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Residuals of beta-hexachlorocyclohexane, dichlorodiphenyltrichloroethane, and hexachlorobenzene in serum, and relations with consumption of dietary components in rural residents in Japan

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Abstract

To estimate levels of organochlorine residuals in the Japanese population and the contribution of dietary factors to these levels, we determined serum levels of β-hexachlorocyclohexane (β-HCH), hexachlorobenzene (HCB), *p,p'*-dichlorodiphenyldichloroethane (p,p'-DDD), 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (p,p'-DDE) and *p,p'*-dichlorodiphenyltrichloroethane (p,p'-DDT) in 41 volunteers (14 men and 27 women) in a rural area of Northern Japan. These organochlorine levels were measured using gas-chromatography mass-spectrometry. By a self-administered dietary history questionnaire, the usual dietary intake was estimated. Their median levels (range) were as follows: β-HCH, 0.50 (0.05–1.50); HCB, 0.20 (0.02–0.70); and total DDT (p,p'-DDE + p,p'-DDT), 5.0 (0.9–31.0) ng/ml serum. Levels of p,p'-DDD were detected in only seven subjects (0.05–0.6 ng/ml serum). The β-HCH levels were increased with rice and milk intakes, but the least squares means were not simply increased according to the quartile of the intakes. Concerning HCB, fish intake showed a borderline significant correlation (0.20, $P = 0.052$). In terms of total DDT, intakes of meat, fish, vegetable and milk showed a positive relationship, although none of them

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provided statistically significant results. No other statistically significant relation between any organochlorines and any food intakes examined was observed in this study. The present study suggests that organochlorine compounds are transported into the human body via foods in the Japanese population. Their effects on health should thus be investigated and monitored. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Organochlorine; Beta-hexachlorocyclohexane; *p,p'*-Dichlorodiphenyldichloroethane; 1,1-Dichloro-2,2-bis(*p*-chlorophenyl) ethylene; *p,p'*-Dichlorodiphenyltrichloroethane; Hexachlorobenzene; Diet

1. Introduction

Organochlorine compounds released into the environment enter living organisms and become biologically concentrated. They are ultimately transported into the human body by consumption of ordinary foods and accumulate in fatty tissue. Their carcinogenic (Ahlborg et al., 1995; Dich et al., 1997; Jaga and Brosius, 1999; von Muhlendahl, 1999) and endocrine disrupting (Guillette, 2000; Johnson et al., 2000; Tyler et al., 1998) effects have been discussed, but no conclusion has been made as to their effects on human health. In Japan, the use of organochlorine pesticides, including β-hexachlorocyclohexane (β-HCH), dichlorodiphenyltrichloroethane (DDT) and hexachlorobenzene (HCB) has been prohibited for approximately 30 years, but their residuals are still detected in the blood. A recent report estimated dairy dietary intakes of these compounds, which had been decreasing, recently showed stable levels in an urban area (Kuwahara et al., 1997). Other investigators also noted that, in a rural area, HCH and DDT in serum has remained at similar levels during the last decade (Sasaki and Hayashi, 1999). Some previous studies in Western nations have shown organochlorine compounds to be related or unrelated to breast cancer (Wolff et al., 1993, 2000). In Japan, the incidence of breast cancer has been relatively low, although recently it has been increasing. The levels, sources and routes of the contamination in the Japanese population may provide important information to elucidate the etiology of breast cancer.

To estimate residuals of organochlorine pesticides in Japanese, we determined serum levels of β-HCH, *p,p'*-dichlorodiphenyldichloroethane (p,p'-DDD), 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (p,p'-DDE), p,p'-DDT and HCB in volunteers living in a rural area of Northern Japan. To examine the contribution of dietary factors to their levels, we also obtained detailed dietary information.

2. Subjects and methods

2.1. Subjects and sample collection

This study was conducted in 1999 in a farming village in Akita Prefecture, located in Northern Japan.

Forty-one volunteers participated in this study after giving their written informed consent. They were parts of a voluntary cohort (approx. 500 persons) of a dietary intervention study conducted earlier in this area. They consisted of 14 men and 27 women, aged 45–68 (mean: 60) and 41–69 (mean: 60), respectively. Their occupation was mainly farming, and most of the women were housewives. The distribution of their characteristics including Body Mass Index (BMI, kg/m^2) and serum levels of total cholesterol is shown in Table 1.

Blood sampling for all subjects was performed between 17 June and 7 July, 1999. Serum was immediately collected by centrifugation, transferred into a stock tube and stored at −80°C until analyzed.

2.2. Assessment of dietary intakes

We used a validated self-administered diet history questionnaire (DHQ) to obtain usual dietary intakes and anthropometric parameters (body

Table 1
Distribution of subject characteristics and dietary intakes in Japanese rural residents (14 men and 26 women)

Items	Mean (S.E.)	Minimum	Median	Maximum
Age	60 (1)	41	63	69
Body Mass Index (kg/m^2)	23.7 (0.5)	17.3	23.7	29.8
Serum total cholesterol (mg/dl)	212 (5)	155	213	275
Total energy intake (kcal)[a]	1969 (101)	1029	1919	3240
Meats (g/day)[a] (beef, pork, chicken)	37.8 (4.9)	1.8	28.2	122.4
Fish (g/day)[a] (including shellfish)	90.2 (9.9)	19.4	76.2	284.8
Vegetables (g/day)[a]	249.4 (22.8)	15.5	216.8	666.4
Fruits (g/day)[a]	78.4 (9.9)	1.7	58.5	268.3
Rice (g/day)[a]	443.5 (32.7)	140.0	410.0	1131.4
Green tea (g/day)[a]	275.6 (42.6)	0.0	150.0	1080.0
Milk (g/day)[a] (full fat + low fat)	105.1 (13.6)	0.0	104.5	337.5

[a]One woman whose total energy intake was extremely low (581 kcal) was considered to be an outlier and deleted from these calculations.

height and weight). The structure of DHQ has been described in detail elsewhere (Sasaki et al., 1998, 2000). Briefly, it asked about the dietary habits of the previous month and consisted of seven sections and 20 pages. For the computations of nutrient and food intakes, the associated ad-hoc calculation algorithm has been used after a small modification for some local foods in this area. The distribution of total energy and food intakes is also included in Table 1. One woman whose total energy intake was extremely low (581 kcal/day) was considered to be an outlier and deleted from the calculation of dietary information. Men consumed more rice than women. In other food intakes, we observed differences by sex, but they were not statistically significant.

2.3. Analytical methods

The organochlorine residues we measured in serum were β-HCH, HCB p,p′-DDD, p,p′-DDE and p,p′-DDT. Extraction of these compounds from 1-ml serum samples was according to the method of Gill et al. (1996).

Quantitative analysis of the organochlorines was undertaken on a gas-chromatography mass-spectrometry (GC-MS) (QP1000EX, Shimadzu Co., Kyoto, Japan). The GC operating conditions were as follows: methyl silicon capillary column (DB-1, 30 m × 0.25 mm i.d. × 0.25 μm, J&W Scientific, USA); carrier gas, helium with a flow rate of 2 ml/min; splitless injection; inlet temperature, 280°C; column oven temperature program, 70°C (15 min)–15°C/min–280°C. The MS operating conditions were as follows: ionization method, electron impact; ionization voltage, 70 eV; selected ion mode (β-HCH, m/z 181; HCB, m/z 284; p,p′-DDD, m/z 235; p,p′-DDE, m/z 246; p,p′-DDT, m/z 235).

