Kawauchi, those individuals who lived in the village less than 5 d/wk were not eligible to participate in our study's personal external dose monitoring.

One participant from each household was invited to participate in the 24-h food-duplicate survey at the same time as the personal external dose monitoring survey, except in Kawauchi. There, public health nurses invited residents to participate in the survey, as part of their standard health service activities (health guidance, health consultation, and so on). The 24-h food-duplicate samples were analyzed for radiocesium and, in part, radiostrontium.

Evaluation of Personal External Doses. The cumulative doses of H_p (10) in participants from August 1 to September 30, 2012, were evaluated. Optically stimulated luminescence personal dosimeters of aluminum oxide were used (Quixel badge system type S; Nagase Landauer, Ltd.). Dosimeters were calibrated following Japanese Industrial Standards JIS Z 4339 (52). The dosimeters were worn on a neck strap. A radiation dose was detected at above 0.01 mSv. Participants recorded their representative daily behavior, and participants whose radiation dose was relatively high were interviewed. The annual radiation dose was calculated by multiplying the dose measured over the course of 2 mo by the number 6.

The external radiation dose from natural radiation was subtracted using literature data for background radiation in Japan (25, 26). Before the FDNPP accident, terrestrial radiation from the ground was comparable between the Kyoto and Fukushima prefectures (0.34 and 0.35 mSv/y, respectively) (25). Ionizing cosmic rays had levels of 0.28 mSv/y in Kawauchi, 0.26 mSv/y in Haramachi, and 0.25 mSv/y in Kyoto (no data for Tamano) (26). Kawauchi and Tamano are located in a mountain-ringed region ~400 m above sea level. The control measurement in Kyoto was 0.10 mSv/2 mo and was in fair agreement with the sum of terrestrial and cosmic radiation. Therefore, background radiation estimates (0.63 mSv/y in Kawauchi and Tamano; 0.61 mSv/y in Haramachi) were subtracted from the values obtained by the dosimeter.

Food Sampling and Preparation. The food-duplicate samples were collected as previously described (15, 53). Participants prepared an additional portion of all meals and beverages consumed in the 24 h of a single day and provided our research team with a list of what they consumed. Those people who avoided eating any food products from the Fukushima region were excluded from this study. From August 7 to 11, 2012, food-duplicate samples were collected from 79 people living in the village of Kawauchi. In the Tamano and Haramachi areas, a food-duplicate survey was conducted between August 27 and 31, 2012, for 16 and 30 individuals, respectively. The homogenates of food-duplicate samples, including drinks and sweets, were lyophilized.

Registered dietitians recorded the weight of food items in the samples and categorized these items into 18 categories on a food composition table. The amount of food of Fukushima origin was measured using the menu records of places of production. A questionnaire regarding eating habits and preferences was also administered.

Airborne Dust Sampling. Airborne dust samples were collected at three sites in the study areas between August 6 and October 30, 2012. Air sampling was conducted using a high-volume sampler (HV-1000F; Sibata). Whole airborne dust in the air was collected on a quartz membrane filter at a height of 1.5 m from the ground. In the period from August 6 to 12, 2012, more than 80 m^3 of air were inspired at a rate of 1,000 L/min within a day. To collect a sufficient volume of dust, air samplers continued running for a week after the end of sample collection on August 12. An Andersen cascade impactor sampler (AN-200; Tokyo Dylec Co.) connected with a low-volume sampler (flow rate of 28.2 L/min) (SL-30; Sibata) was used to collect dust samples with different aerodynamic diameters. Airborne dust was fractionated onto nine filters, ranging from 100 to 11.4 μm to <0.46 μm. Filters corresponding to fractions <100, <4.9 (considered the respirable fraction), and <1.1 μm were combined and subjected to radiometry.

Determination of ^{137}Cs and ^{134}Cs. Lyophilized food-duplicate samples (200–300 g) were placed in cylindrical polyethylene containers. Quartz fiber filters from dust sampling were pressed into thin polyethylene containers. A high-resolution Ge semiconductor detector was used for determination of radiocesium. Characteristic photon energies of radiocesium were monitored (^{134}Cs: 604.7 and 795.9 keV; ^{137}Cs: 661.7 keV). The intensity ratios of 604.7 keV:661.7 keV were plotted against distances between the detector and sample. A summing effect was observed and corrected for ^{134}Cs. Food-duplicate samples and dust samples were measured for >20,000 and >10,000 s, respectively. The limits of detection (LODs) were determined using Kaiser's method (54). Radioactivities were corrected to the sampling date. Every 20 measurements, procedural blanks were prepared to check contamination by equipment and handling. No detectable contamination was observed in the procedural blanks (n = 6).

Determination of ^{89}Sr and ^{90}Sr in Food-Duplicate Samples. ^{89}Sr and ^{90}Sr measurements were conducted by radiochemical analysis (55). The freeze-dried food samples (75–126 g) were ashed at 450 °C for 24 h. Ashed samples were decomposed with aqua regia and an additional amount of nitric acid, and then dissolved in hydrochloric acid. Strontium was precipitated and separated by fuming nitric acid. The activity of ^{89}Sr was immediately measured with a Cherenkov light and liquid scintillation counter (LSC-6101B; Hitachi-Aloka Medical). After attaining a radioactive equilibrium of ^{90}Sr and ^{90}Y for 2 wk, the activity of the solution was again analyzed using a liquid scintillation counter. The activity of ^{90}Y was calculated by subtracting the activity of ^{89}Sr. The LODs were 0.25 and 0.04 Bq for ^{89}Sr and ^{90}Sr, respectively. Activities were corrected to the sampling date, using the physical half-lives of radiostrontium (^{89}Sr: 50.5 d; ^{90}Sr: 28.9 y).

Estimation of Radiation Dose Levels and Monte Carlo Simulation over the Next 10–50 y. Committed effective doses for ingestion of radiocesium were calculated using effective dose coefficients (0.019 μSv/Bq for ^{134}Cs and 0.013 μSv/Bq for ^{137}Cs) (42). For inhalation exposure, it was assumed that adult (51- to 60-y-old males and females) and child (3- to 5-y-old males and females) residents inhaled 15.7 and 10.1 m^3 air per d, respectively (56). The effective dose coefficients of 0.020 μSv/Bq for ^{134}Cs and 0.039 μSv/Bq for ^{137}Cs in the case of inhalation (absorption type S and aerosol size of 1 μm) by adults and 0.041 μSv/Bq for ^{134}Cs and 0.070 μSv/Bq for ^{137}Cs in the case of inhalation by children (3–7 y) were used to calculate committed effective doses (42). To estimate the inhalation exposure doses, it was assumed that all ^{134}Cs and ^{137}Cs in airborne dust samples collected using the high-volume air sampler were respirable; we considered this to be an appropriate hypothesis because 43–77% of radiocesium was collected in the respirable fraction in this study. The estimates of the annual committed effective dose were made under the assumption that the daily intake and inhalation of radiocesium was approximately constant over the course of a year. Cloudshine was estimated using the dose rate coefficient for a semiinfinite volume source in air to obtain effective doses for adults [0.26 nSv/(Bq/m^3)/h for ^{134}Cs and 0.093 nSv/(Bq/m^3)/h for ^{137}Cs] (57). We then estimated the probability distribution of the observed personal external dose rates, observed radiation dose rates from food, and observed radiation dose rates from the air, as obtained in the three study areas. We further tested for associations among the external radiation dose rates, radiation dose rates from food, and radiation dose rates from the air. When those values did not correlate to one another ($P > 0.05$), we considered them to be independent. If we could discard the null hypothesis of uncorrelatedness at $P = 0.05$, we considered the data to be correlated.

Next, those data were tested to determine whether their distribution can be assumed to be log-normal using the Kolmogorov–Smirnov–Lilliefors test. If the test showed $P > 0.05$, we assumed that values were log-normally distributed and estimated GMs and GSDs. The external dose rates, radiation dose rates from food, and radiation dose rates from the air were simulated using the Monte Carlo method using estimated GMs and GSDs. If dose rates were correlated, we generated correlated values by taking into consideration their associations. The total radiation dose rate for an adult subject was calculated by adding the generated values of external dose rates, radiation dose rates from food, and radiation dose rates from the air. Total radiation dose rates were generated 10^4 times for each study area. Annual doses 10 and 50 y after August 2012 were estimated by assuming the physical decay of radiocesium, with or without reduction factor $r(t)$ for long-term migration of radiocesium into the soil (23):

$$r(t) = 0.34 \times e^{-0.693 \times \frac{t}{1.5}} + 0.66 \times e^{-0.693 \times \frac{t}{50}}.$$

The variation in time of radiocesium in food and aerosols other than physical decay was not considered.

The external dose rate was divided into components attributable to ^{134}Cs and ^{137}Cs, assuming that the external dose coefficients per unit deposit are 2.7×10^{-8} and 1.2×10^{-8} (Sv/y)/(Bq/m^2) for ^{134}Cs and ^{137}Cs, respectively (23), and the ratio of ^{134}Cs:^{137}Cs is 1 on March 11, 2011 (13, 40).

To compare dose rate with background radiation in Japan, reference values were cited from the literatures (24–30). Regional dose rates of external exposure and radon inhalation were estimated from means among 47 prefectures and 7 regions in Japan (25–28). Variation in internal exposure to ^{210}Po and ^{210}Pb was calculated by bootstrapping the dose rate estimates among several cities in Japan into 47 prefectures (1,000 iterations) (29, 30). In each bootstrap dataset, the 5th and 95th percentile dose rates were calculated and their means were presented as a range of the total dose rate from background radiation. Other exposures were assumed to be constant across Japan.

Statistical Analysis. If analytical values were less than the LODs, they were converted to one-half of the LODs. Summary statistics (mean, range, and GM) were calculated for the radiation doses. Pearson's r coefficient was used to evaluate

correlation. *P* values less than 0.05 were considered as statistically significant differences. JMP (Version 4; SAS Institute Inc.) was used for these analyses.

Health Risk Assessment of Cancer Incidence from Radiation Dose Associated with the FDNPP Accident. A health risk assessment was conducted according to a framework used in a recent report by the WHO (50).

Mean dose rate estimated by Monte Carlo simulation was used to calculate LAR with or without a reduction factor for the long-term migration of radiocesium. The effective dose rates from external exposure and the dietary intake of radiocesium of a 1-y-old infant and 10-y-old child were assumed to be the same as those for an adult. The organ (colon, breast, and bone marrow) dose rate was calculated using organ dose-to-effective dose ratios for a 1-y-old infant, 10-y-old child, and 20-y-old adult in the three study areas (57). Whereas the ratios were calculated for an 8-wk old and 7-y old, we used the values for a 1-y-old infant and 10-y-old child, respectively. For the child and infant, the skin dose was regarded as the dose for breast tissue. The lifetime organ dose was estimated from the dose rate from August 2012 to an age of 89 for the three age groups, assuming that the dose rate in 2012 decreases only by physical decay unless otherwise indicated. Infants and children were defined as having age ranges of 1–3 y and 4–15 y, respectively. The cancer-free survival rates of males and females were calculated from age- and sex-stratified all-cause mortality in Japan in 2010, age- and sex-stratified all-cancer mortality (International Classification of Diseases 10 codes: C00–C96) in Japan in 2010 (58), and age- and sex-stratified all-cancer incidence (C00–C96) in Japan in 2008 (48). LBR of solid cancer (C00–C89), leukemia (C91–95), and breast cancer (C50) was calculated by integrating the product of cancer incidence probability and the cancer-free survival rate at each age from the age in 2012 to an age of 89 y. LAR from the annual dose (*D*[Sv]) at each age of exposure (*e*) for each sex (*g*) was calculated as

$$\mathrm{LAR}(D,e,g)=\int_{e+L}^{89}\left[w\cdot\mathrm{EAR}(D,e,a,g)+(1-w)\cdot\mathrm{ERR}(D,e,a,g)\cdot m(a,g)\right]\frac{S(a,g)}{S(e,g)}da,$$

where $m(a,g)$ is the cancer incidence probability in the unexposed population and $S(a,g)$ is the cancer-free survival rate. The minimum latency period, L, was set at 2 y for leukemia and 5 y for breast cancer and solid cancer.

