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Table 1 Samples used in the proficiency testing and their reproducibility

Year	No of Participants	Samples		TEQ (pg/g)	Reproducibility RSD%
1998	6	BCR CRM607	spray-dried milk	3.3	6.6
1999	15	BCR CRM607	spray-dried milk	3.6	11
		CARP-1	homogenized fish	79	8.0
		Nonane solution of standards		23	8.7
2000	10	BCR RM534	spray-dried milk	4.6	18
		Nonane solution of standards		16	9.0
2001	9	Sea bass	freeze-dried	6.1	11
		Spinach	freeze-dried	0.32	31
2002	8	Grey mullet	freeze-dried	7.3	7.1

Table 2 Results of proficiency testing in 2001 Sample: Sea bass

Analyte			Normal statistics			Robust statistics		
	max (pg/g)	min (pg/g)	mean (pg/g)	SD (pg/g)	RSD (%)	mean (pg/g)	SD (pg/g)	RSD (%)
2,3,7,8□TCDD	0.31	0.18	0.24	0.05	19	0.24	0.05	22
1,2,3,7,8□PeCDD	1.08	0.70	0.84	0.13	15	0.84	0.13	16
1,2,3,4,7,8□HxCDD	0.28	0.12	0.18	0.05	28	0.17	0.04	25
1,2,3,6,7,8□HxCDD	0.63	0.46	0.55	0.06	12	0.55	0.07	13
1,2,3,7,8,9□HxCDD	0.13	0.07	0.09	0.02	24	0.09	0.03	28
1,2,3,4,6,7,8□HpCDD	0.70	0.20	0.33	0.16	48	0.29	0.08	26
, ŋ þ ç c	18.50	0.37	2.85	6.33	222	0.67	0.25	38
2,3,7,8□TCDF	1.93	1.27	1.50	0.22	15	1.49	0.22	15
1,2,3,7,8□PeCDF	0.69	0.34	0.46	0.12	26	0.45	0.12	26
2,3,4,7,8□PeCDF	2.49	1.51	1.87	0.28	15	1.84	0.22	12
1,2,3,4,7,8□HxCDF	0.35	0.14	0.20	0.07	35	0.18	0.04	20
1,2,3,6,7,8□HxCDF	0.19	0.14	0.17	0.02	14	0.17	0.03	16
1,2,3,7,8,9□HxCDF	1.04	-	-	-	-	-	-	-
2,3,4,6,7,8□HxCDF	0.33	0.26	0.30	0.03	9	0.30	0.03	10
1,2,3,4,6,7,8□HpCDF	0.57	0.06	0.19	0.17	92	0.14	0.06	45
1,2,3,4,7,8,9□HpCDF	0.14	0.01	0.07	0.05	77	0.07	0.06	87
, ŋ þ ç e	0.29	0.01	0.18	0.11	61	0.18	0.13	69
3,3',4,4'□TCB	75.5	43.7	53.7	9.8	18	52.48	8.02	15
3,4,4',5□TCB	3.32	1.69	2.43	0.53	22	2.43	0.60	25
3,3',4,4',5□PeCB	38.7	20.7	30.0	5.4	18	30.0	6.0	20
3,3',4,4',5,5'□HxCB	14.6	9.4	11.2	1.8	16	11.10	1.71	15
2,3,3',4,4'□PeCB	1180	827	983	106	11	979	112	11
2,3,4,4',5 □PeCB	134.7	51.0	77.9	23.2	30	73.7	12.1	16
2,3',4,4',5□PeCB	3589	2741	3214	310	10	3214	351	11
2',3,4,4',5□PeCB	73.9	33.2	47.4	12.5	26	46.1	11.0	24
2,3,3',4,4',5□HxCB	491	391	435	35	8	435	39	9
2,3,3',4,4',5'□HxCB	140	97	114	14	12	113.2	14.4	13
2,3',4,4',5,5'□HxCB	246	205	221	16	7	221	18	8
2,3,3',4,4',5,5'□HpCB	61.7	45.5	54.9	4.7	9	55.2	4.6	8
TEQ	6.90	5.19	6.19	0.60	10	6.19	0.68	11