The detection limits were as follows: HCH, 0.020 ng/ml; HCB, 0.010 ng/ml; p,p′-DDD, 0.016 ng/ml; p,p′-DDE, 0.012 ng/ml; p,p′-DDT, 0.012 ng/ml.

2.4. Statistical analysis

Because the distribution of serum organochlorine levels was not normal, the values were $\log_{10}$-transformed to improve the normality. Differences in the organochlorine levels between two groups or more were tested by Student's t-test or the analysis of variance. To estimate the relation

between the organochlorine levels and dietary intakes, the food intakes were also log_{10}-transformed. Because total energy intake was significantly correlated with each food intake, an adjustment of total energy intake was performed in multivariate analysis (PROC GLM and PROC REG, SAS, SAS Institute, Inc., Cary, NC). We presented least squares means of the organochlorines according to quartiles of food intakes, P values for differences among the quartile categories and partial correlation coefficients adjusted by total energy intake. A P value less than 0.05 (two-tail) was considered to be statistically significant.

3. Results

Residuals of β-HCH, HCB and p,p′-DDE in serum were detected in all subjects. One woman showed a level below the p,p′-DDT detection limit. For statistical analysis, her level was taken to be half the detection limit. p,p′-DDD was only detected in seven subjects. The maximum p,p′-DDD level detected in serum was 0.6 ng/ml.

Table 2 summarizes the statistics of serum levels of β-HCH, HCB, and total DDT (p,p′-DDE + p,p′-DDT). The distribution tended not to be normal and was skewed.

Table 3 shows the organochlorine levels according to the subject characteristics and their total energy intake. In β-HCH and total DDT, men showed higher average levels compared with women, although the differences were not statistically significant. In β-HCH, the mean levels were increased according to the tertiles of age, but again the differences were not statistically significant. Only β-HCH showed a significant difference between the tertiles of the total cholesterol levels; however, the medium class was highest.

Table 4 shows the relation between serum organochlorine levels and food intake. The β-HCH levels were increased with rice and milk intake, but the least squares means were not simply increased according to the quartile of the intakes. Concerning HCB, fish intake showed a borderline significant coefficient (0.20, $P = 0.052$), and the least squares means according to the quartile of the intake also showed a positive, but not significant trend. In total DDT, intake of meat, fish, vegetable and milk exhibited a positive relation, but there were no statistically significant results. No significant relation between any organochlorine levels and consumption of any other food intakes was observed in this study.

4. Discussion

This study has shown serum levels of major

Table 2
Distributions of serum organochlorine levels in Japanese rural residents (14 men and 27 women, aged 41–69)

Organochlorines (ng/ml)		Mean (S.E.)	Minimum	Median	Maximum
β-HCH	Men	0.56 (0.06)	0.20	0.60	1.00
	Women	0.53 (0.07)	0.05	0.50	1.50
	Total	0.54 (0.05)	0.05	0.50	1.50
HCB	Men	0.28 (0.07)	0.03	0.15	0.70
	Women	0.22 (0.03)	0.02	0.20	0.50
	Total	0.24 (0.03)	0.02	0.20	0.70
p,p′-DDE + p,p′-DDT[a]	Men	7.6 (1.9)	1.5	5.9	31.0
	Women	5.6 (0.7)	0.9	5.0	17.3
	Total	6.3 (0.8)	0.9	5.0	31.0

[a]The p,p′-DDT level in one woman was below the detection limit, and her level was taken to half the detection limit (0.006 ng/ml).

Table 3
Geometric means of serum organochlorine levels according to subject characteristics in Japanese rural residents ($n = 41$)

Items, categories (n)		β-HCH		HCB		p,p′-DDE + p,p′-DDT	
		Geometric mean	P value for difference	Geometric mean	P value for difference	Geometric mean	P value for difference
Sex							
	Men (14)	0.50		0.16		5.8	
	Women (27)	0.41	0.40	0.16	0.99	4.7	0.34
Age							
	41–57 (13)	0.36		0.18		4.4	
	58–63 (13)	0.38	0.12	0.21	0.25	6.8	0.12
	64–69 (15)	0.60		0.12		4.4	
Body Mass Index (kg/m²)							
	17.3–22.0 (12)	0.33		0.19		5.7	
	22.2–24.8 (14)	0.43	0.20	0.13	0.63	5.7	0.32
	24.9–29.8 (15)	0.55		0.18		4.1	
Serum total cholesterol (mg/dl)							
	155–202 (13)	0.50		0.20		5.6	
	207–219 (12)	0.35	0.42	0.13	0.51	4.6	0.76
	219–275 (13)	0.46		0.17		5.4	
Total energy intake (kcal)[a]							
	1029–1694 (12)	0.31		0.19		4.2	
	1698–2181 (14)	0.63	0.04	0.13	0.59	4.8	0.50
	2233–3240 (14)	0.39		0.18		5.7	

[a]One woman whose total energy intake was extremely low (581 kcal) was considered to be an outlier and deleted from this calculation.

organochlorine pesticides and the possible contribution of some food intakes to them in Japanese rural residents, although the sample size was relatively small.

We consider that the subjects in this study do not represent the whole Japanese population, because the lifestyle of residents in this area is different to that in urban areas. Here, industry is mainly farming and the residents generally have traditional dietary habits, i.e. high vegetables and low animal meat, hence, there might be special characteristics in organochlorine contamination. However, because dietary habits in this area were relatively stable compared with those in urban areas, they were considered to be appropriate for the estimation of dietary factors which contributed to organochlorine levels. Levels of serum organochlorines do not reflect recent exposure and may indicate long-term exposure and the accumulation of compounds in the body. Thus, unstable dietary habits with rapid change over time and large inter-individual variation in urban areas may not be suitable for this study.

In Japan, the use of HCH, HCB and DDT has been prohibited for approximately 30 years. However, such compounds are considered to accumulate in fatty tissue, including breast and liver. Thus, they might contribute to carcinogenesis in such organs (Ahlborg et al., 1995; Dich et al., 1997; Jaga and Brosius, 1999; von Muhlendahl, 1999). Moreover, these compounds have been reported to act as endocrine disrupting chemicals and may modify the endocrine system and promote hormone-related carcinogenesis in human. Therefore, monitoring such contamination and its source is considered to provide useful information for further epidemiological studies. Polychlorinated biphenyls are also one of the major organochlorines and have similar characteristics, but in this study we could not measure them because of insufficient sample quantity.