The weights of an EAR model and an excess relative risk (ERR) model for solid cancer and leukemia were equal ($w = 0.5$) and EAR was considered for breast cancer ($w = 1$). The dose and dose rate effectiveness factor was taken as 1.

Risk models for solid cancer (49) are

$$\mathrm{ERR}(D,e,a,g)=\beta_1\cdot D\cdot\exp\left[\tau\cdot(e-30)+\upsilon\cdot\ln(a/70)\right](1+\sigma g),$$

where $\beta_1 = 0.4666$, $\tau = -0.01849$, $\upsilon = -1.621$, $\sigma = 0.2465$, and $g = -1$ for male and +1 for female;

$$\mathrm{EAR}(D,e,a,g)=\beta_1\cdot D\cdot\exp[\tau\cdot(e-30)+\upsilon\cdot\ln(a/70)](1+\sigma g),$$

where $\beta_1 = 0.005163$, $\tau = -0.02805$, $\upsilon = 2.406$, $\sigma = 0.1622$, and g = –1 for male and +1 for female.

Risk models for leukemia (59) are

$$\mathrm{ERR}(D,e,a,g)=(\alpha\cdot D+\beta\cdot D^2)\exp[\kappa_1\cdot\ln(a)],$$

where $\alpha = 864.552$, $\beta/\alpha = 1.18092$, $\kappa_1 = -1.647$;

$$\mathrm{EAR}(D,e,a,g)=(\alpha\cdot D+\beta\cdot D^2)\exp[\kappa_1\cdot \mathrm{I}_{s=\mathrm{female}}+\kappa_2\cdot\ln(a-e)],$$

where $\alpha = 7.51650\ 10^{-4}$, $\beta/\alpha = 1.03455$, $\kappa_1 = -0.52526$, $\kappa_2 = -0.6141$, and $\mathrm{I}_{s\,=\,\mathrm{female}} = 0$ for males and +1 for females. Although the model was developed for leukemia mortality, it was considered to be approximate to incidence in the WHO report owing to poor survival in the original population of the model.

The risk model for breast cancer (49) is

$$\mathrm{EAR}(D,e,a,g)=\beta_1\cdot D\cdot\exp\left[\tau\cdot(e-30)+\upsilon\cdot\ln(a/70)\right],$$

where $\beta_1 = 0.0009257$, $\tau = -0.04543$, and $\upsilon = 1.725$.

AR_{15} from 2012 was calculated as

$$\mathrm{AR}_{15}(D,e,g)=\int_{e+L}^{e+15}\left[w\cdot\mathrm{EAR}(D,e,a,g)+(1-w)\cdot\mathrm{ERR}(D,e,a,g)\cdot m(a,g)\right]\frac{S(a,g)}{S(e,g)}da.$$

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Detection of Antibodies to Human T-Cell Leukemia Virus Types 1 and 2 in Breast Milk from East Asian Women

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We investigated the incidence of human T-cell leukemia virus type I (HTLV-1) infection in a total of 17 regions in four countries, including 13 regions in Japan, as well as Korea (Seoul and Busan), China, and Vietnam, by testing breast milk using a particle agglutination assay (PA) and line immunoassay (LIA). Among 266 samples from Japan, 24 (9.0%) were positive on PA and 3 (1.1%) were positive on LIA. Among 50 samples from Seoul, 2 were positive on PA and 1 was positive on LIA. In contrast, all 50 samples from Busan were negative on both tests, suggesting the maldistribution of HTLV-1 infectants in South Korea. The numbers of positive samples were 2/91 on PA and 1/91 on LIA for China and 1/88 on both PA and LIA for Vietnam. In China, one sample with a high probability of HTLV-2 infection was identified by LIA and synthetic peptide enzyme-linked immunosorbent assay (ELISA). We examined HTLV-1 antibody in breast milk samples using commercially available test kits, suggesting the existence of HTLV-1 carriers in endemic areas in Southeast Asia and an HTLV-2 infectant in China. As a part of human ethno-epidemiological research, these results constitute valuable epidemiological data. Further studies on the sensitivity, specificity, and reliability of assays using antibodies to HTLV-1 and 2 in breast milk will be necessary for large-scale epidemiological surveys of HTLV infection.

Key words breast milk; human T-cell leukemia virus type II; line immunoassay; particle agglutination assay

Adult T-cell leukemia (ATL) is a malignant CD4-positive T-cell neoplasm caused by infection with human T-cell leukemia virus type I (HTLV-I). Because HTLV-1 is a retrovirus, its genomic RNA is incorporated into target cells, and DNA is incorporated into the host genome as a provirus *via* reverse transcriptase.[1,2]

The prevalence of ATL is 0.2–0.3%, and a route of infection that poses a particular issue is vertical transmission (mother–child transmission) *via* breast milk.[2,3] ATL is endemic in equatorial Africa, the Caribbean islands, Colombia, Brazil, south India, Papua New Guinea, northeast Australia, and among indigenous people living at the margins of the Andes plateau in South America; in Japan, the infection rate is highest in western Japan, particularly Kyushu.[3–5]

In addition to blood transfusions and sexual intercourse, HTLV-1 infection can be transmitted from mother to child *via* breast milk, with the cause of vertical transmission being infection *via* lymphocytes in breast milk.[1–3] Pregnant women who are carriers must switch to infant formula in order to prevent infection. HTLV-1 testing is required for all pregnant women in Japan, with the main test methods used comprising antigen or antibody testing of blood.[4] Recently, ATL provirus DNA testing using automated nucleic acid purifiers has also been investigated.[6] In developing countries, however, blood testing may not always be adequate.

HTLV-2 has also been isolated from individuals other than leukemia patients, and its association with disease remains unclear. Although HTLV-2 carriers have been reported to be common in Central and South America, the regional distribution of HTLV-2 has not been investigated in detail.[7]

In our preceding paper,[8] we reported for the first time that the gelatin particle agglutination (PA) method can be used to measure HTLV-1 antibody in breast milk, which can be sampled non-invasively and is the major source of infection. This PA method is comparatively easy to use as needed, in both the laboratory and the field. For confirmatory testing, HTLV-1 was examined using the Innogenetics™ Inno-lia™ HTLV I/II Score. A combination of PA and line immunoassay (LIA) was used on breast milk.

The purpose of this study was to develop a screening assay for the detection of antibodies to HTLV-1/2 in breast milk from East Asian women and to further examine HTLV-1 and HTLV-2 incidence using the Innogenetics™ Inno-lia™ HTLV I/II Score and synthetic peptide enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Specimens A total of 545 breast milk samples (Beijing, 91; Hanoi, 88; Seoul, 50; Busan, 50; Okinawa, 33; Nagasaki 28, Yamaguchi, 20; Okayama, 20; Kochi, 10; Hyogo, 20; Wakayama, 15; Kyoto, 20; Fukui, 20; Gifu, 20; Tokyo, 20; Miyagi, 20; and Hokkaido, 20) were collected between 2004 and 2010, and were archived in the Kyoto University Human Specimen Bank. Written informed consent was obtained from all participants. The bank project was reviewed[9] and approved

The authors declare no conflict of interest.

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by the Ethics Committee of the Kyoto University Graduate School of Medicine on 14 November 2003 (E25).

PA Screening tests were performed using 545 breast milk samples and a commercially available SERODIA HTLV-1 test kit (Fujirebio Inc., Tokyo, Japan) for *in vitro* diagnosis.[10] The kit includes HTLV-1 antigen-coated gelatin particles that agglutinate in the presence of HTLV-1 antibody in human serum or plasma. Test samples (25 μL) and positive control serum were prepared by 2-fold dilution up to 1:512. After an equal volume of sensitized particles was added, reactions were visually interpreted in duplicate. The agglutination patterns were interpreted according to the following criteria for an antibody titer of 1/8: negative (−), particles concentrated in the shape of a button with a smooth round outer margin; inconclusive (±), particles concentrated in the shape of a compact ring with a smooth round outer margin; positive (+), peripheral agglutination of the particles in a definite large ring with a rough multiform outer margin; and strongly positive (++), a film of agglutinated particles spread out uniformly on the bottom of the well.

LIA PA-positive breast milk was assayed for the presence of HTLV-1 antibodies using INNO-LIA™ HTLV I/II Score assays (Innogenetics N.V., Gent, Belgium), which were originally designed for testing serum or plasma. Milk samples (100 μL) were incubated in troughs containing LIA strips at 25°C overnight for 16h. This incubation was followed by three washing steps with washing buffer before the addition of an alkaline phosphatase anti-human immunoglobulin conjugate. Samples were then incubated for 30min at 25°C. Three washing steps were again performed, followed by incubation with the chromogen 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium for 30min at 25°C. The results were compared to a positive control.

Synthetic Peptide-ELISA (sp-ELISA) LIA-positive breast milk was assayed by sp-ELISA[11] using three synthesized peptides (corresponding to the Gag p19 protein 100 to 130 aa, the Env gp46 protein 175 to 199 aa, and the Env gp46 288 to 317 aa) derived from HTLV-1 genome sequence ATK-1. The peptide corresponding to the region of the Env gp46 protein 175 to 199 aa (gp46-175) allowed the discrimination of HTLV-1/2 antibodies.

RESULTS AND DISCUSSION

We carried out preliminary screening of HTLV-1 antibody using the PA method with a total of 545 samples: 266 from 13 locations in Japan, and 279 samples from China, Vietnam, and South Korea (Seoul and Busan). Of these, 29 samples showed positive or inconclusive patterns of agglutination. LIA of these 29 samples revealed that three were indeterminate for HTLV-1 infection, four were inconclusive, and two were positive. Of the two positive samples, one from China also indicated positivity for HTLV-2, which is an extremely interesting result. Table 1 shows the overall results for PA and LIA. In the agglutination reaction, 516 of the 545 (94.7%) breast milk samples were negative. Confirmatory testing was required for 29 samples (5.3%), comprising 11 in which particles formed a compact ring with a smooth outer margin (±), eight in which

Table 1. Screening Assay for Antibodies to HTLV-1 in Breast Milk Samples Using PA and LIA

Country	Sampling area	Number of samples	Positive on PA	Positive on LIA
Japan	Okinawa	33	7	0
	Nagasaki	28	3	0
	Yamaguchi	20	0	0
	Okayama	20	0	0
	Kochi	10	0	0
	Hyogo	20	3	0
	Wakayama	15	0	0
	Kyoto	20	0	0
	Fukui	20	0	0
	Gifu	20	0	0
	Tokyo	20	0	0
	Miyagi	20	3	2
	Hokkaido	20	8	1
Japan total		266	24	3
China	Beijing	91	2 ±[a] ±[b]	1 −[a] ±[b]
Korea	Seoul	50	2 +[c] ±[d]	1 +[c] −[d]
	Busan	50	0	0
Vietnam	Hanoi	88	1 ±[e]	1 ±[e]
Total		545	29	6

Japanese data were reported by Matsubara *et al.*[8] Year of collection and age of sample donors were as follows: *a*) Sep. 2008, 27; *b*) Feb. 2009, 26; *c*) Jan. 2010, 31; *d*) Feb. 2010, 31; *e*) Sep. 2008, 19.

there was peripheral agglutination of the particles in a clear large ring with a rough multiform outer margin (+), and 10 in which a film of agglutinated particles was spread out uniformly on the bottom of the well (++). Table 1 also shows the donor locations of these 29 breast milk samples: seven from Okinawa, three from Nagasaki, three from Hyogo, three from Miyagi, and eight from Hokkaido were positive. Samples from all other Japanese regions were negative. Five samples (1.8%) from the other countries required confirmation, with two from Beijing, one from Hanoi, and two from Seoul being positive.

HTLV-1 and HTLV-2 were examined using the Innogenetics™ Inno-lia™ HTLV I/II Score using 10-fold quantities of samples according to the manufacturer's standard protocol. As shown in Table 1, two samples were positive and four had a deferred pattern. As shown in Fig. 1, a positive pattern for HTLV-2 was observed in one sample from China, and the sample did not react to gp46-175, suggesting that the donor may be an HTLV-2 carrier. Although it is difficult to distinguish between HTLV-1 and HTLV-2 using the PA method, Berini *et al.*[12] reported that the PA sensitivity of HTLV-2 in the blood of carriers is equal to that of HTLV-1.