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Enzyme-Linked Immunosorbent Assay for Monitoring Toxic Dioxin Congeners in Milk Based on a Newly Generated Monoclonal Anti-Dioxin Antibody

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To develop an enzyme-linked immunosorbent assay (ELISA) for monitoring the toxicity due to polychlorinated dibenzo-*p*-dioxins and dibenzofurans contaminated in human breast milk, we have generated novel monoclonal antibodies using some haptenic derivatives linked to bovine serum albumin via the C-1 or C-2 position on the dioxin skeleton. BALB/c or A/J mice were repeatedly immunized with the immunogen, and spleen cells were fused with P3/NS1/1-Ag4-1 myeloma cells. After five fusion experiments, a hybridoma clone was established that secretes an antibody D9-36 group specifically recognizing the major toxic congeners, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin, and 2,3,4,7,8-pentachlorodibenzofran. An ELISA is developed on the basis of the competitive and labeled-antigen format. The toxic congeners extracted from butter or milk specimens by a novel extraction cartridge and a peroxidase-labeled dioxin analogue were sequentially reacted with a fixed amount of D9-36 in the presence of Triton X-100. The bound fraction was captured on a microtiter plate, immobilizing a second antibody, and the enzyme activity was colorimetrically determined. This ELISA afforded a practical sensitivity (measurable range, 1−100 pg/assay; detection limit, 1.0 pg/assay as 2,3,7,8-TCDD equivalent). The assay values for milk and butter samples were in reasonable accordance with the sum of the toxicity-equivalent quantity of each congener, which had been determined by a high-resolution gas chromatography/high-resolution mass spectrometry method.

Environmental chemical contaminants have been concerns of the public and the government for many years. Particularly, polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are persistent pollutants because of their high lipophilicity that exhibit potential teratogenic, reproductive, immunotoxic, and carcinogenic effects. Therefore, PCDDs and PCDFs (PCDD/Fs) are the subject of surveillance by regulatory agencies. To evaluate the toxicity to newborn children, it is particularly important to monitor the trace amount of dioxins that may contaminate human breast milk. Gas chromatography/mass spectrometry (GC/MS) has conventionally been used as the reference method of determining these compounds. This method offers high sensitivity and specificity and has the potential for the simultaneous determination of multiple isomers having very similar structures. However, the GC/MS method requires complicated and time-consuming sample cleanup procedures, resulting in a poor performance of treating a number of samples. Furthermore, the equipment is available at relatively few research institutes and requires well-trained operators. Any feasible and quicker method which is therefore suitable for the routine analysis of PCDD/Fs in all laboratories is in demand, particularly for large-scale epidemiological studies or long-term monitoring.

Enzyme-linked immunosorbent assay (ELISA) is expected to be suitable for this purpose when the incorporated antibody shows enough affinity and specificity to target dioxin congeners. Several immunoassay procedures for PCDD/Fs have already been reported;[1-9] however, most of them have required a large amount of environmental samples because of insufficient sensitivity that

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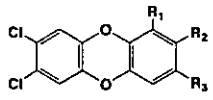
is attributed to lack of antibody affinity. We recently established a practical ELISA system for PCDD/Fs using a rabbit polyclonal antibody that is group-specific to some toxic dioxin congeners (unpublished data). Modern immunoassays, however, require the use of monoclonal antibodies, which are obtainable by hybridoma technology.[10] These monoclonal antibodies can be constantly supplied while maintaining well-defined and unique binding properties and are consequently able to standardize assay performance. From these points of view, we generated novel monoclonal antibodies, one of which was group-specific to the toxic dioxin congeners, which enabled the development of an ELISA system for monitoring the toxicity of dioxin contaminants in milk and butter samples. This ELISA provided assay values that are in good accordance with the toxicity-equivalent quantity (TEQ) of PCDD/Fs, which had been determined by a high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) method.