In the statistical analysis of the relation between organochlorine levels and food intakes, we adjusted only total energy intake because it was related to intake of all food items except for green tea. The theoretical background of energy

Table 4
Relation between serum organochlorine levels and food intakes in Japanese rural residents (14 men and 26 women, aged 41–69)

	Organochlorine levels[a]					Partial correlation coefficients[a]	
	Least squares means (ng/ml)				*P* value for difference	*R*	*P* value
	Q1	Q2	Q3	Q4[b]			
β-HCH							
Meats	0.35	0.48	0.56	0.35	0.37	−0.21	0.44
Fish	0.54	0.37	0.48	0.34	0.63	−0.23	0.44
Vegetables	0.52	0.56	0.34	0.34	0.29	−0.21	0.45
Fruits	0.59	0.56	0.34.	0.29	0.14	−0.36	0.06
Rice	0.33	0.47	0.29	0.70	0.02	0.18	0.09
Green tea	0.34	0.63	0.45	0.34	0.20	0.03	0.66
Milk	0.32	0.29	0.70	0.48	0.08	0.22	0.03
HCB							
Meats	0.22	0.18	0.10	0.17	0.41	−0.20	0.24
Fish	0.10	0.13	0.14	0.37	0.23	0.20	0.05
Vegetables	0.14	0.13	0.18	0.21	0.75	−0.05	0.88
Fruits	0.14	0.18	0.13	0.21	0.74	0.16	0.17
Rice	0.28	0.14	0.17	0.11	0.19	−0.31	0.06
Green tea	0.15	0.09	0.17	0.28	0.11	0.17	0.30
Milk	0.15	0.17	0.13	0.17	0.97	−0.003	0.87
p,p′-DDE + p,p′-DDT							
Meats	4.5	5.3	4.7	6.1	0.79	0.09	0.38
Fish	4.9	3.1	5.1	8.3	0.05	0.06	0.39
Vegetables	5.6	4.0	4.5	6.5	0.42	0.08	0.41
Fruits	5.4	6.7	4.1	4.6	0.44	0.01	0.71
Rice	6.4	4.2	5.5	4.9	0.52	−0.24	0.16
Green tea	5.8	4.2	6.4	4.0	0.36	−0.21	0.23
Milk	5.1	4.2	9.4	5.9	0.82	0.13	0.40

Values of organochlorine levels and food intakes were log-transformed in these statistical analyses.
[a]Multivariate analysis was used for adjustment of total energy intake.
[b]Q1–Q4: Quartiles of food intakes.

intake has been discussed elsewhere (Willett and Stampfer, 1989). The accumulation of organochlorines was suspected to have increased in relation to the period of their contamination. Previous studies have observed the relation between age and serum organochlorine residuals (Ahlborg et al., 1995; Deutch and Hansen, 2000). Gender was reported to affect organochlorine levels. Lactation is well known to contain organochlorines (Ahlborg et al., 1995) and decreases the residuals in the female body. A previous study reported higher levels of HCB, DDT and DDE in males (Stehr-Green, 1989). The present study also showed higher levels of β-HCH and total DDT in men. BMI correlates closely with body density and skin thickness (Last, 2001), and reportedly showed a weak correlation with organochlorine levels (Deutch and Hansen, 2000), probably because of the fat content in the body. Although smoking status was previously reported to be related with organochlorine levels (Deutch and Hansen, 2000), in the present study the number of smokers was only five, and no significant effect of smoking was found (data not shown). Since we observed no statistically significant effect of such factors, we did not adjust these factors in the statistical analysis of the relation between organochlorines and foods.

Studies originating outside Japan have reported the levels of β-HCH, HCB and DDT in blood (DeVoto et al., 1998; Frank et al., 1988; Leoni et al., 1989; Lommel et al., 1985; Mes, 1992; Murphy and Harvey, 1985; Needham et al., 1990; Stehr-Green, 1989). However, the units used in some studies were different from ours, making an exact comparison difficult. In the United States in the late 1980s (Stehr-Green, 1989), the mean concentrations in serum were reportedly as follows: β-HCH, 0.84 ng/ml; HCB 0.27 ng/ml; and DDE, 9.9 ng/ml. Another US study around the same time reported the median concentrations in serum as follows: β-HCH, 0.54 ng/ml; HCB, 0.19 ng/ml; and DDE, 12.7 ng/ml. On the other hand, in a recent study in Greenland in 1997–1998 (DeVoto et al., 1998), the levels were: β-HCH, 0.15–0.56 ng/ml; HCB, 0.80–1.45 ng/ml; and total DDT, 3.79–11.1 ng/ml. The organochlorine levels observed in our study were similar. These are interesting findings because the incidence of breast cancer in Japan has been much lower than in Western countries (Parkin et al., 1997). However, the relationship between organochlorine contamination and breast cancer is still controversial (von Muhlendahl, 1999; Wolff et al., 2000). Epidemiological studies of breast cancer in the Japanese population using information on organochlorine residuals may well provide useful information to elucidate a possible relationship between organochlorine contamination and breast cancer.

In a recent report from the United States, the serum HCH level was related to the consumption of beef and lamb (DeVoto et al., 1998). The present study was conducted in a rural farming area, and meat consumption was much lower (28.2 g/day in median). Thus, such dietary habits may make it difficult to assess the association between HCH and meat consumption. In the present study, a relationship with rice and milk consumption is suggested. HCH contamination of rice has been reported outside of Japan (Kaphalia et al., 1990), but we have not found any domestic reports of such contamination. A survey of organochlorine pesticide contents in the average daily diet of Japanese suggested that milk and other daily products were major dietary sources (Kuwahara et al., 1997), and recent results from monitoring pesticide residuals in foods showed low but constant HCH contamination in milk (Toyoda and Ikarashi, 1997). Our observation is consistent with these survey results.

HCB is considered to be contained in animal foods including meat, fish and milk (The International Programme on Chemical Safety, 1997). In this study, HCB shows a possible relation with fish intake, in agreement with a previous report in Greenland (Deutch and Hansen, 2000) in which marine food intake showed a correlation coefficient of 0.25 after the adjustment of several confounders. Sala et al. (1999) reported high HCB exposure and its outcome in an occupational setting. The mean serum HCB level was reported to be 36.7 ng/ml, and no increased odds ratios for cancer and reproductive disorders were observed. Judging from this report, the HCB levels observed in the present study would not seem to cause cancer or reproductive disorders. However, HCB has been pointed out to act as a dioxin-like compound, and the effects of low-dose exposure remain unclear.

DDT was the compound first noted to be a biologically accumulated organochlorine. DDE is a major metabolite of DDT, and usually showed higher levels than DDT. In the present study, DDE and DDT were combined for statistical calculation, as described elsewhere (Deutch and Hansen, 2000). DDT accumulates in vegetable products and in animal fat (Jaga and Brosius, 1999). Our observation is consistent with the latter report, but we did not obtain statistically significant results. Because DDE levels were relatively high compared with other organochlorines, we should take into account the residual levels of DDE or total DDT in future epidemiological studies of related cancer. Estrogenic effects of DDT have been reported in experimental animals (Bitman et al., 1978; Fry and Toone, 1981; Morozova et al., 1997; vom Saal et al., 1995). Thus, information on estrogen-related receptor should also be considered with DDT levels in epidemiological studies.

In conclusion, we found that the serum levels of β-HCH, HCB and DDT among Japanese living in a rural area were similar to levels previously

reported in some Western countries. Only one food item showed a statistically significant relationship with the organochlorine level. However, our study suggested organochlorine compounds are transported into the human body via foods. Further study is needed to monitor the contamination by organochlorines and contributing factors.