The incidence of HTLV-1 infection is high in western Japan, particularly in Kyushu,[3–5,13] with some cases also found in Hokkaido,[14] and it has also been reported in equatorial Africa, the Caribbean islands, Colombia, Brazil, south India, Papua New Guinea, northeast Australia, and among indigenous people living at the margins of the Andes plateau in South America, which represent endemic areas of the virus.[2] As shown Table 1, with the exception of Japan, HTLV-1-endemic areas are often in developing countries, where blood testing may not be feasible. The PA method uses a freeze-dried product that is prepared when required, and determination is made by diluting the sample and visually observing agglutination images; thus, it can easily be used for preliminary screening and is effective for use in developing countries.

It seems that the PA positivity ratio in breast milk is higher than that in the blood, but the results of the LIA method in the present study were equivalent to those obtained with blood samples. Thus, although the PA method using breast milk is useful as a first screen, follow-up testing is necessary.

The sp-ELISA method uses ELISA-coated synthetic peptides corresponding to the immunodominant regions of HTLV-1 structural proteins,[11] and for this study we used p19 gag protein (100 to 130 aa), gp46 protein (175 to 199 aa), and gp46 (288 to 317 aa). The peptide corresponding to the region of the Env gp46 protein, 175 to 199 aa (gp46-175), allows the discrimination of HTLV-1/2 antibodies.

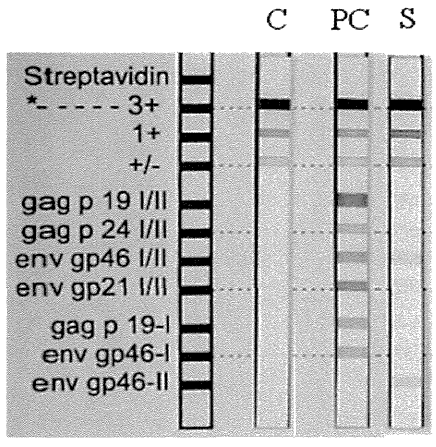

Fig. 1. Positive LIA Reactions of Milk Samples

C, control; PC, positive control; S, milk sample (HTLV-2 (±)).

HTLV-2 has been isolated from individuals other than leukemia patients, and its association with disease remains unclear. It has also been found in locations including Central and South America,[7] Sweden,[15] Spain,[16] and Brazil,[17] and its roots are believed to lie in the indigenous peoples of areas such as the Florida peninsula of the United States, the Yucatan peninsula of Mexico, and Panama.[5] In this study, it was interesting that we detected an HTLV-2-positive infectant in China.

In countries such as Japan, the United States, France, and the Netherlands, blood donations are routinely checked for a variety of infectious diseases. Recently, China[18] and Korea[19] have reported epidemiological analyses of HTLV-1 and -2 infection, but in other Asian countries, screening has only been established in the last few years, if at all.[20] High-performance antibody screening, such as that on donated blood, is required to assess the status of HTLV infection and to prevent further infection. While it is useful to demonstrate the validity of screening breast milk as opposed to blood, it is important not only to facilitate simpler screening but also highly accurate screening. Further studies on the sensitivity, specificity, and reliability of assays using antibodies to HTLV-1 and -2 in breast milk are necessary for large-scale epidemiological surveys of HTLV infection.

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Temporal trend and age-dependent serum concentration of phenolic organohalogen contaminants in Japanese men during 1989–2010

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ABSTRACT

The temporal trend in serum concentrations of phenolic organohalogen contaminants (POCs) were investigated in two age groups of men from Kyoto, Japan, from 1989 to 2010. These concentrations and trends were compared with neutral contaminants including polychlorinated biphenyls (PCBs) and pesticides. Serum concentrations of pentachlorophenol (PenCP) and 4-hydroxy-PCB187 were age-dependent and decreased to approximately one-half during the two decades, whereas no contamination trends were observed for 2,4,6-tribromophenol (TriBP), tetrabromobisphenol A (TBBPA) and 6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (6-OH-BDE47). 6-OH-BDE47 was found in all samples (up to 3000 pg/g wet weight), whereas TBBPA was detected in 17 of 60 serum samples (up to 950 pg/g wet weight). The concentrations of TriBP, TBBPA and 6-OH-BDE47 were not correlated to those of PenCP or 4-OH-PCB187 in either age group, suggesting the different kinetics on exposure routes and fate between these brominated and chlorinated POCs.

1. Introduction

Despite the use of persistent organic pollutants, such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), having either been phased-out or restricted, they are still found in the environment, in both neutral and phenolic forms. Phenolic organohalogen contaminants (POCs) of concern may include pentachlorophenol (PenCP), hydroxylated PCBs (OH-PCBs) and hydroxylated PBDEs (OH-PBDEs) and brominated flame retardants (BFRs), such as 2,4,6-tribromophenol (TriBP) and tetrabromobisphenol A (TBBPA). These POCs have endocrine-disrupting effects and may influence neurological, reproductive and sexual development (Lans et al., 1993; Meerts et al., 2000; Montaño et al., 2013; Suzuki et al., 2008). However, limited information is available on human exposure to these POCs in contrast to traditional neutral organohalogens.

PenCP, which is the predominant POC in human serum, has similar concentrations to persistent pesticide i.e., 4,4′-DDE (Meijer et al., 2008; Rylander et al., 2012). PenCP has been used as a herbicide in agricultural chemicals and preservatives in Japan until 1990 (Suzuki et al., 2008). The usage of PenCP in the past might have led to its ubiquitous presence because of its persistence and, consequently, to contamination of the indoor environments (Ge et al., 2007; Inoue et al., 2006; Suzuki et al., 2008). OH-PCBs are another group of POCs that have been commonly detected in human tissues. PenCP and certain OH-PCBs have shown the capability of interacting in human blood with thyroid hormones (Meerts et al., 2002; Otake et al., 2007), which play an important role in fetal growth and development (Zheng et al., 2012). Previous studies have suggested that PenCP and OH-PCBs are responsible for adverse effects on thyroid or sex hormone homeostasis (Meerts et al., 2002) and are transferred from mother to fetus via the placenta during pregnancy (Meijer et al., 2008).

Of brominated POCs, TriBP and TBBPA are now used as flame retardants in many industrial and computer products. In Asian countries, the total production of both BFRs accounted for 85,900 tons in 1999 of which 36% are produced in Japan (Watanabe and Sakai, 2003). TriBP is not used directly as a BFR but as an intermediate for brominated epoxy resins made from TBBPA (9500 tons/year in 2001) (Suzuki et al., 2008; Watanabe and Sakai, 2003). TriBP is also naturally produced by marine bacteria or plants and has been detected in fish (Haraguchi et al., 2010; Whitfield et al., 1999). Household materials containing TriBP may exist as indoor sources, whereas seafood may be a dietary source of TriBP. TBBPA, in contrast, is a reactive BFR that is released into the environment via

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many household electrical products and their disposal. Because of the short half-life of these phenolic BFRs in air, water, sediment and in humans (Covaci et al., 2009), their environmental distribution has been limited. However, phenolic BFRs have been reported to be thyroid hormone agonists and estrogenic *in vitro* (Kitamura et al., 2002; Meerts et al., 2001), and thus have potential for posing a threat to human health even at a low-dose exposure.

Exposure to hydroxylated PBDEs (OH-PBDEs) may be a more recent concern. Two congeners, e.g., 2′-OH-BDE68 and 6-OH-BDE47, have been frequently detected in blood of fish (Marsh et al., 2004), marine mammals (Marsh et al., 2005) and humans (Eguchi et al., 2012; Fujii et al., 2012a,b). The ortho-substituted OH-PBDEs are proposed to be of natural origin, whereas meta- or para-substituted OH-PBDE congeners detected in other studies may be possible metabolites of PBDEs (Athanasiadou et al., 2008). OH-PBDEs have been shown to have a higher affinity for human transthyretin (TTR) than PBDEs in liver (Meerts et al., 2000). However, the formation process and exposure routes of OH-PBDEs remain unclear.

Previous studies have suggested that the body burden of legacy neutral contaminants, such as PCBs or chlorinated pesticides, has decreased from 1973 to 1996 (Masuda et al., 2005), but there is much less information available on the temporal trends of POCs in human tissues. Therefore, retrospective monitoring of POCs is required to judge the development of pollution and to assess the fate of contaminants in humans.

The aim of the present study was to investigate the temporal trends of serum POC concentration in Japanese populations from 1989 to 2010. Target chemicals consisted of representative congeners, 1) PenCP and 4-OH-PCB187; 2) TriBP and TBBPA; and 3) 6-OH-BDE47. To avoid possible variation of body burdens by age and gender, this study was restricted to male subjects in two groups, aged in their 20s (21–29 years) and >50 years of age, at different sampling periods. Furthermore, the temporal trends in serum POC concentrations were compared with those of legacy neutral contaminants including α-endosulfan.

2. Materials and methods

2.1. Sample collection

Human serum samples were obtained from the Kyoto University Human Specimen Bank using a standardized protocol (Koizumi et al., 2005). Individual serum samples (1–2 mL each, $n = 60$) were collected from healthy volunteers in the Kyoto area that consisted of two groups of males, aged in their 20s and >50 years of age, in 1989, 1999 and 2010 (Table 1). The Ethics Committee of Kyoto University approved the protocol of the present study (E25), and appropriate written informed consent was obtained from all of the participants. Samples were stored in clean screw-capped plastic containers at −20 °C until the time of analysis.

2.2. Chemicals

Three internal standards, $^{13}C_9$-labeled α-endosulfan, $^{13}C_6$-labeled PenCP, and $^{13}C_{12}$-labeled 4-hydroxy-2,2′,4,4′,5,5′-hexachlorobiphenyl (4-OH-[$^{13}C_{12}$]PCB187), were used for the determination of target POCs. Pesticide standard solution (unlabeled pesticide mix #1037; 2 μg/mL) was purchased from Kanto Chemical Co., Ltd., (Tokyo, Japan). The standards of two PCB congeners (PCB-153 and PCB-187) and α-endosulfan were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). PenCP, TriBP, 2,2-bis(3,5-dibromo-4-hydroxyphenyl)propane (TBBPA), 4-hydroxy-PCB187, 6-hydroxy-BDE47 and their O-methylated analogs were purchased from Wellington Laboratories Japan Inc. (Tokyo, Japan). These standards were used for the calibration, recovery, and quantification of the target compounds. Silica-gel (Wako gel S-1) used for purification was obtained from Wako Pure Industries Ltd. (Osaka, Japan) and was heated at 130 °C for 3 h prior to use. All solvents used (i.e., ethanol, diethylether, *n*-hexane and dichloromethane) were of pesticide-grade quality, and purchased from Kanto Chemical Co. Inc. Japan. Diazomethane was prepared by 1-methyl-3-nitro-1-nitrosoguanidine (Kanto Chemical Co., Inc. Japan).

Table 1
Information of serum samples collected from volunteers in Kyoto during 1989–2010.