MATERIALS AND METHODS

Reagents. 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin was purchased from AccuStandard (New Haven, CT). 2,7-Dichrolo-, 2,3,7-trichrolo-, 1,3,6,8-tetrachloro-, 1,2,4,6,8-/1,2,4,7,9-pentachloro-, and 1,2,3,4,6,7-hexachlorodibenzo-*p*-dioxins, polybrominated dibenzo-*p*-dioxins/dibenzofrans, and polychlorinated biphenyls (PCBs) were obtained from Cambridge Isotope Laboratories (Andover, MA). All the other PCDD/Fs, including 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin (TMDD) and 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), were purchased from Wellington Laboratories (Ontario, Canada). Bovine serum albumin (BSA), horseradish peroxidase (HRP; EC 1.11.1.7, type IV) (250–330 U/mg), and Triton X-100 were obtained from Sigma Chemical Co. (St Louis, MO). Freund's complete adjuvant (FCA) and incomplete adjuvant (FIA) were purchased from DIFCO (Detroit, MI). AffiniPure rabbit anti-mouse IgG + IgM antibody (the second antibody in the following ELISA) was obtained from Jackson ImmunoResearch (West Grove, PA). Protein G Sepharose 4 Fast Flow Lab Packs were purchased from Amersham Biosciences (Piscataway, NJ). 96-Well EIA/RIA plates (No. 3590) and plastic cell culture wares were obtained from Costar (Cambridge, MA). Reagents for the cell culture, including media, fetal bovine serum, hybridoma cloning factor (HCF), and poly(ethylene glycol) (PEG) were previously described.[11,12] Benzene, $CHCl_3$ (for PCB analysis), MeOH, EtOH, *n*-hexane (for dioxin analysis), and Presep phthalocyanine immobilized silica gel cartridges (Presep cartridges) were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents and solvents were of analytical grade.

Haptenic Derivatives and Their BSA Conjugates. Five kinds of dioxin haptens used for preparing the immunogenic conjugates or enzyme-labeled antigens were those having a chemical linker on the C-1 (Ia, Ib, Ic) or C-2 (IIa, IIb) position of the dioxin skeleton[13] (Figure 1). The synthesis of haptens Ib[1], Ic[7], and IIb[7] has already been reported. The BSA conjugates of the haptens Ia, Ib, Ic, and IIa were prepared according to a reported method.[13] The hapten/BSA molar ratios in the conjugates were 11 (Ia), 16 (Ib), 11 (Ic), and 14 (IIa).



Haptenic derivative	R_1	R_2	R_3
Ia	$NHCO(CH_2)_3COOH$	H	Cl
Ib	$NHCO(CH_2)_4COOH$	H	Cl
Ic	CH=CHCOOH	Cl	Cl
IIa	H	$O(CH_2)_3COOH$	Cl
IIb	H	CH=CHCOOH	Cl

Figure 1. Structures of haptenic derivatives of dioxin used for antibody production and enzyme-labeling.

Buffers. The following were the buffers used: buffer A, 0.05 M sodium phosphate buffer (pH 7.4); buffer B, buffer A containing 0.9% NaCl; and buffer C, buffer B containing 0.1% gelatin.

Preparation of Enzyme-Labeled Antigen. The *N*-succinimidyl ester of the dioxin hapten Ib, Ic, or IIb was synthesized according to the reported method[14] and was reacted with HRP (4 mg) in 5 mM $NaHCO_3$ (200 μL) at room temperature for 30 min. The molar ratio of the activated ester to the enzyme in this reaction was adjusted to 5. To remove the unreacted haptens, the reaction mixture was submitted to gel filtration chromatography using Sephadex G-25, and the effluent showing the HRP activity was collected and stored at 4 °C for a few days. After the solution had become cloudy due to the remaining unreacted dioxins, the solution was centrifuged (1000*g*, 10 min), and the supernatant (1 mL) was washed three times with $CHCl_3$ (each 3 mL). After centrifugation, the resulting aqueous solution containing the enzyme-labeled antigen was diluted with buffer B and stored at 4 °C until use.