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ORIGINAL ARTICLE

Urinary bisphenol A and plasma hormone concentrations in male workers exposed to bisphenol A diglycidyl ether and mixed organic solvents

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Aims: To evaluate effects of exposure to bisphenol A diglycidyl ether (BADGE) on urinary excretion of bisphenol A, and plasma gonadotrophic hormones and testosterone in male epoxy resin sprayers.
Methods: Cross sectional study of 42 workers whose job was to spray epoxy resin hardening agents including BADGE and mixed organic solvents, and 42 matched control workers without BADGE use in the same machine plants.
Results: Concentrations of urinary bisphenol A were higher in the epoxy resin sprayers (median 1.06 μmol/mol creatinine) compared with the controls (median 0.52 μmol/mol creatinine). Urinary metabolite concentrations of organic solvents used were all higher in the epoxy resin workers compared with the controls. Endocrinological examination showed different concentrations of follicle stimulating hormone (FSH) between the epoxy sprayers (median 5.3 mIU/ml) and the controls (median 7.6 mIU/ml). FSH showed a mild correlation with urinary bisphenol A, but not with the metabolites of organic solvents. Luteinising hormone and free testosterone concentrations did not differ between the two groups.
Conclusion: BADGE may generate bisphenol A endogenously. Results suggest that bisphenol A may disrupt secretion of gonadotrophic hormones in men. The clinical significance of endocrine disrupting effects by bisphenol A should be further investigated in male workers exposed to bisphenol A.

Bisphenol A diglycidyl ether (BADGE) is a member of the glycidyl ether group. Glycidyl ethers have been widely used as basic components of epoxy resins since the late 1940s. BADGE and its oligomers are major components of epoxy resins. Occupational exposure to BADGE occurs in its production, epoxy resin production, and various uses of epoxy products.[1] Apart from allergic reaction,[2-5] its health effects and metabolism in humans have rarely been reported. An oestrogen receptor binding capacity of BADGE was reported to be low in a previous in vitro study.[6] However, if bisphenol A is generated from BADGE through a metabolic transformation in the body, the endogenous bisphenol A may affect endocrinological function. In mice orally exposed to BADGE, the amount of bisphenol A excretion was reported to be low, but no observations have been reported in exposed humans. Animal experiments have shown effects of bisphenol A on male reproductive function or generative organs,[7-9] but no effects have been reported in men.

To evaluate the effects of occupational exposure to BADGE on urinary excretion of bisphenol A, and the effects of endogenous bisphenol A on plasma gonadotrophic hormones and testosterone in male workers, we conducted a cross sectional study of epoxy resin workers who sprayed BADGE with mixed organic solvents in plastic plants. Their urinary bisphenol A and plasma hormones (including luteinising hormone (LH), follicle stimulating hormone (FSH), and free testosterone) were examined.

Main messages

- BADGE may generate bisphenol A endogenously.
- Bisphenol A may disrupt secretion of gonadotrophic hormones in men.

Policy implication

- Clinical significance of endocrine disrupting effects by bisphenol A should be further investigated in male workers exposed to bisphenol A.

SUBJECTS AND METHODS

Subjects and sample collection

Forty two male workers, whose work was to spray BADGE with mixed organic solvents as an epoxy resin hardening agent in a machine plant and two related plants located in Aichi Prefecture, served as the exposed workers in this study. They included all workers using BADGE in these plants. According to the product specification sheets of seven hardening agents used, they contained 10–30% BADGE. The product sheets indicated that these agents also contained toluene (0–30%), xylene (0–20%), 2-ethoxyethanol (0–20%), 2-butoxyethanol (0–20%), and methyl isobutyl ketone (0–30%). The workers used the above agents in spray rooms which were not completely airtight. They were in charge of the spraying at least three hours a day, and wore protection devices during the spraying, but their workplace atmosphere was polluted.

To select control subjects, 82 male workers whose age was similar to that of the epoxy resin workers, were randomly selected from 1202 assembly workers who did not use BADGE in the same plants. None refused to be recruited for this study. Forty two workers were finally selected as the controls to be matched to each epoxy resin worker by age (±3) and number of cigarettes/day.

Abbreviations: BADGE, bisphenol A diglycidyl ether; FSH, follicle stimulating hormone; LH, luteinising hormone; HPLC, high performance liquid chromatography; ECD, electrochemical detector; BDL, below detection limit

This study was conducted according to the Declaration of Helsinki. All subjects participated in the study as volunteers, and gave their written informed consent.

Mean age (37±9, 38±10), percentage of current smokers (86%, 86%), and number of cigarettes/day among smokers (21±7, 21±6) were comparable between the epoxy resin sprayers and the controls. The percentage of alcohol drinkers was significantly lower in the epoxy resin sprayers (43%) than in the controls (57%) ($p = 0.031$).

Urine and blood samples were collected at periodic health examinations conducted in June and July, 1999, but not after a night shift. Urine samples were collected in the morning (10–12 am), but not on the first day of the week. Peripheral blood was collected in an EDTA-2Na tube at 10–12 am on the same day as urine sampling. Plasma was collected by centrifugation. A simple questionnaire was used to obtain lifestyle information, including smoking and alcohol drinking habits.

Analytical methods

Urinary bisphenol A was measured by high performance liquid chromatography (HPLC) with an electrochemical detector (ECD, Model 5600A CoulArrey Detector, ESA, Inc., Chelmsford, MA) using modifications of methods reported previously.[10 11] Briefly, 50 µl of β glucuronidase (Wako Chemicals, Osaka, Japan) and 200 µl of 0.1 M sodium acetate buffer (pH 5.0, 0.1% ascorbic acid, 0.01% EDTA) were added to 200 µl of urine. Dimethylbutylidene bisphenol (Wako Chemicals) was added to this solution as an internal standard. After incubation at 37°C for three hours, 1.2 ml ethanol was added to the solution. One ml of supernatant was obtained by centrifugation at 12 000 *g* for 15 minutes, and was evaporated by a vacuum evaporator. Residue was resolved by 0.3 ml of 50% methanol. After filtration, 40 µl of the solution was injected into the HPLC system. The analytical conditions of the HPLC–ECD system were as follows: octadecil column (ODS250, 250×4.6 mm, MC Medical, Inc., Tokyo, Japan); mobile phase (A), acetonitrile–water–phosphoric acid (20:79.8:0.2); mobile phase (B), acetonitrile–water–phosphoric acid (80:18.8:0.2); linear gradient programme, 25%B (0–28 min), 25%B–100%B (28–37 min); ECD detector voltages, 360, 400, 440, 480, 520, 560, 600, and 640 mV. The peak of bisphenol A was confirmed using profiles of reactions in these eight channels. The bisphenol A detection limit in this system was approximately 10 pg (0.05 pmol). The bisphenol A concentration was determined by a linear regression line of standard bisphenol A (Tokyo Kasei Co., Tokyo, Japan), and was adjusted by a recovery rate of the internal standard. The recovery rates of bisphenol A and the internal standard were approximately 100%. The coefficient of variation of the measure was <10%. A blank sample containing water (Milli-Q SP VOC, Millipore Co., MA) instead of urine was treated using the same method, and analysed. The value of the blank sample (0.5 pmol/ml) was subtracted from the values of the samples. If the value of a sample was below the blank level, the sample level was considered "not detected". The water was confirmed to contain no bisphenol A. The bisphenol A concentration was adjusted by urinary creatinine concentration.

We analysed the concentrations of the following urinary metabolites: *o,m,p*-cresol, *o,m,p*-methylhippuric acid, 2-ethoxyacetic acid, 2-butoxyacetic acid, and methyl isobutyl ketone.

Urinary *o*-cresol was measured using the method of Taguchi and colleagues.[12] Briefly, the hydrolysed sample was extracted with ethyl acetate. The extracted analyte was measured using a high performance liquid chromatograph (HPLC, Model L-7000, Hitachi Ltd, Ibaraki, Japan) equipped with a fluorescence spectrophotometer (Ex270/Em290 nm, Model F-1050, Hitachi Ltd).