Sample group	Region	M/F	*n*	Year of sampling	Mean age (range)
Serum89A	Kyoto	M	10	1989	20.6 (20–22)
Serum89B	Kyoto	M	10	1989	51.0 (50–52)
Serum99A	Kyoto	M	10	1999	24.1 (21–28)
Serum99B	Kyoto	M	10	1999	54.2 (50–59)
Serum10A	Kyoto	M	10	2010	21.5 (21–23)
Serum10B	Kyoto	M	10	2010	68.0 (65–69)

2.3. Extraction, clean-up and fractionation procedures

The methodology used to analyze neutral and phenolic contaminants in serum samples was based on lipid extraction, gel permeation chromatography (GPC) and silica-gel column cleanup, and gas chromatography-mass spectrometry with electron capture negative ionization (GC/MS/ECNI) analysis as previously described (Fujii et al., 2012a). Briefly, each 1-mL serum sample was spiked with three internal standards, namely [$^{13}C_6$]PenCP and 4-OH-[$^{13}C_{12}$]PCB187 for phenolic analytes (0.2 ng of each) and α-[$^{13}C_9$]endosulfan for neutral OCs (2 ng). The sample was extracted with *n*-hexane, after adding formic acid (0.1% v/v), ethanol, and diethylether. A combined extract was dissolved in dichloromethane (DCM):*n*-hexane (1:1), and then subjected to GPC with a Bio-Beads S-X3 column (Bio-Rad Laboratories, Hercules, CA, USA). The gel material (35 g) was packed in a glass column (55 cm × 27 m i.d.) with DCM:*n*-hexane (1:1) as the eluting solvent at a flow rate of 4 mL/min. The first 96-mL fraction of the eluate contained lipids and was discarded. Subsequently, the next 68 mL fraction was collected. The eluate was concentrated and partitioned between 1 M KOH:ethanol (7:3) and *n*-hexane. After acidification, the phenolic contaminants in the KOH solution were back-extracted twice with 20% diethylether in *n*-hexane. The phenolic fraction was derivatized to O-methylated analogs by diazomethane in diethylether. The neutral and methylated phenolic fractions were purified with a silica-gel column (0.2 g, Wako gel S-1) by an elution of 15 mL DCM:*n*-hexane (12:88, v/v). Each fraction was concentrated to 200 μL and spiked with ^{13}C-labeled PCB-153 to determine the recoveries of internal standards and to correct the volume of samples prior to GC/MS analysis.

2.4. Instruments and quantification

Fifteen analytes were measured by GC/MS/ECNI using an Agilent GC/MSD 5973i (Agilent Technologies, Santa Clara, CA, USA) coupled with a 6890N gas chromatograph. The GC/MS conditions and target ions for the determination of analytes are summarized in Supplementary Table S1. Quantification of the compounds was based on signals in the mass chromatograms and on comparison with ^{13}C-PCB153 that was used as a syringe standard. The concentrations of all analytes were reported as picogram per gram wet weight (pg/g ww).

2.5. Quality control and quality assurance

The extraction, cleanup, and fractionation steps were evaluated by measuring the absolute recoveries of the compounds ($^{13}C_{12}$-labeled internal and native surrogate standards). For recovery tests, two concentrations (2.0 and 10.0 ng/mL) of 11 analytes were spiked into cow milk, passed through the entire analytical procedure and determined based on GC/MS-selected ion monitoring. Procedural blanks were analyzed simultaneously with every batch of 10 samples to check for interference or contamination from solvents and glassware. The recoveries were between 81% and 94% with relative standard deviations of <10% ($n = 5$). The limits of quantification (LOQ), defined as 10-fold that of the noise level, ranged from 1 to 50 pg/g ww (Supplementary Table S1). When the concentrations of the target chemicals were less than their LOQs, we allocated half of the LOQ as the value for analysis. The calibration (2–100 ng/mL of each analyte) was linear and characterized by good correlation coefficients (>0.99). The quality of the method under validation was verified by Standard Reference Materials (non-fortified human serum, SRM1974, NIST) for PCBs and selected pesticides. Data from the current study were within 15% of the certified values of SRM1974.

2.6. Statistical analysis

The data were analyzed using SPSS version 16.0 for Windows 2007 (SPSS Inc., Chicago, IL, USA). Steel–Dwass test and Mann–Whitney *U*-test were used to examine differences in the target chemical concentrations in three sampling years and between two age groups. Spearman's rank correlation coefficients were used to evaluate the relationship between neutral and phenolic OCs. Probability values of less than 0.05 were considered to indicate statistical significance.

3. Results and discussion

3.1. Legacy persistent organohalogen pollutants (POPs)

Within the current study group of legacy organohalogens, serum concentrations of the representative congeners, 4,4′-DDE, PCB-153, *trans*-nonachlor, hexachlorobenzene (HCB) and α-

endosulfan are summarized in Table 2. During the two decades from 1989 to 2010, the mean serum concentrations were significantly decreased from 5620 to 1620 pg/g ww for 4,4′-DDE, from 1070 to 680 pg/g ww for PCB-153, and from 180 to 86 pg/g ww for *trans*-nonachlor. These contaminants were significantly higher in the >50 years of age group than in the 20 years of age group ($p < 0.01$) in each sampling year. For legacy POPs, older people in 2010 has been exposed to these contaminants for more decades, and when the contamination was decreasing, age-difference may influence the trend. Such age-dependency in the concentrations of legacy POPs has also been observed in blood samples in 1999 ($n = 151$) where the concentrations of pesticides were positively correlated with age (20s–50s) (Masuda et al., 2005).

α-Endosulfan was detected in all serum samples (mean, 160 pg/g ww in 1989) at comparable concentrations to *trans*-nonachlor, and then were gradually reduced to 85 pg/g ww in 2011. The concentrations of α-endosulfan were positively correlated to those of PCBs, *trans*-nonachlor and HCB ($p < 0.01$) but not to those of 4,4′-DDE (Table 3). Unlike 4,4′-DDE, no decreasing trend was observed for α-endosulfan between 1999 and 2010. This trend is most likely due to continuous local usage of endosulfans until its agricultural registration had expired in 2010. A recent dietary survey has reported an exponential increase in α- and β-endosulfans in diets from China and Korea (Desalegn et al., 2011). However, no such increasing trend appeared in foods from Japan in that study. The present concentrations of endosulfans and residue profiles of pesticides in serum of Japanese men may be correlated to those in Japanese breast milk which has been contaminated with endosulfans at similar concentrations to other pesticides (Fujii et al., 2012b).

3.2. PenCP and OH-PCBs

Serum PenCP concentrations ranged from 89 to 1670 pg/g ww with a mean of 650 pg/g ww in 1989 and then decreased to 140 pg/g ww in 2010 (Table 2). No age-dependency in these concentrations was observed. The mean serum PenCP concentrations were at comparable concentrations to *trans*-nonachlor but not significantly correlated to each other (Table 3). Based on previous studies, the current concentrations are lower than those of European women (Glynn et al., 2011; Meijer et al., 2008; Rylander et al., 2012).

Unlike other pesticides, such as 4,4′-DDE and *trans*-nonachlor, PenCP concentrations in the 20 years of age group in 1989 ranged from 37 to 1670 pg/g ww, which were an order of magnitude greater than those of the >50 years of age group (Fig. 1). Such large variation of PenCP in young ages has also been observed in Norwegian children (Thomsen et al., 2002). This may be attributed to the combined exposure routes, dietary ingestion of contaminated seafood (Ge et al., 2007; Guvenius et al., 2003; Sjödin et al., 2001), and dust ingestion via inhalation (Inoue et al., 2006; Suzuki et al., 2008). It should not be excluded that PenCP may exist as a demethylated metabolite of pentachloroanisole (Ikeda and Sapienza, 1995) or as a hydroxylated metabolite of hexachlorobenzene in serum (To-Figueras et al., 1997).

For the monitoring of hydroxylated PCBs, we measured 4-OH-PCB187 that has been detected as one of major components in human blood (Rylander et al., 2012; Sandau et al., 2002). Serum concentrations of 4-OH-PCB187 in both age groups ranged from <LOQ to 800 pg/g ww (mean 120 pg/g ww), which are comparable to previous results from Japanese women (15–43 pg/g ww, Kawashiro et al., 2008; 12–370 pg/g ww, Nomiyama et al., 2010), and those from eastern Slovakia (273 pg/g ww, Park et al., 2009), Latvia (50 pg/g ww), and Sweden (120 pg/g ww, Sjödin et al., 2001).

The concentration of 4-OH-PCB187 was positively correlated to that of the parent compound PCB-187 ($r = 0.747$, $p < 0.01$, Fig. 2) but not significantly to that of PenCP ($r = 0.178$, $p > 0.05$, Table 3). This finding was also observed in other studies (Rylander et al., 2012). No significant differences in the ratio were observed between the 20 and >50 years of age groups. The mean concentration ratio of OH-PCB187 to PCB-187 was 1.4 in 1989, but it declined to 0.6 in 1999 and 2010. These findings suggest that OH-PCBs may be eliminated faster than the parent PCBs in individuals with lower concentrations of PCBs. This association indicates that the variation of 4-OH-PCB187 would depend on the extent of dietary exposure to the parent PCBs. This same phenomenon has been observed by Dirtu et al. (2009).

3.3. TriBP and TBBPA

Serum TriBP concentrations ranged from 46 to 960 pg/g ww (mean 248 pg/g ww) and showed slightly increasing trends in both age groups during the two decades (Fig. 1). Unlike the chlorinated

Table 2
Serum concentrations in Japanese men (20 and>50 age groups) from Kyoto during 1989–2010.

Contaminants	LOQ(pg/g ww)	Freq (%)	AM concentration (pg/g wet weight)						
			Sampling year			p value	Age		p Value
			1989 (n = 20)	1999 (n = 20)	2010 (n = 20)		20s (n = 30)	>50s (n = 30)	
Neutral OCs									
4,4′-DDE	50	100	5620[a] (330–12000)	3260 (390–8890)	1620 (470–6000)	<0.001	1970 (330–10900)	5030[a] (620–13000)	<0.001
HCB	2.0	100	490[a] (130–1230)	230 (120–450)	260 (43–660)	<0.001	250 (43–710)	400[a] (130–1230)	0.009
PCB-153	10	100	1070 (100–2830)	640 (200–1390)	680 (100–2140)	0.051	390 (100–1050)	1200[a] (330–2830)	<0.001
PCB-187	10	100	150 (13–560)	104 (21–240)	135 (20–480)	0.383	60 (13–200)	200[a] (49–560)	<0.001
trans-nonachlor	10	100	180 (10–714)	90 (12–190)	86 (4.1–530)	0.051	41 (4.1–190)	200[a] (42–710)	<0.001
α-endosulfan	10	100	160 (28–600)	59 (14–110)	85 (39–150)	0.004	61 (42–710)	140[a] (14–600)	0.002
Phenolic OCs									
PenCP	1.0	100	650[a] (89–1670)	300 (150–1060)	140 (37–850)	<0.001	430 (37–1670)	290 (50–1060))	0.917
4-OH-PCB187	20	92	220 (23–800)	67 (<LOQ -300)	76 (<LOQ -340)	0.792	42 (<LOQ -140)	200 (6.6–800)	0.241
TriBP	2.0	100	150 (46–370)	240 (110–380)	350[a] (62–960)	<0.001	250 (46–960)	250 (67–500)	0.149
TBBPA	50	28	NR (5%) (<LOQ-940)	NR (45%) (<LOQ-950)	NR (35%) (<LOQ-420)	NR	NR (33%) (<LOQ-950)	NR (23%) (<LOQ-420)	NR
6-OH-BDE47	20	100	200 (52–680)	550 (25–1590)	610 (34–3075)	0.032	360 (25–1580)	550 (52–3080)	0.182
Ratio									
4-OH-CB187/ PCB-187			1.4	0.6	0.6		0.7	1.0	

AM, arithmetic mean; LOQ, limit of quantification; *n*, number of samples; Freq%, % proportion of numbers that were >LOQ.
Concentrations below LOQ were treated as 1/2 LOQ for arithmetic mean. NR: not reported due to low detection frequency (<50%).
[a] Significantly higher than the other group ($p < 0.05$, Steel–Dwass test and Mann–Whitney *U*-test). *p* values, Spearman's rank correlation.

Table 3
Spearman's rank correlation coefficients between concentrations of analytes in human serum ($n = 60$).

	HCB	PCB-153	PCB-187	*trans*-nonachlor	α-Endosulfan	TriBP	PenCP	4-OH-PCB187	TBBPA	6-OH-BDE47
4,4′-DDE	0.519*	0.778**	0.651**	0.683**	0.329	−0.241	0.388	0.646*	−0.260	−0.204
HCB		0.588*	0.516*	0.585*	0.367	−0.089	0.226	0.518*	0.069	−0.186
PCB-153			0.951**	0.770**	0.585*	0.053	0.091	0.793**	−0.115	−0.069
PCB-187				0.736**	0.538*	0.191	−0.067	0.747**	−0.020	−0.026
trans-nonachlor					0.455*	0.132	0.160	0.629**	0.011	0.014
α-endosulfan						0.162	−0.007	0.474*	−0.140	−0.144
TriBP							−0.411	−0.105	0.412	0.340
PenCP								0.178	−0.159	−0.245
4-OH-PCB187									−0.258	−0.082
TBBPA										0.131

*$p < 0.05$, **$p < 0.01$.