Immunization. Female BALB/c and A/J mice (each 8 weeks of age; 5 each) (Japan SLC; Hamamatsu, Japan) were immunized at three-week intervals with one of the hapten-BSA conjugates. The conjugate (50 μg) was subcutaneously injected with an emulsion of FCA (primary immunization) or FIA (booster immunizations) and sterile saline (1:1; 0.2 mL) into the footpads and at multiple sites on the back. Seven days after the fifth booster injection, blood was collected from the retrobulbar plexus, and the binding ability of the serum antibodies (diluted to 1:25 000) (50 μL) to the HRP-labeled dioxin (12.5 ng; 50 μL) was determined by the ELISA procedure (see below). The BSA conjugate (50 μg) in sterile saline (0.5 mL) was intraperitoneally injected into three BALB/c mice and two A/J mice that showed a high immune response, from which spleen cells were prepared 3 days later.

Monoclonal Antibody Production. The cell fusion experiment was performed according to previous papers.[11,12] Briefly, the immune spleen cells (1×10^8 cells) and 1/5 numbers of P3/NS1/1-Ag4-1 myeloma cells[15] were fused with 0.4 g/mL PEG 4000 in

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a sterile phosphate-buffered saline containing 10% (v/v) DMSO and 10 μg/mL poly-L-arginine·HCl solution (1 mL). The fused cells were suspended in a HAT medium supplemented with 10% HCF and cultured in 96-well cluster dishes (100 μL/well) overnight under 5% CO_2/95% air at 37 °C. After further culture for approximately 2 weeks in HAT medium, the hybridoma supernatants were submitted to screening by the ELISA described below. The antibody-secreting hybridomas were expanded in HT medium, cloned by limiting dilution, and were grown until confluence was reached. These hybridomas were inoculated into pristane-treated male BALB/c or BALB/c nude mice (8 weeks of age), and the relevant monoclonal antibodies were prepared on a large scale as ascitic fluids. The contained antibodies were purified using the Protein G column, and were used in the following ELISA.

ELISA Procedure for Hybridoma Screening. A solution of the second antibody diluted with buffer A (1.0 μg/mL; 100 μL) was distributed into each well of the EIA/RIA plates, which were left overnight at 4 °C. After washing twice with buffer B, the wells were blocked with a 5 mg/mL BSA solution in buffer B (200 μL) at room temperature for 2 h. The wells were washed twice with buffer B, to which the HRP-labeled hapten (20 ng/well) and the culture supernatant (1:5 dilution), each of which had been diluted with buffer C (each 50 μL), were then added. After incubation at room temperature for 1 h, the solutions were aspirated off, and the wells were washed three times with buffer B. The bound enzyme activity was colorimetrically measured using a substrate solution (100 μL) containing 0.05% *o*-phenylenediamine·2HCl and 0.01% H_2O_2. After incubation at room temperature for 1 h, the enzyme reaction was terminated by the addition of 3 M H_2SO_4 (50 μL), and the absorbance at 490 nm was measured using a BL 312e microplate reader (Bio-Tek Instruments; Winooski, VT).

Cleanup Procedure of Milk and Butter Samples for ELISA. A commercially available and ordinal butter or milk to which a known amount of dioxin [2,3,7,8-TCDD, 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PeCDD), or 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PeCDF)] had been added was suspended in 5-fold (milk) or 10-fold (butter) volumes of EtOH, and saponified with KOH (final KOH concentration, 1 M) at 30–40 °C over 2 h. The resulting mixture was diluted with an equal volume of water and extracted three times with *n*-hexane (10 mL each). The organic layer was washed with H_2SO_4 (1 mL per each round) until the H_2SO_4 layer became clear. The organic solvent was evaporated and the residue was dissolved in H_2SO_4 (1 mL), which was left at room temperature for more than 1 h. This mixture was extracted with *n*-hexane (3 mL), and the organic layer was washed with 5% $NaHCO_3$ and water (each 3 mL) and then applied to the Presep cartridge. The cartridge was washed with *n*-hexane (3 mL) and *n*-hexane/benzene (9:1) (1 mL), then eluted with *n*-hexane/benzene (7:3) (1 mL). This solution was submitted to the ELISA as shown below.