The methylhippuric acid concentration was determined according to Hasegawa and colleagues.[13] Briefly, a diluted urine sample was measured using an HPLC (Model L-7000, Hitachi Ltd) with UV detector (230 nm, Model L-4000, Hitachi Ltd).

Urinary 2-ethoxyacetic acid and 2-butoxyacetic acid were measured according to the method of Smallwood and colleagues.[14] Briefly, a urine sample was extracted by methylene chloride. The methylene chloride layer was injected into a gas chromatograph (GC) equipped with a flame ionisation detector (Model G-3000, Hitachi Ltd).

Methyl isobuthyl ketone in urine was analysed by the head space method. Briefly, urine was heated, then the head space air inside the vial was injected into a GC equipped with a flame ionization detector (Hitachi Model 263–50, Hitachi Ltd).

Detection limits were as follows: cresol 4 ng/ml; methylhippuric acid 1 µg/ml; 2-methoxyacetic acid 2 µg/ml; and 2-butoxyacetic 1 µg/ml.

Luteinising hormone (LH), follicle stimulating hormone (FSH), and free testosterone in plasma were measured by radio immunosolvent assay in a commercial laboratory (SRL Inc., Tokyo, Japan). The reference values for these determinations provided by the laboratory were 1.8–5.2 IU/ml, 2.9–8.2 IU/ml, and 14–40 pg/ml, respectively. The coefficients of variation for these measures were <5%.

Statistical analysis

Values, including urinary bisphenol A, metabolites, and plasma hormones, were log transformed for statistical analysis because their distribution was not normal. Differences between the two groups (BADGE workers and controls) were tested by paired *t* test. Pearson's correlation coefficient was calculated to assess the degrees of relations between the metabolites and plasma hormones. To adjust for age and alcohol drinking habits, possible confounders, a multiple linear regression analysis was used to assess relations between the metabolites and the hormones. Differences in proportions concerning basic characteristics between BADGE workers and controls were tested by the χ^2 method. A p value less than 0.05 (two tailed) was considered significant.

RESULTS

Urinary metabolites of organic solvents were detected more frequently in the epoxy resin workers compared with the controls: *o*-cresol (81%, 33%), methylhippuric acids (62%, 12%), and 2-butoxyacetic acid (62%, 0%). Median concentrations (range) were as follows: *o*-cresol, 33 (below detection limit (BDL) to 816) µmol/mol creatinine; methylhippuric acids, 1.7 (BDL to 23) µmol/mol creatinine, and 2-butoxyacetic acid, 0.6 (BDL to 70) mmol/mol creatinine in the epoxy resin sprayers. In controls, *o*-cresol was BDL (BDL to 467) µmol/mol creatinine and methylhippuric acids were BDL (BDL to 4) µmol/mol creatinine. Concentrations of urinary 2-butoxyacetic acid in the controls were below the detection limit. Urinary metabolite concentrations were all higher in the epoxy resin workers compared with the controls. Urinary 2-ethoxyacetic acid and MIBK were not detected in either group.

Figure 1 shows the concentrations of bisphenol A in urine. A significant difference in bisphenol A concentrations was observed between the epoxy resin sprayers (median 1.06; range: not detected to 11.2 µmol/mol creatinine) and the controls (median 0.52; range: not detected to 11.0 µmol/mol creatinine) ($p = 0.002$, average difference = 2.5; 95% confidence interval (CI) 1.4 to 4.7). We could not detect bisphenol A for three epoxy resin sprayers and one control.

Table 1 shows the average plasma hormone concentrations. Free testosterone concentrations did not differ between the epoxy resin sprayers and controls ($p = 0.74$, average difference = 1.0; 95% CI −1.1 to 1.1). No difference in LH concentrations between the two groups was observed ($p = 0.41$, average difference = 1.1; 95% CI −1.1 to 1.3). However, we observed a significant difference in FSH concentrations between the sprayers (median 5.3 mIU/ml) and the controls (median 7.6 mIU/ml) ($p = 0.022$, average difference = −1.3; 95%CI −1.5 to −1.0).

FSH showed a mild correlation with urinary bisphenol A (correlation coefficient −0.20, $p = 0.071$, fig 2), but not with

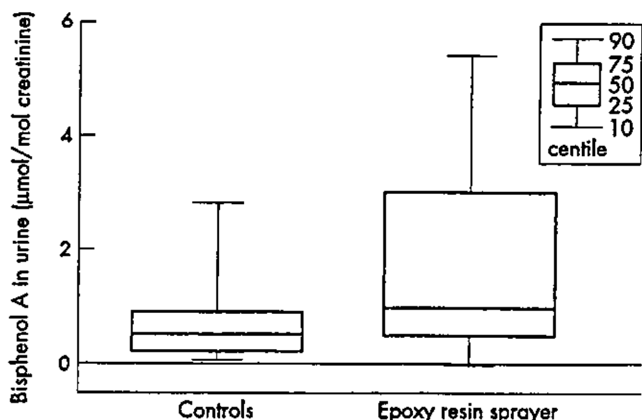

Figure 1 Concentrations of bisphenol A in urine of epoxy resin sprayers who used bisphenol A diglycidyl ether (n = 42) and the control workers (n = 42). The difference between the two groups was statistically significant (p = 0.002).

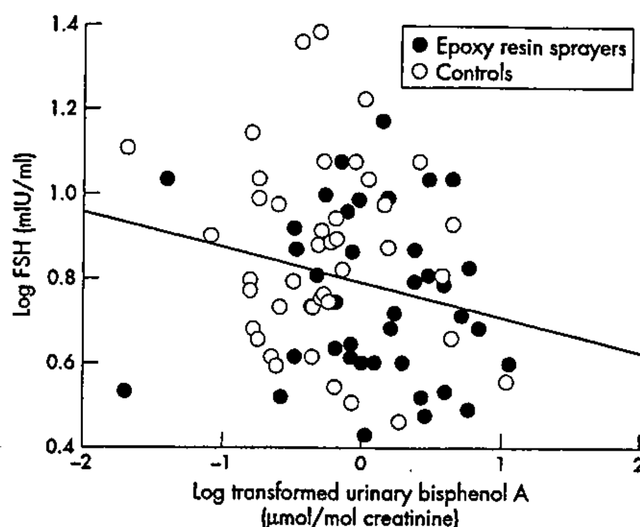

Figure 2 Relation between urinary bisphenol A concentrations and plasma FSH in all subjects (n = 84, correlation coefficient −0.20, p = 0.071).

Table 1 Median (interquartile range) of plasma hormone levels in workers using bisphenol A diglycidyl ether and control workers

Hormones	Exposed sprayers (n=42)	Control workers (n=42)
LH (mIU/ml)	4.0 (4.0–5.0)	4.0 (3.0–6.0)
FSH (mIU/ml)	5.3 (4.0–8.3)	7.6 (5.4–11.0)
Free testosterone (pg/ml)	15.2 (12.7–18.7)	15.1 (12.5–17.1)

the metabolites of organic solvents. The multivariate analysis showed a statistically significant relation between FSH and bisphenol A, adjusted for alcohol drinking habits (p = 0.045). No significant correlations were observed between other hormones and urinary metabolites (table 2).

DISCUSSION

In this cross sectional study, we observed an increased excretion of bisphenol A and decreased plasma FSH in the male workers who sprayed epoxy resin hardening agents containing BADGE compared to the matched controls.