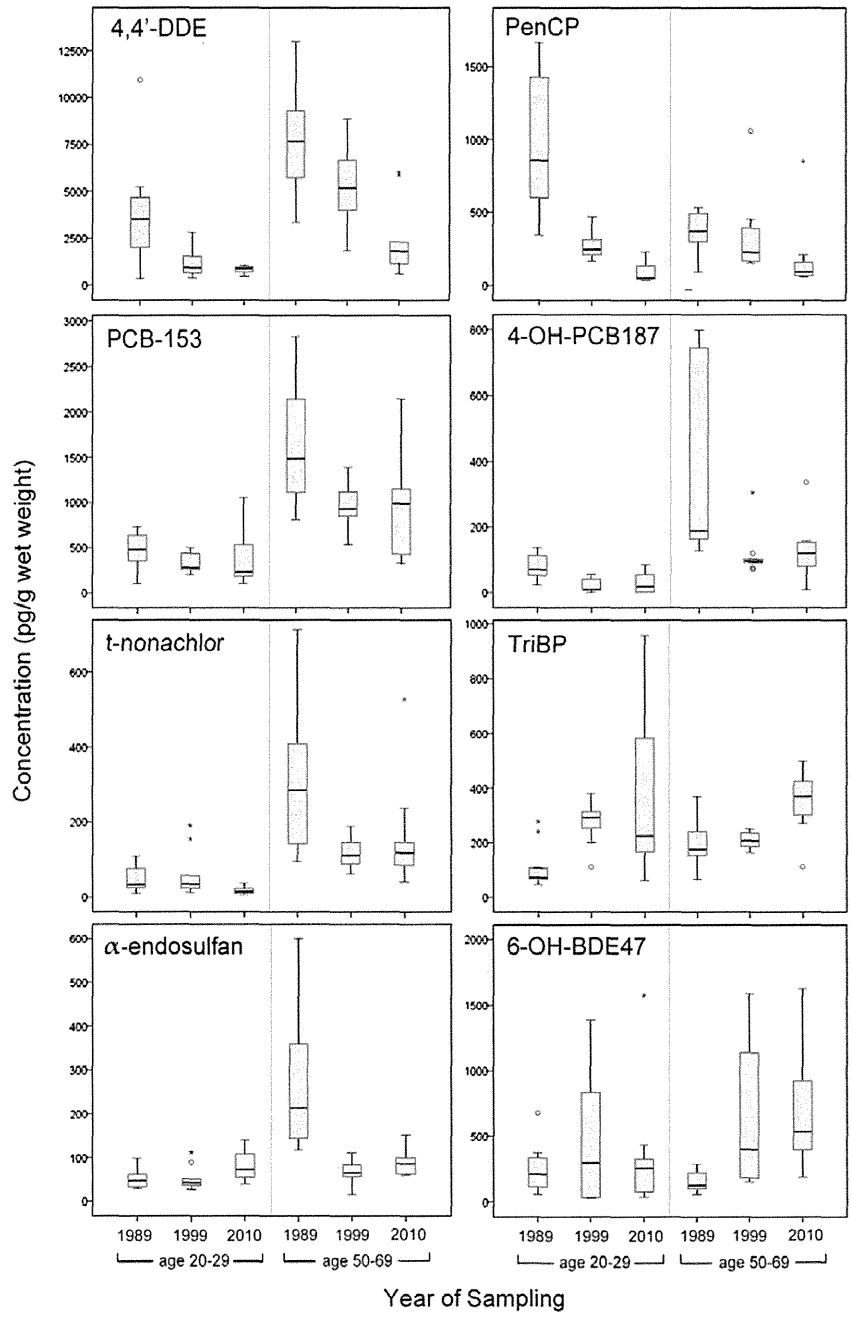

Fig. 1. *Temporal trend in serum concentration (pg/g wet weight) of organohalogens in two age groups during 1989–2010 from Kyoto. Box plot: box, 25th–75th percentiles; center line, median value; whiskers, min–max values; dots, outliers; circle, extremes.*

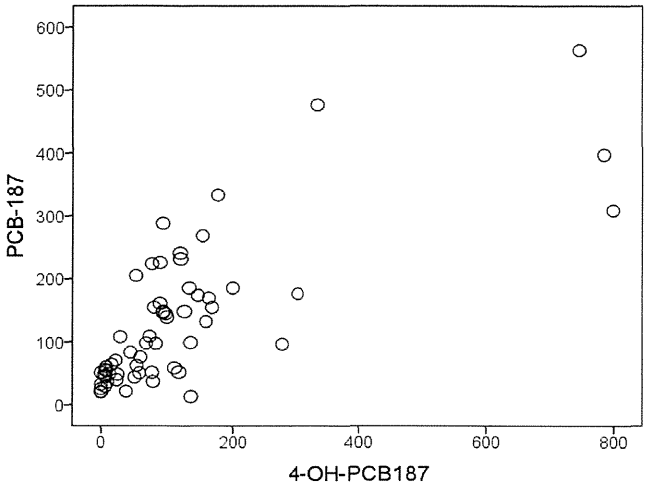

Fig. 2. Correlation between PCB-187 and 4-OH-PCB187 in serum concentration (pg/g wet weight) from Kyoto. Spearman's rank correlation coefficient, $r = 0.747$, $p < 0.01$.

pesticide, no age-dependency was observed for TriBP. Compared with other studies, the current TriBP concentrations were one order of magnitude greater than those of pregnant women (mean 22 pg/g ww; Kawashiro et al., 2008) or those of Norwegians during 1977–1999 (0.077–26 pg/g ww; Thomsen et al., 2002), whereas concentrations were comparable to those of Indians (72–1200 pg/g ww, mean 360 pg/g ww, Eguchi et al., 2012).

Serum TriBP concentrations were not correlated to those of the other POCs (Table 3). This may be explained by the combined exposure to TriBP from different sources. One possible exposure source would be via dietary seafood because TriBP is a naturally produced flavor in marine algae and fish (Haraguchi et al., 2010; Whitfield et al., 1999), which can be bioconcentrated in higher trophic organisms (Whitfield et al., 1999). Another possible source may be house dust contaminated with TriBP, which was used as a BFR intermediate and released into the air by leakage (Watanabe and Sakai, 2003) and indoor dust (Suzuki et al., 2008). Because of the lack of data on other TriBP exposure routes, we have no conclusion regarding the relative importance of diet or house dust.

TBBPA was detected in 28% of the studied samples (17 out of 60) with concentrations ranging from <LOQ (50 pg/g ww) to 950 pg/g ww (Table 2). The highest concentration was observed in the 20 years of age group in 1999. None of volunteers were occupationally exposed to TBBPA. The mean concentration of TBBPA in the detected samples ($n = 17$) was 98 pg/g ww, which is higher than that of the Fukuoka residents investigated in 2000 (7 pg/g ww, Nagayama et al., 2000) and that of pregnant women from Japan (26 pg/g ww, Kawashiro et al., 2008). As the present mean values of TBBPA were based on only 17 samples (28% detection frequency), the present TBBPA concentrations may be overestimated. However, our results in 1999 and 2010 are comparable to the serum concentrations from Belgium (80 pg/g ww; Dirtu et al., 2008) or France (154 pg/g ww; Cariou et al., 2008). This is the first study to report the temporal trend of TBBPA using human blood samples in Japan. This study could not clarify the relationship between concentrations of TriBP and TBBPA because of the small sample size utilized in this study (Table 3).

The exposure routes of TBBPA are possibly via diet and house dust, which is similar to that of TriBP. The Norwegian population was exposed to TBBPA via diet as a major source (Thomsen et al., 2001). However, plasma concentrations of TBBPA were higher in the workers at the dismantling plant (Thomsen et al., 2002) than that of the non-occupationally-exposed workers. Consistent with its phenolic structure, TBBPA can be rapidly conjugated in human liver and subsequently excreted in bile (Schauer et al., 2006). No age-dependency in serum concentrations of TBBPA may be because of its short half-life (two days) in human plasma (Hagmar et al., 2000). Therefore, the occurrence of TBBPA in human serum is likely to reflect recent exposure rather than past exposure (Covaci et al., 2009).

3.4. OH-PBDEs

Of phenolic PBDE congeners, 6-OH-BDE47 was predominantly detected in all serum samples investigated. The concentrations ranged from 25 to 3075 pg/g ww (mean 451 pg/g ww), which was 20-fold greater than those of BDE-47 (Table 2). The 6-OH-BDE47 concentrations were comparable to those of PenCP or 4-OH-PCB187. It was not significantly correlated to those of BDE-47 or to the other chlorinated OCs (data not shown). No age- or time-related differences were observed from 1988 to 2010 (Fig. 1). Other isomeric hydroxy- and methoxy-PBDEs have been found in human blood from India and Nicaragua (Athanasiadou et al., 2008; Eguchi et al., 2012), but we could not detect any metabolites in our samples. The *ortho*-substituted 6-OH-BDE47 detected in this study is considered to be of natural origin, although it can be also formed by human P460 *in vitro* (Erratico et al., 2013). The *meta*- and *para*-substituted OH-tetraBDEs detected in the other studies are most likely metabolites of parent PBDEs (Athanasiadou et al., 2008). The occurrence of 6-OH-BDE47 in serum in this study may be a result of dietary exposure to 6-OH-BDE47 itself via seafood (Marsh et al., 2004; Wang et al., 2012) or demethylation of 6-MeO-BDE47 by microsomes of mammals (Wan et al., 2009). The toxicological implication of human exposure to OH-PBDEs remains unknown, but some studies have shown that 6-OH-BDE47 exhibits thyroid-disrupting effects and a higher affinity to human TTR than BDE-47 *in vitro* (Liu et al., 2011; Meerts et al., 2001).

4. Conclusions

Serum concentrations of legacy neutral contaminants in Japanese men showed a decreasing trend in two age groups from 1989 to 2010. Although the concentrations of PenCP and 4-OH-PCB187 showed a decreasing trend, TriBP and 6-OH-BDE47 appeared to be slightly increasing. TBBPA was detected in 28% of serum samples, whereas 6-OH-BDE47 was present at the highest concentrations of POCs investigated in all samples. No age-dependency was observed for all POCs tested. The concentrations of TriBP and 6-OH-BDE47 were not correlated with those of PenCP, OH-PCBs and chlorinated pesticides. These findings suggest that the kinetics on exposure routes and fate of the studied brominated POCs are different from chlorinated POCs and legacy POPs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2013.11.002.

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Dietary exposure to phenolic and methoxylated organohalogen contaminants in relation to their concentrations in breast milk and serum in Japan

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ABSTRACT

This study investigated human exposure to neutral, phenolic, and methoxylated organohalogen contaminants (OHCs) in a duplicate diet study to evaluate their concentrations in breast milk and serum of Okinawan people from Japan during 2004–2009. Dietary intakes of phenolic OHCs were predominantly 2,4,6-tribromophenol (TriBP), followed by tetrabromobisphenol A (TBBPA), and 6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (6-OH-BDE47). After exposure, TriBP and TBBPA were transferred to breast milk, whereas 6-OH-BDE47 was selectively retained in serum. Despite a lower dietary exposure to pentachlorophenol and 4-hydroxy-CB187, both were retained in serum. For the methoxylated OHCs, 2,4,6-tribromoanisole (TriBA) and 6-methoxy-BDE47 were the predominant dietary contaminants, of which TriBA was present in both breast milk and serum, whereas 6-methoxy-BDE47 was selectively transferred to breast milk. These findings suggest that dietary exposure to phenolic and methoxylated OHCs may result in differential partitioning between breast milk and serum with different pharmacokinetic or exposure routes.