Optimized ELISA Procedure for Determination of Dioxins in Milk and Butter Samples. Calibration curves were mainly obtained by using 2,3,7,8-TCDD. In some experiments, a nontoxic analog TMDD[7] was used as a "surrogate standard antigen," because this shows almost the same reactivity against the antibody D9-36 as 2,3,7,8-TCDD. The standard solution (2,3,7,8-TCDD or TMDD) (0–12.5 ng/mL in MeOH) (100 μL) or the effluent containing dioxins recovered from the fat or milk (see above) (1 mL) was mixed with 0.05% Triton X-100 in MeOH (50 μL), and the solvent was evaporated off. Buffer C containing the antibody D9-36 (0.5 ng/assay; 125 μL) was added to the residue, mixed, and left at room temperature for 30 min. An aliquot of the resulting solution (50 μL) and buffer C containing the HRP-labeled hapten Ic (20 ng/assay; 50 μL) were added to the second antibody-coated microtiter plate prepared as above, mixed using a plate mixer, and left overnight at 4 °C. The solution was then aspirated, and the plate was washed three times with buffer B. The bound HRP activity was measured as described above. Using the software installed in the microplate reader, sigmoidal dose–response curves were fitted to the following four-parameter logistic equation,

$$y = \{(A - D)/[1 + (x/C)^B]\} + D$$

where A is the maximum absorbance at zero concentration, B is the curve slope at the inflection point, C is the x analyte concentration giving 50% inhibition, and D is the minimum absorbance (background signal) at infinite concentration.[16]

HRGC/HRMS Analysis. HRGC/HRMS was carried out on a Hewlett-Packard (Palo Alto, CA) 5890-II gas chromatograph equipped with a Supelco 2331 column (60 m × 0.25 mm i.d.) combined with a JEOL (Tokyo, Japan) JMS-700 mass spectrometer (electron ionization) at a resolution R = 10 000 in the selected ion monitoring mode. Details of the conditions were as follows: (HRGC) column temperature program, 130 °C (2 min), 130–200 °C (15 °C/min), 200–260 °C (3 °C/min), 260 °C (30 min); carrier gas, He; column head pressure, 168 Kpa; injection temperature, 270 °C; injection volume, 2 μL (splitless): (HRMS) ion source temperature, 270 °C; ionizing current, 600 μA; ionizing energy, 38 eV; and accelerating voltage, 10 KV.

Safety Considerations. As is well-known, some dioxin congeners are highly toxic and we should consider the probability that any dioxin-related compound might be revealed to be toxic in the future. On the basis of such recognition, all of the handling of the dioxin-related compounds was performed in a draft chamber by well-trained researchers who used extreme caution as listed below. The researchers wore protective gloves, a laboratory coat, safety glasses, and a protective mask containing a carbon powder layer. The laboratory glassware was decontaminated by treatment with KPEG reagent (potassium poly(ethylene glycol)ate) at 150 °C for 12 h to remove chlorine atoms from the dioxin skeleton. Any contaminated site on the laboratory bench was cleaned up by a thorough wiping using wet cloths with an adequate organic solvent (acetone, toluene, or methoxymethanol) followed by UV irradiation to degrade the dioxin-related compounds.[17,18]

RESULTS

Monoclonal Antibody Production. To enlarge the opportunity for obtaining antibody-secreting hybridomas, we im-