A previous in vivo study reported many types of urinary metabolites in mice which had orally ingested BADGE.[15] According to the study, bisphenol A was one of the minor metabolites, and the amount was reported to be small.[16] The experiment by Climie *et al* used radiolabelled BADGE, so the results of their study seemed to be generally reliable. Nevertheless, we observed an increased mean concentration of bisphenol A in the exposed sprayers.

In this study, we could not analyse major metabolites of BADGE, for example, bis-diol of BADGE,[15] because their standard reagents were not available. Thus, we could not directly confirm exposure to BADGE in the sprayers. However, increased metabolites of some organic solvents in the epoxy resin hardening agents supported the hypothesis that BADGE also entered their body during work. The vapour pressure of BADGE is unknown, but as it is generally used as a liquid, workers can intake the mist. Consumption of canned beverages, a suspected potential source of bisphenol A exposure in daily life,[17] was comparable between the epoxy resin sprayers and the matched controls, but few subjects consumed them in either group. Urinary bisphenol A was detected in most of the controls, therefore other unknown sources of bisphenol A intake cannot be excluded. However, no specific source contributed to bisphenol other than in BADGE in the sprayers, because a study of urinary bisphenol A concentrations in rural residents in Japan has shown that most of the general population have concentrations similar to those of the controls in the present study (manuscript in preparation). It is reasonable to consider that bisphenol A was generated endogenously by occupational exposure to BADGE.

One control showed a high concentration of bisphenol A as shown in fig 1. One possibility is that he was exposed to BADGE passively near the spraying works. Similar phenomena were observed in urinary metabolites of organic solvents. Use of protective devices and passive exposure might cause a selection bias in this survey.

Effects of bisphenol A on the endocrinological system have been reported. Its oestrogen receptor binding capacity has been observed in earlier reports.[18] [19] Concerning genital function in males, vom Saal *et al* observed decreased sperm production,[7] although a negative result has also been reported,[20] and Takao *et*

Table 2 Standardised partial regression coefficients between selected serum hormones and urinary metabolites in spray workers and controls

Metabolites	Hormones: LH (mIU/ml) r	LH p	FSH (mIU/ml) r	FSH p	Free testosterone (pg/ml) r	Free testosterone p
Bisphenol A (µmol/mol creatinine)	−0.05	0.65	−0.23	0.045	−0.15	0.17
o-Cresol (µmol/mol creatinine)	−0.05	0.64	−0.03	0.82	−0.13	0.23
Methylhippuric acids* (µmol/mol creatinine)	−0.01	0.90	−0.07	0.56	0.19	0.08
2-butoxyacetic acid* (mmol/mol creatinine)	−0.02	0.88	−0.18	0.12	−0.06	0.61

Log translated values were used for metabolites and hormone levels. Standardised partial regression coefficients were adjusted for age and alcohol drinking habits.
*Half the detection limit was used for a subject whose urinary metabolite was not detected.

al observed decreased free testosterone and LH in male mice exposed to bisphenol A.[8] Furthermore, an experiment by Gupta *et al* showed that bisphenol A decreased epididymal weight in male offspring following maternal exposure.[9] In men, LH and FSH are involved in sperm production and androgen synthesis. In the present study, LH and free testosterone were not different between the exposed sprayers and the controls, but we observed decreased FSH in the former. The controls in this study were clinically healthy persons confirmed by a periodical health examination. The reference values for FSH determination also supported the fact that they were clinically normal although some showed relatively high concentrations.

In endocrinological studies, timing of blood sampling in the daytime is an important factor for estimation of hormone concentrations. A "cross sectional" evaluation is a major limitation of our study. However, in this study, the timing of blood sampling was almost similar, and was not after the night shift, for all subjects; a previous study observed that diurnal rhythm for FSH was less notable than that for LH and testosterone in men.[21] This may explain why there was no observable relation between LH and bisphenol A.

We speculate that endogenous bisphenol A bound to oestrogen receptors in the pituitary body, and FSH secretion was directly suppressed. Oestrogen receptors have been found in the human pituitary body,[22] and Finkelstein *et al* showed that oestradiol directly inhibited gonadotrophin secretion at the pituitary level in men.[23] On the other hand, absence of a feedback effect of testosterone on FSH in men has been reported,[24] a finding that may also support our speculation.

Effects of chemical exposure on male reproduction have been a concern from the late 1800s in occupational medicine. Some organic solvents have been reported to be potential modifiers of male reproductive function.[25] Morck *et al* observed increased FSH concentrations in male workers exposed to toluene.[26] In the present study, BADGE was used with organic solvents. Thus, one possibility is that mixed organic solvents contained in the epoxy resin hardening agents decreased FSH concentrations. The decreased FSH is believed to be caused by effects on the central nervous system, because such effects of organic solvents are well known. However, levels of exposure to the organic solvents were relatively low. It cannot reasonably be assumed that the effects of organic solvent exposure are causative.

Recently, some chemicals have been noted as endocrine disrupting agents. In this context, environmental effects on male fertility have been discussed elsewhere.[27] According to our observation, serum gonadotrophic hormones may be a sensitive marker for evaluation of the effects of endocrine disrupting chemicals in men. In a previous study, decreased free testosterone was observed in mice,[8] but we observed no difference between the sprayers and the controls. The difference of FSH observed in the present study occurred within clinically normal conditions. Thus, the clinical significance is still unclear.

Conclusions

- BADGE may generate bisphenol A endogenously.
- Bisphenol A may disrupt secretion of gonadotrophic hormones in men.
- Clinical significance of the endocrine disrupting effects of bisphenol A should be further investigated in male workers exposed to bisphenol A.

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Cytochrome P450 1B1 mRNA levels in peripheral blood cells and exposure to polycyclic aromatic hydrocarbons in Chinese coke oven workers

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Abstract

Cytochrome P450 1B1 (*CYP1B1*) is induced through the Ah receptor and is involved in the activation of polycyclic aromatic hydrocarbons (PAHs). To determine the validity of a quantitative analysis of *CYP1B1* mRNA in peripheral human blood cells for the estimation of PAH exposure, a real-time quantitative polymerase chain reaction method was used to measure the relative levels of *CYP1B1* mRNA in 37 Chinese coke oven workers and 13 control workers. A large inter-individual difference in the levels was observed. The average level of the *CYP1B1* mRNA in workers at the top work site, where the PAH exposure level from the coke ovens was highest, was significantly higher than in workers at the middle site ($P<0.01$) or the controls ($P=0.02$). A non-significant positive correlation was found between the *CYP1B1* mRNA levels and urinary 1-hydroxypyrene ($R=0.22$, $P=0.13$), and a significant correlation between these mRNA levels and urinary cotinine ($R=0.33$, $P=0.02$). It was interesting that a significant positive correlation between *CYP1B1* mRNA and 1-hydroxypyrene was observed in subjects with the *Leu/Leu* type of *CYP1B1 Leu*432*Val* polymorphism ($R=0.33$, $P=0.02$, $n=38$) and a non-significant correlation in subjects with the *Leu/Val* and *Val/Val* types ($R=-0.36$, $P=0.25$, $n=12$), although the number of subjects in this strata analysis was small. Our preliminary study suggests that PAH exposure in coke ovens and smoking maybe associated with *CYP1B1* mRNA levels in peripheral blood cells although mRNA is generally unstable and could be expressed following exposure to other agents.