1. Introduction

Organohalogen contaminants (OHCs) include a diverse group of phenolic chemicals, such as pentachlorophenol (PenCP), 2,4,6-tribromophenol (TriBP), tetrabromobisphenol A (TBBPA) and hydroxylated analogs of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs). These compounds are persistent, and bioaccumulative, and have been distributed in wildlife and humans (Fujii et al., 2012; Marsh et al., 2004). Recent studies have shown that these phenolic OHCs can cause carcinogenic, thyrotoxic, estrogenic, and neurotoxic effects in experimental animals and humans (Meerts et al., 2001; Otake et al., 2007; Saegusa et al., 2009). Of the phenolic pesticides, PenCP is a ubiquitous thyroid-disrupting compound (Zheng et al., 2011) and contributes to the human transthyretin (TTR) binding potency of OHCs in indoor dust (Suzuki et al., 2008), but data regarding its body burden are limited (Hong et al., 2005). PCBs and PBDEs are metabolized to hydroxy-CBs and hydroxy-BDEs, respectively, by cytochrome P450 in mammalian liver (Erratico et al., 2013). Some of these metabolites are selectively retained in blood (Sandau et al., 2002), but there are no data regarding their accumulation in adipose tissues (Nomiyama et al., 2010). TriBP and TBBPA are phenolic brominated flame retardants (BFRs) and have been detected separately from PBDEs in humans (Thomsen et al., 2002). However, accumulation and exposure kinetics of these phenolic BFRs are not largely understood.

Phenolic OHCs may be in part methylated to their anisoles by marine bacteria or fungi in the marine food web (Allard et al., 1987; Whitfield et al., 1997). For example, TriBP and PenCP are known to be biotransformed to 2,4,6-tribromoanisole (TriBA) and pentachloroanisole (PenCA), respectively, both of which have been distributed in marine biota (Watanabe et al., 1983a). Microbial O-methylation may also be observed for TBBPA, which leads to its mono- or dimethylether derivatization in the marine environment (George and Häggblom, 2008; Watanabe et al., 1983b). Furthermore, naturally occurring hydroxy-BDEs have been produced in marine algae or sponges, together with their methoxylated PBDEs (Haraguchi et al., 2011), suggesting that the possible microbial methylation of phenolic OHCs occurs in marine biota. Such methoxylated analogs may increase the probability of bioaccumulation in the food chain because of the addition of a hydrophobic methyl group (Glickman et al., 1977), whereas the phenolic OHCs have short half-lives owing to their rapid elimination (Covaci et al., 2009; Hagmar et al., 2000).

Although the relative importance of the various potential routes of exposure to phenolic and methoxylated OHCs remains unknown, it has been suggested that food, water, house dust, and airborne sources may all be significant (Sjödin et al., 2001). Chronic human exposure to phenolic OHCs is most likely the result of the long-term intake of contaminated foods, including drinking water (Shi et al., 2009).

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Therefore, it is important to survey the dietary intake of phenolic and methoxylated OHCs as it relates to their contamination status in breast milk or blood, and to investigate the dietary health risk for the general population and infants.

The aim of the present study was to investigate the association between the levels of phenolic and methoxylated OHCs in diet, breast milk, and serum. We selected five representative phenolic OHCs and their methoxylated analogs for comparison, together with legacy persistent organohalogen pollutants, such as PCBs, PBDEs or chlorinated pesticides, which were investigated by duplicate diet sampling during 2004 and 2009 in Okinawa, Japan.

2. Materials and methods

2.1. Sample collection

Diet samples from the Kyoto University Human Specimen Bank (Koizumi et al., 2005) were used for the chemical analysis. At the time of collection, participants were requested to donate the same duplicate samples as all food and drink items that they consumed over a 24-h period. Ten duplicate 24-h diet samples were collected in Okinawa in 2004. An additional 10 duplicate 24-h diet samples (i.e., a typical day's worth of food and drink for consumers) were purchased from markets in Okinawa in 2009. This study provided 20 duplicate 24-h diet samples. All food and drink samples in each duplicate sample were combined, homogenized, and stored as a dietary homogenate.

Okinawan breast milk and serum samples were obtained from the Kyoto University Human Specimen Bank using a standardized protocol (Koizumi et al., 2005). Human breast milk samples (5–10 mL each, n = 9) were obtained from healthy women in Okinawa between 2005 and 2006 (average age 31 years old). Individual serum samples (1–2 mL each, n = 10) were collected from healthy volunteers in the Okinawa area in 2006 (average age 44 years old). The Ethics Committee of Kyoto University approved the protocol of the present study (E25), and appropriate written informed consent was obtained from all of the participants. Samples were stored in clean screw-capped plastic containers at −20 °C until the time of analysis. The study populations are provided in Table 1.

2.2. Chemical reagents

Four ^{13}C-labeled standards, PenCP [$^{13}C_6$], 4-hydroxy-2,2′,3,4′,5,5′,6-heptachlorobiphenyl (4-OH-CB187[$^{13}C_{12}$]), 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB-153[$^{13}C_{12}$]) and α-endosulfan[$^{13}C_9$] were purchased from Wellington Laboratories Inc. (Guelph, Canada). Another internal standard, 4′-methoxy-2,3′,4,5′,6-pentabromodiphenyl ether (4′-MeO-BDE121) was kindly provided by Göran Marsh (Stockholm University, Sweden). Target standards were purchased from AccuStandard Inc. (New Haven, CT, USA) for calibration, recovery, and quantification (Supplementary Table S1). Silica-gel (Wako gel S-1), which was used for purification, was obtained from Wako Pure Industries (Osaka, Japan) and heated at 130 °C for 3 h prior to use. All solvents used were of pesticide-grade quality.

Table 1
Information on dietary homogenate, human breast milk and serum samples in Okinawa, Japan.

Sample	City	M/F	n	Sampling year	Mean age
Diet 2004 (duplicate diet study)	Okinawa	Male	4	2004	32.2 (20–50)
		Female	6		
Diet 2009 (duplicate diet study)	Okinawa	Male	10	2009	36
Breast milk	Okinawa	Female	9	2005–2006	31.4 (26–39)
Serum	Okinawa	Female	10	2006	43.7 (40–47)

2.3. Extraction procedure

The methodology used to analyze phenolic and methoxylated contaminants in samples was based on lipid extraction, gel permeation chromatography (GPC), fractionation, derivatization of phenolic compounds, silica-gel column cleanup, and gas chromatography/mass spectrometry with electron capture negative ionization (GC/MS/ECNI) as previously described (Fujii et al., 2012). Briefly, 10 g dietary homogenate, 10 mL breast milk and 1 mL serum sample were spiked with the four internal standards, PenCP[$^{13}C_6$] and 4-OH-CB187[$^{13}C_{12}$] for phenolic analytes (0.2 ng of each), and α-endosulfan[$^{13}C_9$] (2 ng) and 4′-MeO-BDE121 (0.5 ng) for neutral analytes. The sample was extracted with *n*-hexane, after adding formic acid (0.1% v/v), ethanol, and diethyl ether. A combined extract was dissolved in dichloromethane (DCM):*n*-hexane (1:1), and then subjected to GPC with a Bio-Beads S-X3 column (Bio-Rad Laboratories, Hercules, CA, USA). The gel material (35 g) was packed in a glass column (55 cm × 27 m i.d.) with DCM:*n*-hexane (1:1) as the eluting solvent at a flow rate of 4 mL min^{-1}. The first 96-mL fraction of the eluate contained lipids and was discarded. Subsequently, the next 68-mL fraction was collected. The eluate was concentrated and partitioned between 1 M KOH:ethanol (7:3) and *n*-hexane. After acidification, the phenolic contaminants in the KOH solution were back-extracted twice with 20% diethylether in *n*-hexane. The phenolic fraction was derivatized to O-methylated analogs by diazomethane in diethylether. The residues in both neutral and methylated phenolic fractions were purified with a silica-gel column (0.2 g Wako gel S-1) by elution with 15 mL of DCM:*n*-hexane (12:88, v/v). Each fraction was concentrated to 200 μL prior to GC/MS analysis.

2.4. Instruments and quantification

Twelve analytes were measured by GC/MS/ECNI using an Agilent GC/MSD 5973i (Agilent Technologies, Santa Clara, CA, USA) coupled to a 6890N gas chromatograph. The GC/MS conditions and target ions for determination of analytes are summarized in Supplementary Table S1. Quantification of the compounds was based on signals in the mass chromatograms and in comparison with CB-153[$^{13}C_{12}$], which was used as a syringe spike. The concentrations of chemicals are reported as picogram per gram (pg g^{-1} wet weight for serum, pg g^{-1} lipid weight for breast milk).

2.5. Quality control and quality assurance

The extraction, cleanup, and fractionation steps were evaluated by the measurement of the absolute recoveries of the compounds (^{13}C-labeled internal and native surrogate standards) that were spiked and passed through the entire analytical procedure. Procedural blanks were analyzed simultaneously with every batch of 10 samples to evaluate for interference or contamination from solvents and glassware. For recovery tests, two levels (2.0 and 10.0 ng g^{-1}) of the 11 analytes were spiked into cow milk and determined based on GC/MS-selected ion monitoring (GC/MS-SIM). The recoveries were between 87 and 99% with a relative standard deviation of <10% (n = 5). The limits of quantification (LOQ), defined as 10-fold that of the noise, ranged from 1 to 200 pg g^{-1} (Supplementary Table S1). When the levels of the target chemicals were less than their LOQs, we allocated half of the LOQ as the value for analysis. The calibration (0.1 to 5 ng mL^{-1} of each analyte) was linear and characterized by good correlation coefficients (>0.99) for all of the studied compounds. The quality of the method under validation was verified by Standard Reference Materials (non-fortified human serum, SRM1974, NIST) for PCBs and selected pesticides. Data from the current study were within 12% of the certified values of SRM1974.

2.6. Statistical analysis

The data were analyzed using SPSS version 18.0 for Windows 2007 (SPSS Inc., Chicago, IL, USA). Spearman's rank correlation coefficients were used to test the relationship among concentrations of analytes. Probability values of less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Duplicate diet study

Daily intakes (ng day^{-1}) of neutral, phenolic and methoxylated OHCs in the duplicate diet studies from Okinawa in 2004 and 2009 are shown in Table 2. For the analyzed neutral OHCs, 4,4′-DDE was the predominant contaminant in both sampling years, followed by α-endosulfan, and then hexachlorobenzene (HCB). Some of the dietary homogenates were contaminated with higher levels of BDE-99 than BDE-47 in 30% of samples. The maximum intake of BDE-99 was estimated to be 914 ng day^{-1} in the 2009 study, although the median intake of BDE-47 exceeded that of BDE-99 in both sampling years. The dietary exposure to BDE-99 in most cases could originate from commercial penta-BDE products that have been previously used as a BFR in Japan until 1999, whereas the unusual high levels of BDE-99 may be from another source, i.e. indoor environment.

For the phenolic OHCs, we detected PenCP and TriBP in all analyzed samples, whereas TBBPA, 4-OH-CB187 and 6-OH-BDE47 were detected at frequencies of 80, 20 and 35%, respectively, from the dietary samples. Of the methoxylated OHCs, PenCA and TriBA were found in all samples, whereas 6-MeO-BDE47 was present at a 50% frequency. Methoxy-CBs and dimethoxylated TBBPA were not detected in any of the dietary homogenates.

The mean dietary intake of phenolic OHCs in the 2004 diet study was higher in the order of TriBP > TBBPA > PenCP > 6-OH-BDE47 > 4-OH-CB187, whereas those in the 2009 was higher in the order of TriBP > 6-OH-BDE47 > TBBPA > PenCP >4-OH-CB187 (Table 2). For the methoxylated analogs, the dietary intake in the 2004 study was higher in the order of TriBA > 6-MeO-BDE47 >PenCA, whereas those in the 2009 was higher in the order of TriBA > PenCA > 6-MeO-BDE47. The ratios of methoxylated and phenolic OHCs were estimated to be 0.15 for TriBA/TriBP, 0.33 for PenCA/PenCP, and 0.69 for 6-MeO-BDE47/6-OH-BDE47 in the 2004 diet study (Table 2).