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Table 1. Cross-Reactivity of Monoclonal Antibodies D2-37, D9-36, and D35-42 with Various PCDD/F Congeners in the ELISA[a]

congener[b]	cross-reactivity D2−37	D9−36	D35−42	TEF
2,3,7,8-TCDD	1.00	1.00	1.00	1.0
1,2,3,7,8-PeCDD	0.19	0.48	0.96	1.0
1,2,4,6,8-PeCDD		0.008		
1,2,4,7,9-PeCDD		0.008		
1,2,3,4,6,7-HxCDD		0.003		
1,2,3,4,7,8-HxCDD	0.10	0.07	0.0001	0.1
1,2,3,6,7,8-HxCDD	0.07	0.04	0.0002	0.1
1,2,3,7,8,9-HxCDD	0.12	0.06	0.0014	0.1
1,2,3,4,6,7,8-HxCDD	0.08	0.006	0.0001	0.01
1,2,3,4,6,7,8,9-OCDD	0.03	<0.0001	<0.0001	0.0001
2,3,7,8-TCDF	0.16	0.14	0.0001	0.1
1,2,3,7,8-PeCDF	0.08	0.03	0.0003	0.05
2,3,4,7,8-PeCDF	0.16	0.17	0.0004	0.5
1,2,3,4,7,8-HxCDF	0.06	0.02	0.0002	0.1
1,2,3,6,7,8-HxCDF	0.13	0.08	0.0001	0.1
1,2,3,7,8,9-HxCDF	0.05	0.07	0.002	0.1
2,3,4,6,7,8-HxCDF	0.07	0.06	0.0001	0.1
1,2,3,4,6,7,8-HpCDF	0.04	0.002	0.0001	0.01
1,2,3,4,7,8,9-HpCDF	0.03	0.002	0.0001	0.01
1,2,3,4,6,7,8,9-OCDF	0.02	<0.0001	0.0001	0.0001
2,7-DiCDD		0.05		
2,3,7-TrCDD		0.16		
1,2,3,4-TCDD	<0.001	0.0002	<0.001	
1,3,6,8-TCDD		0.0004		
TMDD	0.74	0.87	0.61	

[a] ELISA conditions are described in Figure 3. [b] Abbreviations: DiCDD, dichlorodibenzo-*p*-dioxin; TrCDD, trichlorodibenzo-*p*-dioxin; HxCDD, hexachlorodibenzo-*p*-dioxin; HpCDD, heptachlorodibenzo-*p*-dioxin; OCDD, octachlorodibenzo-*p*-dioxin; HxCDF, hexachlorodibenzofuran; HpCDF, heptachlorodibenzofuran; OCDF, octachlorodibenzofuran.

munized five BALB/c mice that are the commonest spleen donors in cell fusion and also five A/J mice using one of four kinds of haptens (Ia, Ib, Ic, and IIa) (Figure 1) conjugated with BSA. Thus, a total of forty mice, each of which was numbered, were immunized. The A/J mouse strain was selected here because it often shows a better immune response than the BALB/c strain against some lipophilic small molecules, such as steroids (e.g., the active form of vitamin D_3[11]). The haptens used here are the derivatives of 2,3,7,8-TCDD having a chemical bridge at the C-1 or C-2 position on the dioxin skeleton. The relationship between the number of mice and the strain/immunogen used is summarized in Figure 2. After the fifth booster immunization, small amounts of serum were collected from all these mice, and the titer of the anti-dioxin antibody was compared in the ELISA using the HRP-labeled hapten Ib, Ic, or IIb. The selection of these three HRP-labeled antigens was intended to avoid a false negative due to the inadequate combination of the haptenic derivatives used for immunization and enzyme labeling.[19] In site heterologous combination, for example, the combination such as an antibody derived from the hapten Ia and the enzyme-labeled antigen prepared with the hapten IIa, the anti-hapten antibody sometimes recognizes the difference in the position of the bridge and consequently does not show enough binding to the labeled antigen.