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Keywords: Cytochrome P450 1B1; Polycyclic aromatic hydrocarbons; Coke oven; 1-hydroxypyrene; Cotinine

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1. Introduction

Cytochrome P450 1B1 (*CYP1B1*) belongs to a multigene superfamily of monomeric mixed-function monooxygenases responsible for phase 1 metabolism (Sutter et al., 1994), and is involved in the activation of a broad spectrum of procarcinogens, especially polycyclic aromatic hydrocarbons (PAHs). *CYP1B1* is induced by the Ah receptor (AhR) which is also characteristic of *CYP1A1* (Tang et al., 1996).

CYP1B1 is expressed in many normal human tissues including peripheral blood cells (Sutter et al., 1994). Recently, Spencer et al. (Spencer et al., 1999) demonstrated a dose-dependent induction of *CYP1B1* in peripheral lymphocytes by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in in vitro studies, and suggested the possibility of using *CYP1B1* mRNA levels in peripheral blood cells as a marker of AhR ligands. However, few studies have explored the relationship between exposure to PAHs, known to be AhR ligands, and *CYP1B1* mRNA levels in peripheral blood cells of subjects exposed to PAHs at high levels.

To determine the validity of a quantitative analysis of *CYP1B1* mRNA in peripheral blood cells for the estimation of PAH exposure, this preliminary study analyzed *CYP1B1* mRNA levels in Chinese coke oven workers exposed to PAHs at high levels using a real-time quantitative polymerase chain reaction.

2. Subjects and methods

2.1. Study population and subject selection

The subjects for this study were 37 coke oven workers and 13 controls who worked in the Angang Iron Steel Company, Anshang, China. They were from among 229 subjects in a cross-sectional study conducted in 1998–1999. The 229 subjects were selected from the top (high exposure), middle (medium exposure) and bottom (low exposure) work sites of coke oven plants, and control plants. These groups were matched for age and smoking status. The details of the selection method were similar to those of our previous study (Pan et al., 1998). Among the 229 subjects, cDNA in peripheral blood cells were successfully obtained from 54. Ultimately, there were 50 subjects (top 9, middle 16, bottom 12, control 13) for whom no data was lacking, and those 50 were assigned to this study. These subjects had no clinical disease. Basic characteristics including mean age, frequency of smoking, and exposure indices were comparable between the 50 subjects and the remaining subjects.

2.2. Questionnaire survey

An interview investigation by two trained interviewers using a structured questionnaire identified personal characteristics including smoking habits, alcohol consumption, indoor air pollution (coal heating and cooking), frequency of eating roasted foods, lifetime occupational history, use of personal protectors, and disease history.

The cumulative BaP exposure dose (CBED, $\mu g/m^3 \times$years) was calculated by an industrial hygienist according to the lifetime work history of each worker and the established matrix of job title-time period-BaP concentration (Xu et al., 1996).

2.3. Blood and urine sampling

Blood sampling was completed within 1–2 weeks of urine sampling. Seven milliliters of peripheral venous blood was collected into an EDTA-2Na tube, and fractionated into a buffy coat, plasma and red blood cells by centrifugation. The samples were stored at −70 °C until further treatment. White blood cells (WBCs) were collected from the buffy coat by osmotic hemolysis before RNA and DNA extraction.

Urine samples were collected on the morning of the last day of a work shift (after the second night of the work shift) for the shift workers, and at the end of the last working day for the day-time and control workers.

2.4. Urinary 1-hydroxypyrene (1-OHP) and cotinine

The 1-OHP concentration was determined using the method described by Hara et al. (Hara et al., 1997). Briefly, the conjugated metabolite in a urine

sample was hydrolyzed with β-glucuronidase, and the 1-OHP was quantitated using high-performance liquid chromatography with a fluorescence detector.

Urinary cotinine was determined using a method originally described by Horstmann (Horstmann, 1985).

2.5. RNA extraction and reverse transcription

Total RNA was isolated from WBCs using a commercial RNA isolation reagent (Trizol Reagent, Gibco Life Technologies, Inc., Rockville, MD) according to the manufacturer's instructions. Isolated RNA was resuspended in 10 μl of diethylpyrocarbonate-treated water.

The prepared RNA was reverse-transcribed to synthesize cDNA using avian myeloblastosis virus reverse transcriptase XL and random primers (Takara Biochemicals, Osaka, Japan). The RT reaction mixtures were incubated at 30 °C for 10 min and at 50 °C for 15 min, and then chilled at 5 °C.

2.6. Real-time polymerase chain reaction (PCR)

For quantitative comparison of *CYP1B1* mRNA levels, we used real-time TaqMan technology with an ABI Prism 7700 Sequence Detector (PE Biosystems, Foster City, CA). The principle of this technique was described elsewhere (Gibson et al., 1996; Heid et al., 1996). Using this technology, real-time PCR amplification data on the template cDNA could be obtained. The amount of template cDNA was expressed by the threshold cycle (C_T) determined by the amplification curve (exponential phase) and by the threshold level of PCR product detection. One C_T was equal to a two-fold difference in the initial template.

A comparison of *CYP1B1* mRNA levels among subjects was made by the comparative C_T method using separate tubes, as described elsewhere (User Bulletin #2, PE Biosystems). Briefly, the individual level of initial target cDNA was expressed as the difference in C_T between the target and an appropriate endogenous control (ΔC_T). Calibration was achieved by subtracting a subject's ΔC_T from a calibrator's ΔC_T ($\Delta\Delta C_T$). The calibrator was an optional sample for this assay. The relative amount of target in a subject, normalized to an endogenous control and relative to a calibrator, was finally given by $2\text{-}\Delta\Delta C^T$. In this report, the relative level of *CYP1B1* mRNA of each subject is expressed as a divisor by the median level because values of the relative amount mentioned above were small.

A specific primer and a specific TaqMan probe of *CYP1B1* were designed for exon 2 to exon 3 according to a published sequence (GenBank U56438); forward (GCA GTG GCT GCT CCT CC), reverse (TGG TCA CCC ATA CAA GGC AGA), and probe (5′-FAM-TGC AGG CAG AAT TGG ATC AGG TCG-TAMRA-3′). For *CYP1B1*, the PCR reaction was carried out with 25 μl of 2×TaqMan Universal PCR Master Mix, forward and reverse primers (final concentration 400 nM), probe (final concentration 138 nM), approximately 1 μg of cDNA, and 21 μl of RNase/DNase-free water. The PCR products of a selected sample were confirmed to be cDNA by a sequencer (ABI Prism 377 DNA Sequencer, PE Biosystems). We used the β-actin gene as an endogenous control gene. For β-actin, the PCR reaction was carried out according to the manufacturer's instructions (Pre-Developed TaqMan Assay Reagent, Human β-actin, PE Biosystems). The PCR conditions were as follows: (a) 50 °C for 2 min; (b) 95 °C for 10 min and (c) 50 cycles of 95 °C for 15 s and 60 °C for 1 min.

The average C_T difference between duplicate analyses was 0.4 C_T (approx. 1.3 times the difference in the initial template amount). All measurements were made without information about the origin of the samples and were performed in duplicate.

