concentrations ($r = 0.37$, $p < 0.01$). Further, a significant correlation was observed between RBC-Hg and plasma DHA in fetus ($r = 0.35$, $p < 0.01$). These results confirm that both MeHg and DHA which originated from fish consumption transferred from maternal to fetal circulation and existed in the fetal circulation with a positive correlation. Pregnant women in particular need not give up eating fish to obtain such benefits. However, they would do well to at least consume smaller fish, which contains less MeHg, thereby balancing the risks and benefits from fish consumption.

Introduction

Methylmercury (MeHg) is a well-known and widespread environmental neurotoxicant. In the natural course of events, most human exposure to MeHg is through fish and sea mammal consumption. Generally, the larger fish and sea mammals at the top of the food chain, such as shark, tuna, and whale, contain higher levels of MeHg than the smaller ones. Fetuses are known to be a high-risk group for MeHg exposure (*1*–*3*) since the susceptibility of the developing brain itself is high (*3*–*5*) and higher MeHg accumulates in cord blood than in maternal blood (*6*–*10*). Therefore, the effect of MeHg exposure on pregnant women remains an important issue for elucidation, especially in populations which consume much fish and sea mammals (*3*, *11*–*14*). Serum or plasma is known as a good biomarker of elementary or mercuric Hg exposure (*15*). On the other hand, mercury concentration in red blood cells (RBC-Hg) is the best biomarker of MeHg exposure (*3*, *16*–*18*). Additionally, more than 90% of that in RBC is known to be in the methyl form in high-fish-consuming populations (*19*). Further, hematocrit (Htc) values are quite different between mother and fetus at parturition (Table 1). Therefore, we used total Hg concentrations not in whole blood but in RBCs to reveal the MeHg levels in mothers and fetuses in the present study.

On the other hand, human intake of the *n*-3 longer chain of polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, C20:5*n*-3) and docosahexaenoic acid (DHA, C22:6*n*-3), is also known to occur through marine products, mainly from fish consumption. Both of these fatty acids are very beneficial for human health (*20*, *21*). Especially, DHA is known to be an important *n*-3 PUFA for normal brain development and function (*22*–*25*). Rapid brain growth occurs primarily during the third trimester in humans (*26*, *27*), and the amount of these fatty acids increases dramatically during the period (*25*). This period corresponds to when the human brain is most susceptible to MeHg (*27*), and also a high accumulation of MeHg in the brain may occur during the period (*5*).

We conducted a study to determine the relationship between RBC-Hg and plasma fatty acid concentrations in fetus to evaluate the risks and benefits of maternal fish consumption by comparing 63 maternal–fetal pairs of blood samples.

Materials and Methods

Sixty-three healthy Japanese pregnant women, ranging in age from 21 to 41 yr (average 29.6 ± 4.4 yr), planning to deliver in Munakata Suikokai General Hospital, Munakata City, Fukuoka, Japan, gave informed consent to take part in the present trial. Blood samples were collected from the mothers and umbilical cord. The samples included 13 mL of venous umbilical cord blood at birth and 10 mL of venous maternal blood 1 day after parturition before breakfast. Both blood samples were obtained by venipuncture with a small amount of heparin–Na and centrifuged at 3000 rpm for 10 min to separate into RBCs and plasma. Samples were stored at −80 °C until analysis. This study was approved by the Ethics Committee of the National Institute for Minamata Disease (NIMD).

Total Hg in 0.5 g of RBC was determined by cold vapor atomic absorption spectrophotometry (CVAAS) according to the method of Akagi and Nishimura (*29*). The method

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