The sera affording significant positive signals (from mice nos. 2, 7, 9, 10, 14, 17, 18, 21, 26, 29, and 35) (Figure 2) were further assessed for their affinity to dioxins by comparing the inhibition of the bound enzyme activity when fixed amounts (1, 10, and 100 pg) of TMDD were added (data not shown; the cross-reactivity of TMDD to the antibodies, see Table 1). On the basis of these results, mice nos. 2, 9, 14 (the BALB/c mice immunized with the hapten Ia, Ib, and Ic, respectively), 26, and 35 (A/J mice immunized with the hapten Ic and IIa) were selected as spleen donors. Five fusion experiments were performed using the spleen cells prepared from each of these mice. About 10 days after the cell fusion, hybridomas were observed in over 95% of the microwells. The ELISA screening of the anti-dioxin antibody-secreting hybridomas and subsequent cloning by limited dilution allowed establishing three hybridoma clones, D2-37, D9-36, and D35-42. Antibodies D2-37 and D9-36 were each derived from the BALB/c mouse no. 2 (immunized with the hapten Ia) and the BALB/c mouse no. 9 (immunized with the hapten Ib), respectively; and D35-42 was from the A/J mouse no. 35 (immunized with the hapten Ic). These monoclonal antibodies were prepared as ascitic fluids, purified using the protein G column, and used in the following ELISA.

Characterization of Monoclonal Antibodies and Optimization of ELISA Conditions. The isotypes of the heavy and light chains of each monoclonal antibody were as follows: D2-37 (γ2a, κ), D9-35 (γ1, κ), and D35-42 (γ2a, κ). The binding characteristics of these antibodies were investigated by the competitive ELISA system using the HRP-labeled hapten Ic that showed satisfactory reactivity against all these antibodies. Because of the very strong hydrophobicity of the dioxins, we predicted that a suitable solubilizing agent (carrier proteins, detergents, or organic solvents) must be contained in the assay buffer. After several examinations (data not shown), we found that Triton X-100 works effectively for this purpose. Tween 20 and poly(vinyl alcohol) were also tested but were much less effective. A defined amount of Triton X-100 was added to the solution of the standard compounds or the extract fat or milk, and then the solvent was evaporated. The resulting residue [the mixture of dioxin(s) and Triton X-100] was dissolved in the assay buffer containing the monoclonal antibody. This procedure had to be done in a glass tube (not plastic tube or plate), and was important in order to increase the immunoreactivity of the dioxin(s) in the aqueous buffer.

To compare the assay sensitivity obtainable using each antibody, dose−response curves were constructed for 2,3,7,8-TCDD in combinations with the HRP-labeled hapten Ic (Figure 3). These assays have been done in the assay buffer containing 0.01% (v/v) Triton X-100 as the final concentration (the procedure for adding Triton X-100 is as described above). The optimum dilution rate of the antibody was arbitrarily determined as the dilution ratio affording the B_0 enzyme activity corresponding to 0.6−0.8 absorption units by a 1-h enzyme reaction. Although every antibody afforded a dose−response curve having an acceptable sensitivity, the curves of D2-37 and D9-36 were obviously more sensitive than D35-42.

We separately confirmed that the final concentration of Triton X-100 employed above (0.01%) is optimum by comparing the dose−response curves obtained at various concentrations of this detergent (Figure 4). In these experiments, antibody D9-36 was used employing TMDD as the competitor instead of 2,3,7,8-TCDD because TMDD provided dose−response curves almost equal to