2.7. *CYP1B1 Leu432Val* polymorphism

DNA extracted from the buffy coat using a commercial kit (Wako Chemical Co., Osaka, Japan) was used for the determination of *CYP1B1 Leu432Val* polymorphism. This polymorphism in exon 3 was measured by the PCR-restriction fragment length polymorphism method using BfrI enzyme described by Fritsche, et al. (Fritsche et al., 1999). Subjects were divided into three groups: *Leu/Leu* (wild-type), *Leu/Val* (heterozygous type), and *Val/Val* (mutant-homo type). In this

Table 1
Basic characteristics and exposure indices of the study subjects

	Coke oven workers ($n=37$)	Controls ($n=13$)	P value for difference
Age (mean, range)	38 (25–51)	37 (26–53)	0.61
Smoker (%)	62	62	0.97
Urinary 1-hydroxypyrene (μmol/mol creatinine) (mean, range)	10.9 (2.6–84.9)	0.5 (0.1–2.8)	<0.0001
Urinary cotinine (mmol/mol creatinine) (mean, range)	0.18 (0.003–2.35)	0.11 (0.003–1.85)	0.51
Cumulative benzo(*a*)pyrene exposure dose ($\mu g/m^3 \times$ years) (mean, range)	79.4 (8.4–746.4)	0.6 (0.2–1.5)	<0.0001
Cytochrome P450 1B1 *Leu*/432*Val* polymorphism in exon 3 (% *Leu*/*Leu* type)	76	77	0.93

report, *Leu*/*Val* and *Val*/*Val* genotypes were combined for calculation.

2.8. Statistical analysis

Values, including *CYP1B1* mRNA, urinary 1-OHP, cotinine, and the CBED were normalized by log10-transformation. Differences in proportions were tested by the chi-square method or, when the expected values were small, by Fisher's exact test. Means in different subgroups were compared by Student's *t*-test or analysis of variance. To assess associations between *CYP1B1* mRNA and other variables, correlation coefficients were computed. A P value less than 0.05 was considered statistically significant. SAS and StatView statistics software programs (SAS Institute Inc., Cary, NC) were used for the analyses.

3. Results

Table 1 shows the basic characteristics and exposure indices of the 50 subjects in the coke ovens and the control plants. Mean age and smoking status were comparable in the two groups. Significant differences in urinary 1-OHP levels were observed between the coke workers and the controls ($P<0.0001$), although urinary cotinine levels were comparable between the two groups. The CBED, which was significantly related with 1-OHP ($R=0.85$, $P<0.0001$) and age ($R=0.31$, $P=0.03$), differed significantly between the two groups, whereas the frequency distribution of *CYP1B1 Leu*432*Val* polymorphism did not. There was no difference in average 1-OHP levels ($P=0.81$) or cotinine levels ($P=0.68$) between the *CYP1B1* genotype groups.

Fig. 1 shows the *CYP1B1* mRNA levels according to the work sites of the subjects. The average level in workers at the top work site, where PAH exposure level was highest in the coke ovens, was significantly higher than in workers at the middle site ($P<0.01$) or the controls ($P=0.02$). A non-significant positive correlation was found between the mRNA levels and 1-OHP ($R=0.22$, $P=0.13$, Fig. 2), and a significant correlation between the mRNA levels and urinary cotinine levels ($R=0.33$, $P=0.02$, Fig. 2). When 1-OHP and cotinine were included in the same multiple regression model, a similar non-significant association between the mRNA and 1-OHP and a significant association between the mRNA and cotinine were still observed (data not shown).

When the subjects were divided into two genotypes of the *Leu*432*Val* polymorphism, a statistically significant correlation between the mRNA levels and urinary 1-OHP was found with the *Leu*/*Leu* type ($R=0.33$, $P=0.02$, $n=38$) whereas none was observed with the *Leu*/*Val* and *Val*/*Val* type ($R=-0.36$, $P=0.25$, $n=12$). Looking at urinary cotinine, correlation's in both genotype groups were similar: *Leu*/*Leu* type, $R=0.27$, $P=0.10$; *Leu*/*Val* and *Val*/*Val* type, $R=0.58$, $P=0.05$.

CYP1B1 mRNA showed no statistically significant correlation with CBED levels, roasted food consumption, or indoor exposure to coal smoke.

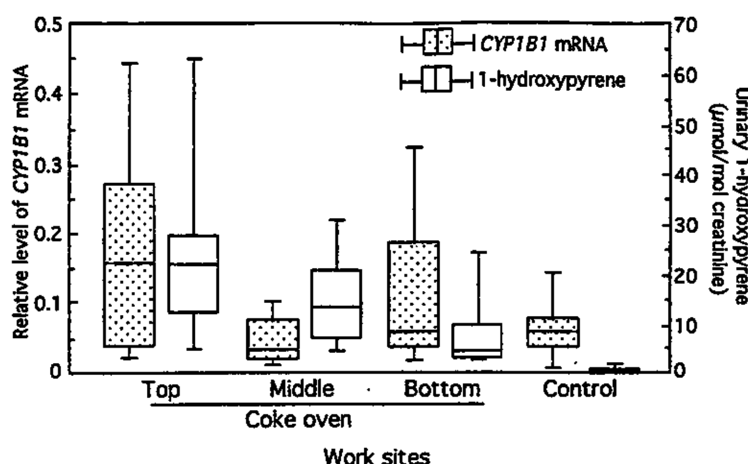

Fig. 1. Levels of urinary 1-hydroxypyrene and *CYP1B1* mRNA in peripheral blood cells according to the work sites in the coke ovens and controls. The number of subjects; top, 9; middle, 16; bottom, 12; and control, 13. The left axis shows a relative level of *CYP1B1* mRNA. The unit is described in the text. The right axis shows urinary 1-hydroxypyrene level.

4. Discussion

Although the sample size of these study was relatively small, our observations suggest that *CYP1B1* mRNA level in peripheral blood cells may be a marker of PAH exposure. The analyses were based on 50 subjects for whom sufficient cDNA specimens were obtained. Because of the low quantity of total RNA and cDNA, we excluded three-fourths of the subjects from our previous cross-sectional study. Although there was no difference in basic characteristics, including age, smoking status and the *CYP1B1 leu432Val* polymorphism, between the 50 subjects and the subjects excluded, the statistical power of the present study might be weakened due to the low number of subjects.

CYP1B1 mRNA is constitutively expressed in many human extrahepatic tissues, and the expression pattern is similar to that for *CYP1A1*. An in vitro study demonstrated the dose-dependent induction of *CYP1B1* in mitogen-stimulated peripheral lymphocytes by TCDD. Although *CYP1B1* mRNA levels in mitogen-stimulated lymphocytes were higher than those in uncultured lymphocytes, they showed a large interindividual variation of the mRNA levels even in uncultured lymphocytes as shown in the present study (Spencer et al., 1999).

Peripheral blood cells are not a target organ. However, some previous studies have also suggested that a quantitative assessment of the mRNA expression of *CYP1A1* and AhR in peripheral blood cells could provide information on PAH exposure (Cosma et al., 1992; Hayashi et al., 1994; Vanden Heuvel et al., 1993). In bronchial epithelial cells, a higher expression of *CYP1B1* was observed in smokers than non-smokers (Willey et al., 1997), so a simultaneous estimation of *CYP1B1* mRNA levels in peripheral blood cells and target tissues may further our understanding of the *CYP1B1* expression in peripheral blood cells.

We observed that the mRNA levels in workers at the top work site was relatively high compared with workers at other sites, however, the relationship between the mRNA levels and PAH exposure might not be linear. Adaptation or adoptive response could modify the expression of the mRNA in some individuals exposed to PAHs at high level. A large inter-individual variance in *CYP1B1* mRNA levels may also be explained in part by adaptation.