The estimated daily intakes (EDIs; ng kg body $weight^{-1}$ day^{-1}) of neutral and phenolic OHCs via diet for adults are shown in Supplementary Table S2. The average EDIs of organohalogens for adults were lower by at least a factor of 1000 than the intake guidelines established by World Health Organization (van Oostdam et al., 1999).

The correlations in concentrations among selected contaminants are shown in Supplementary Tables S3 and S4. Lipids (%) in the diet were independent of the contaminant levels. For phenolic OHCs, TriBP was positively associated with TBBPA ($r = 0.809$, $p < 0.01$) and α-endosulfan ($r = 0.619$, $p < 0.01$) but not with the other contaminants. TBBPA was associated with PenCA ($r = 0.531$, $p < 0.01$), but not PenCP ($p > 0.05$). 6-Meo-BDE47 was not associated with 6-OH-BDE47 and the other contaminants. For neutral OHCs, BDE-47 were significantly associated with BDE-99 ($r = 0.988$, $p < 0.01$) but not with the other neutral OHCs.

3.2. Breast milk

The concentrations of neutral, phenolic and methoxylated OHCs in breast milk from Okinawa in 2005 are shown in Table 3. The concentrations of neutral contaminants were in the order of 4,4′-DDE > HCB > α-endosulfan. The concentrations of phenolic OHCs were present in the order of TriBP (mean, 1167 pg g^{-1} lipid) > TBBPA (1035 pg g^{-1} lipid) > PenCP (577 pg g^{-1} lipid) > 4-OH-CB187 (29 pg g^{-1} lipid), whereas 6-OH-BDE47 was below the LOQ in all samples. The methoxylated analogs were present in the order of TriBA (445 pg g^{-1} lipid) > 6-MeO-BDE47 (249 pg g^{-1} lipid) > PenCA (37 pg g^{-1} lipid). The ratios of TriBA/TriBP and PenCA/PenCP in breast milk were 0.38, and 0.06, respectively (Table 3). The EDIs of the analytes via human milk for nursing infants

Table 2
Daily intake of contaminants (ng day^{-1}) in the Okinawa area of Japan.

	Freq	2004 (N = 10)			2009 (N = 10)		
	(%)	Median	Mean ± SD	Range	Median	Mean ± SD	Range
Age		32.0	32.2 + 8.7	20–50	36.0	36.0 ± 0.0	36.0–36.0
Total homogenate (g)		2340	2360 ± 230	2060–2850	1690	1700 ± 403	1075–2452
Fat (%)		2.6	3.0 ± 1.5	1.2–5.6	3.7	4.0 ± 2.4	0.7–9.3
Halogenated compounds							
4,4′-DDE	100	165	168 ± 69	48–303	82	132 ± 134	30–457
HCB	100	22.1	26.2 ± 10.1	18.0–44.7	19.4	27.9 ± 23.3	9.2–86.6
trans-Nonachlor	100	1.2	1.2 ± 0.6	0.3–2.4	1.6	2.7 ± 2.8	0.6–9.4
α-Endosulfan	100	36.3	45.0 ± 26.1	28.8–118	28.2	30.3 ± 12.5	14.6–53.5
CB-153	100	19.3	20.0 ± 9.6	7.1–32.9	12.8	27.4 ± 30.5	5.0–91.6
CB-187	100	2.0	2.6 ± 1.5	1.2–4.7	1.9	5.2 ± 7.1	0.2–21.1
BDE-47	85	7.47	7.51 ± 3.25	1.04–12.1	2.47	16.8 ± 40.3	<LOQ–131
BDE-99	30	0.14	5.9 ± 10.6	<LOQ–30.4	0.09	93.2 ± 288	<LOQ–914
BDE-153	10	0.12	0.71 ± 1.88	<LOQ–6.07	0.09	3.95 ± 12.2	<LOQ–38.7
Phenolic OHCs							
PenCP	100	5.2	7.0 ± 5.0	2.3–12.4	1.8	2.2 ± 1.8	0.2–5.6
4-OH-CB187	20	0.06	0.07 ± 0.03	<LOQ–0.14	0.05	0.40 ± 0.72	<LOQ–2.26
TriBP	100	165	181 ± 83	60–373	48	52 ± 26	13–110
TBBPA	80	10.6	15.6 ± 12.3	3.9–40.2	2.7	3.0 ± 2.8	<LOQ–7.5
6-OH-BDE47	35	0.12	0.12 ± 0.01	<LOQ–0.14	1.83	11.1 ± 22.5	<LOQ–72.6
Methoxylated OHCs							
PenCA	100	1.4	1.5 ± 0.88	0.45–3.7	0.77	1.1 ± 0.71	0.43–2.4
4-MeO-CB187	0	<LOQ	<LOQ		<LOQ	<LOQ	
TriBA	100	5.1	7.3 ± 6.8	1.8–22.8	20.3	21.5 ± 14.7	3.6–44.9
diMeO-TBBPA	0	<LOQ	<LOQ		<LOQ	<LOQ	
6-MeO-BDE47	50	0.12	1.0 ± 2.4	<LOQ–7.6	7.08	9.48 ± 11.1	<LOQ–35.1
Ratio							
PenCA/PenCP		0.27	0.27		0.43	0.50	
TriBA/TriBP		0.03	0.04		0.42	0.41	

Daily intake was calculated as total content (ng) in 24-h diet concentrations of analytes (ng g^{-1} wet) × total content of homogenate (g). Concentrations below the LOQ were treated as 1/2 LOQ for the mean and median values.

Table 3
Concentrations of organohalogen contaminants in human breast milk and serum in Okinawa.

Analytes	Breast milk, ng g^{-1} lipid (n = 9)				Serum, pg g^{-1} wet weight (n = 10)			
	n[a]	Median	Mean ± SD	Range	n[a]	Median	Mean ± SD	Range
Halogenated compounds								
4,4′-DDE	9	76.1	91.1 ± 51.1	30.9–177	10	1850	1950 ± 1490	250–5250
HCB	9	7.15	7.0 ± 3.2	2.77–12.6	10	134	136 ± 47	59–214
trans-Nonachlor	9	52.4	66.9 ± 35.9	29.3–144	10	83	110 ± 71	27–254
α-Endosulfan	9	1.03	1.11 ± 0.56	0.35–1.95	10	133	157 ± 83	96–373
CB-153	9	16.0	13.9 ± 6.35	4.19–20.8	10	235	215 ± 66	64–286
CB-180	9	9.53	7.78 ± 4.20	2.30–13.9	10	133	123 ± 45	23–180
CB-187	9	4.44	3.88 ± 1.96	1.02–6.29	10	38	35 ± 14	9–56
CB-183	9	1.00	0.83 ± 0.35	0.23–1.20	10	9	10 ± 6.9	4–27
BDE-47	9	0.57	0.87 ± 0.74	0.093–1.99	5	1	1.5 ± 0.4	<LOQ–3
BDE-99	8	0.33	0.54 ± 0.51	<LOQ–1.13	0	<LOQ	<LOQ	
BDE-153	9	0.40	0.38 ± 0.18	0.13–0.65	0	<LOQ	<LOQ	
Phenolic OHCs								
PenCP	9	0.63	0.58 ± 0.37	0.087–1.09	10	143	216 ± 166	87–633
4-OH-CB187	2	<LOQ	0.029 ± 0.59	<LOQ–0.16	10	42	45.3 ± 21.3	21–79
TriBP	9	1.06	1.17 ± 0.75	0.13–2.73	10	26	40.2 ± 30.1	18–100
TBBPA	9	0.72	1.04 ± 0.65	0.39–2.22	3	1.0	40.5 ± 78.0	<LOQ–238
6-OH-BDE47	0	<LOQ	<LOQ		9	90	172 ± 184	<LOQ–542
Methoxylated OHCs								
PenCA	9	0.033	0.037 ± 0.018	0.007–0.068	10	2.5	5.4 ± 8.6	1–29
4-MeO-CB187	0	<LOQ	<LOQ		0	<LOQ	<LOQ	
TriBA	9	0.39	0.45 ± 0.25	0.028–0.85	10	10.5	22.1 ± 28.1	4–87
diMeO-TBBPA	0	<LOQ	<LOQ		0	<LOQ	<LOQ	
6-MeO-BDE47	8	0.19	0.25 ± 0.19	<LOQ–0.53	0	<LOQ	<LOQ	
Ratios								
PenCA/PenCP		0.05	0.06			0.02	0.02	
TriBA/TriBP		0.37	0.38			0.40	0.55	

Concentrations below the LOQ were treated as 1/2 LOQ for the mean and median values.
[a] Number detected.

were calculated, based on 5-kg infants and 750 mL of daily dietary milk (Supplementary Table S2). The average EDIs via milk of all analytes were less than one-tenth of the acceptable daily intake (ADI) and provisional tolerable daily intake (PTDI) established by WHO (van Oostdam et al., 1999), except for *trans*-nonachlor, which was a relatively high concentration (mean 52% of ADI).

3.3. Serum

Serum concentrations of neutral, phenolic and methoxylated OHCs in Okinawan females are shown in Table 3. The neutral contaminants were present in the order of 4,4′-DDE > CB-153 > HCB > α-endosulfan. The phenolic OHCs in serum were quantified in the order of PenCP (216 pg g^{-1} wet weight) > 6-OH-BDE47 (152 pg g^{-1} wet weight) > 4-OH-CB187 (45 pg g^{-1} wet weight) > TriBP (41 pg g^{-1} wet weight) > TBBPA (40 pg g^{-1} wet weight). The methoxylated analogs were detected at the levels of 22 pg g^{-1} wet weight for TriBA and of 5.4 pg g^{-1} wet weight for PenCA, whereas the levels of 6-MeO-BDE47 were below the LOQ in all samples. 4-MeO-CB187 and TBBPA dimethyl ether were not detected in any of the samples. The ratios of TriBA/TriBP and PenCA/PenCP in serum were 0.55 and 0.02, respectively (Table 3).

3.4. Association between diet and serum/milk

Dietary intake of phenolic and methoxylated OHCs are illustrated in Fig. 1. Their profiles and concentrations in serum and breast milk are collectively illustrated in Fig. 2. Dietary PenCP and PenCA were present at a ratio of 0.03 and 0.27, respectively. The concentration ratios of TriBP and TBBPA between breast milk and serum were higher than those of the other phenolic OHCs. 6-OH- and 6-MeO-BDE47 were at similar levels (1:1 and 1:4 ratios in the dietary homogenates), respectively, whereas 6-MeO- and 6-OH-BDE47 were selectively partitioned between breast milk and serum, respectively (Table 2, Fig. 2).

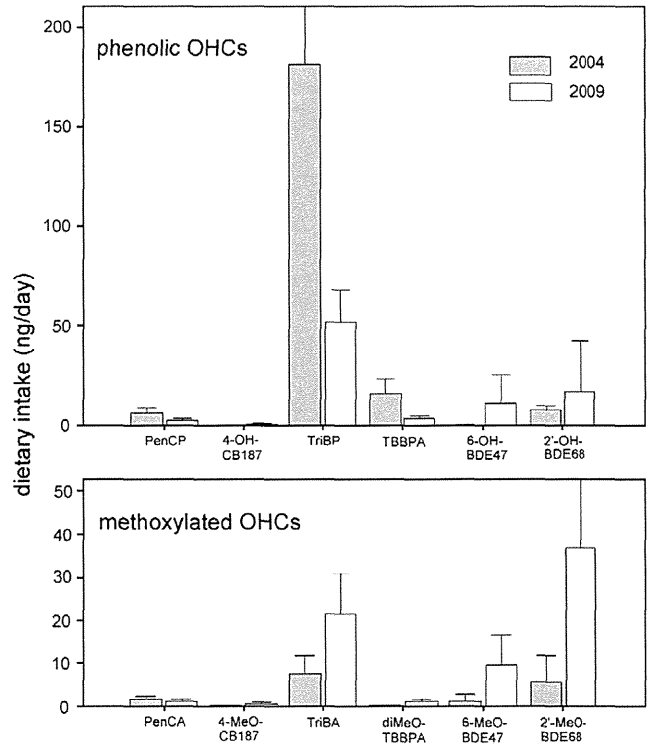

Fig. 1. Daily intake of phenolic and methoxylated organohalogens investigated in a duplicate diet study. PenCP = pentachlorophenol; PenCA = pentachloroanisole; TriBP = 2,4,6-tribromophenol; TriBA = 2,4,6-tribromoanisole; TBBPA = tetrabromobisphenol A; diMeO-TBBPA = TBBPA-dimethylether.

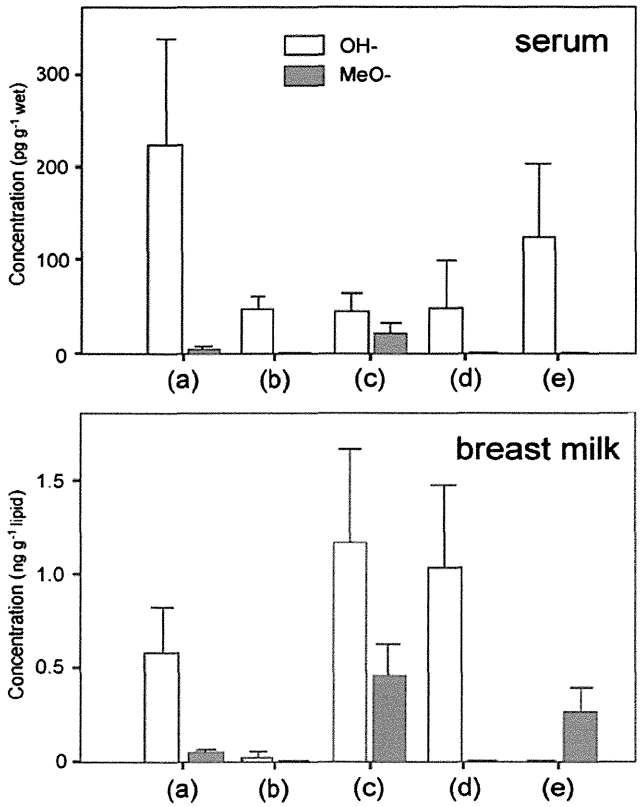

Fig. 2. Comparison of concentrations of phenolic and methoxylated organohalogens between human breast milk and serum in Okinawa. (a) = PenCP and PenCA; (b) = 4-OH-CB187 and 4-MeO-CB187; (c) = TriBP and TriBA; (d) = TBBPA and diMeO-TBBPA; (e) = 6-OH-BDE47 and 6-MeO-BDE47.

4. Discussion

4.1. PenCP, PenCA, and OH-CB

PenCP has been used as an herbicide in agricultural chemicals and preservatives in Japan until 1990 (Suzuki et al., 2008). The present data demonstrate that the dietary intake of PenCP was low compared with other OHCs. The reduced dietary exposure to PenCP may result in the lower levels in breast milk. In fact, the current levels (577 pg g^{-1} lipid) are much lower than those in a Chinese study (Hong et al., 2005). In contrast, the serum concentrations of PenCP ranged from 87 to 633 pg g^{-1} wet weight (mean 216 pg g^{-1} wet weight), as high as CB-153. Selective retention of PenCP in serum is most likely because of the specific binding potency to human TTR (Suzuki et al., 2008). The current values are still lower than those in European countries (Glynn et al., 2011; Guvenius et al., 2003; Meijer et al., 2008; Rylander et al., 2012). The primary source is considered to be dietary because PenCP has been reported in fishery products from China (Ge et al., 2007). Indoor air has been also reported as a possible source of PenCP in Japan (Suzuki et al., 2008), but the intake rates from both exposure routes are unknown.

PenCA, in contrast, has been reported as a microbiological transformation product from PenCP in marine fish, shellfish, and sediments in Japan (Watanabe et al., 1983a). This compound is expected to be more persistent than PenCP and has been found in rainbow trout (Glickman et al., 1977). However, the PenCA/PenCP ratios (0.27 and 0.50) in the dietary homogenate resulted in lower ratios in serum (0.02) and breast milk (0.06). This may be explained by the exposure to PenCP from other sources e.g., indoor environment (Harrad et al., 2010), which may have led to the increased ratios in the body tissues.

Hydroxylated PCBs (OH-CBs) are formed by cytochrome P450-mediated oxidation in hepatic microsomes after dietary ingestion of PCBs (Rylander et al., 2012). In this study, we selected 4-OH-CB187 to monitor the contamination status of phenolic PCBs in diet, serum and milk because this metabolite has been commonly observed at a high level in humans (Sandau et al., 2002). 4-OH-CB187 was detected in 20% of samples, at a 0.03:1 ratio of 4-OH-CB187:CB-187 in the diet, which was not negligible. 4-OH-CB187 was retained in blood at the second highest level. This is because of the binding affinity of phenolic OHCs to human TTR (Lans et al., 1993), a property which is in common with PenCP. The current serum concentrations of 4-OH-CB187 (45 pg g^{-1} wet weight) may reflect the contamination status of phenolic PCBs in humans because these levels are comparable to those of Japanese women (Kawashiro et al., 2008; Nomiyama et al., 2010). The serum levels of 4-OH-CB187 were well correlated with those of CB-187 ($r = 0.774$, $p < 0.01$, Supplementary Table S3), indicating that the variation of 4-OH-CB187 in serum depends on dietary exposure to parent PCBs and the individual metabolic capacity for PCB.

4.2. TriBP, TriBA, and TBBPA

Dietary intake of TriBP varied between 2004 and 2009. The large variation suggests diverse exposure routes of TriBP and food preference (i.e. deviated habitual consumption of organic foods or seafood). Dietary TriBP would be most likely derived from marine biota, i.e., ocean fish (Whitfield et al., 1999) and marine algae via the food chain (Haraguchi et al., 2010). The release of TriBP from BFR may be another source (Watanabe and Sakai, 2003). In the body, TriBP was more abundant in breast milk compared with serum. This is in contrast to PenCP and 4-OH-CB187, which demonstrated selective retention in blood. The serum levels of TriBP in Okinawa were similar to those from Norway (Thomsen et al., 2002) and India (Eguchi et al., 2012), although these are much higher than those detected in pregnant women in Japan (Kawashiro et al., 2008). Therefore, the thyroid-disrupting effects for populations exposed to TriBP should be addressed in future studies.

TriBA is a known endproduct of microbial O-methylation of TriBP (Allard et al., 1987; Whitfield et al., 1997) and is a major cause of mustiness in food (Whitfield et al., 1997). It has been detected in wine (Giannikopoulos and Whitfield, 2009) and in seafood from Japan at similar levels to PenCP (Watanabe et al., 1983a). In the present study, although the ratio of TriBA/TriBP in the diet was largely different between the 2004 and 2009 diet studies, the ratios (0.42) in 2009 were comparable to those in seafood in 1983 (Watanabe et al., 1983a). Because of the higher lipophilicity of TriBA, higher ratios were expected in the body but were not found to be elevated in either breast milk or serum (0.37–0.40). This may support the hypothesis that TriBA is more lipophilic than TriBP and can transfer to breast milk and that dietary intake is a major contributor of human exposure to TriBA.

The production of TBBPA has been reported to be 31,000 tons in 1999 in Japan, and it has been widely distributed in the environment via many flame retarded consumer products (Watanabe and Sakai, 2003). The present survey demonstrated that the possible source for human exposure to TBBPA is diet. The EDI of TBBPA via diet for adults (50 kg body weight) was calculated as a maximum of 804 pg kg^{-1} body weight day^{-1}, which was 4 orders of magnitude lower than the PTDI values (1 mg kg^{-1} body weight day^{-1}) established by The UK Committee on Toxicity (COT, 2004). The present EDIs of TBBPA (mean, 185 pg kg^{-1} body weight day^{-1}) were lower than those in the total diet study in China in 2009, ranging from 232 to 280 pg kg^{-1} body weight day^{-1} (Shi et al., 2009). However, they are consistent with the EDIs of people from Belgium (Dirtu et al., 2008), France (Cariou et al., 2008) and Norway (Thomsen et al., 2002). The levels of TBBPA (390–2220 pg g^{-1} lipid) in human breast milk were lower compared with those in China (5100 pg g^{-1} lipid) (Shi et al., 2009). The EDIs of TBBPA may predict its concentration in breast milk and serum. In contrast, the milk/serum partition ratio was

relatively higher in TriBP and TBBPA compared with other phenolic OHCs (Table 3), indicating these phenolic BFRs are preferentially transferred from blood to breast milk.

The Asian population is exposed to several BFRs, most likely with food as a major source (Shi et al., 2009). However, higher plasma concentrations of TBBPA have been reported in workers at a dismantling plant (Thomsen et al., 2001), indicating an occupational exposure to TBBPA for these individuals via ingestion of indoor dust. The dietary intakes of TBBPA were significantly associated with those of TriBP but not to the other phenolic OHCs, implying a similar exposure source between TBBPA and TriBP. Because TBBPA has a short half-life (two days) in human plasma (Hagmar et al., 2000), the occurrence of TBBPA in human serum more likely reflects recent exposure rather than past exposure (Covaci et al., 2009; Sjödin et al., 2003).

TBBPA can also be methylated by bacteria in the environment to form mono- or dimethylether derivatives (George and Häggblom, 2008). Because of its lipophilicity, we expected the occurrence of TBBPA dimethylether, but failed to detect it in food, breast milk, and serum. Further research using human serum samples from a larger number of individuals is necessary for a more complete assessment of the exposure kinetics of TBBPA.

4.3. 6-OH-BDE and 6-MeO-BDE

The present study revealed that dietary homogenates were contaminated with both OH- and MeO-BDEs at similar ratios (1:1 to 1:3). The mean serum concentration of 6-OH-BDE47 (172 pg g^{-1} wet weight) was higher than that of 4-OH-CB187, whereas 6-MeO-BDE47 was below the LOQ. In contrast, 6-MeO-BDE47 was more abundant in breast milk, but the hydroxylated analogs were not detected in the serum samples. Therefore, OH-BDEs and MeO-BDEs seem to be differentially partitioned between serum and breast milk, respectively. A possible explanation is that after dietary exposure, OH-BDEs are retained in blood because of an ability to bind to human TTR, whereas MeO-BDEs were preferentially transferred to breast milk because of their lipophilicity. The ortho-substituted OH-BDEs, such as 6-OH-BDE47, are considered to be of natural origin rather than metabolites of PBDEs (Haraguchi et al., 2010). The major source of 6-OH-BDE47 in human exposure is likely seafood from the Japanese coastal water or east Asian islands (Fujii et al., 2012; Zhang et al., 2012) because it has been distributed in marine sponges or algae in the Asia-Pacific region (Haraguchi et al., 2011), and in blood of fish (Marsh et al., 2004). The present serum levels are high compared with those from Chinese and Indian studies (Eguchi et al., 2012; Wang et al., 2012). OH-BDEs may be formed in part by demethylation in hepatic microsomes after exposure of MeO-BDEs (Wan et al., 2009). The toxicological implication of human exposure to OH-BDEs remains unknown, but one study observed a reduction of thyroid hormone levels in vivo (Malmberg et al., 2005).

5. Conclusions

The present study revealed that the Okinawan diet is contaminated with toxic persistent phenolic OHCs and suggests a positive relationship between dietary exposure to both phenolic and methoxylated OHCs via diet, which are positively related to the corresponding methoxylated OHCs making up the body burden of OHCs. The available data indicate that dietary exposure to phenolic OHCs leads to the selective retention of PenCP, 4-OH-CB187 and 6-OH-BDE47 in serum, whereas methylated derivatives transfer to breast milk. In particular, 6-OH-BDE47 and 6-MeO-BDE47 were differentially partitioned between serum and breast milk, respectively. These patterns may be due to the different kinetics between hydroxylated and methoxylated PBDEs. To evaluate the potential for thyroid-disrupting effects caused by phenolic OHCs, continuous monitoring of human exposure to phenolic OHCs should be performed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2013.10.016.

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