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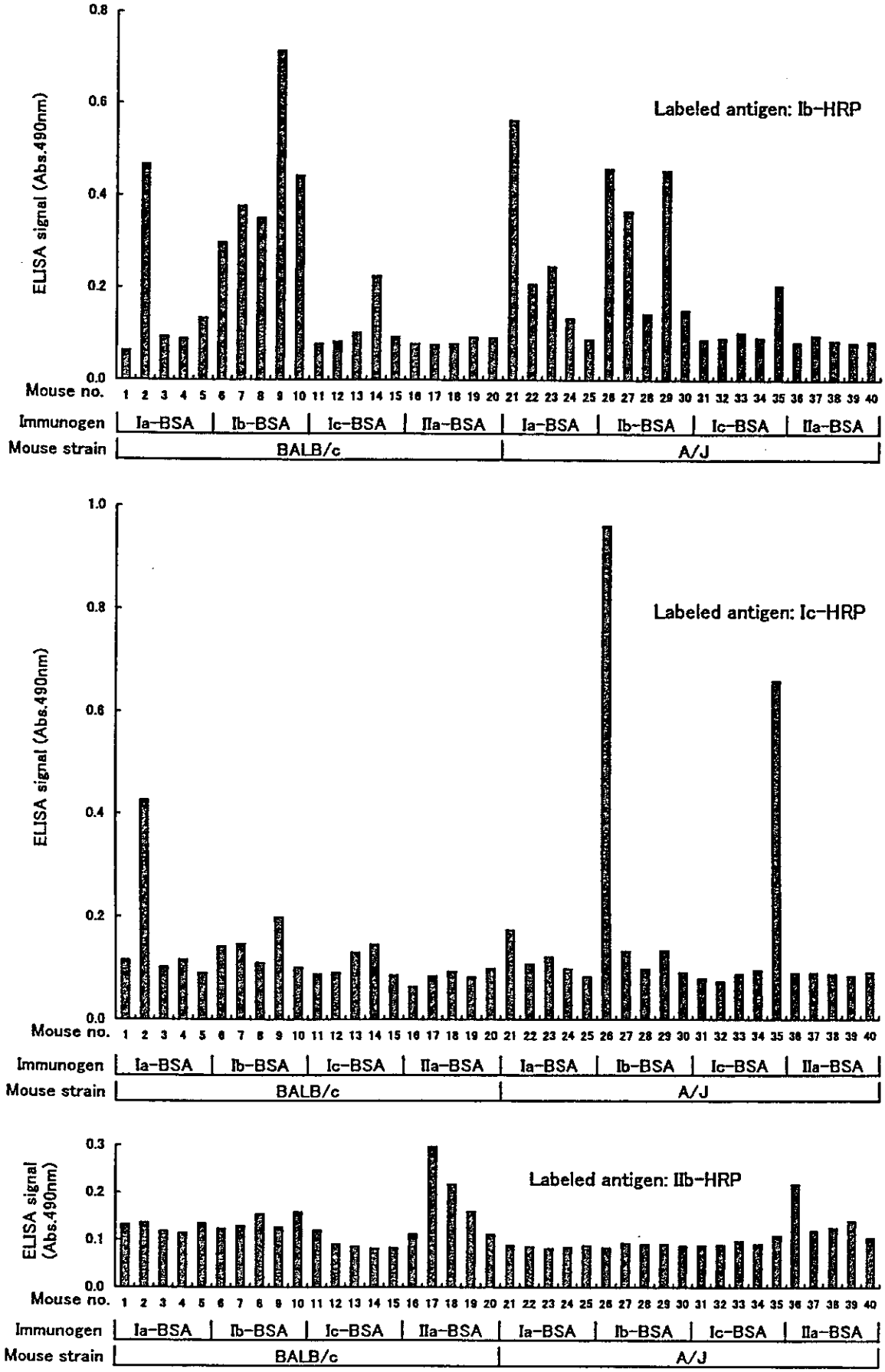

Figure 2. Comparison of the titer of anti-dioxin antibodies in the serum (1:25 000 dilution) obtained from the BALB/c or A/J mice after the fifth immunization with various immunogens (the BSA conjugate of haptenic derivative Ia, Ib, Ic, or IIa). Each bar shows the bound enzyme activity in the ELISA using the HRP-labeled hapten Ib, Ic, or IIb (25 ng/assay).

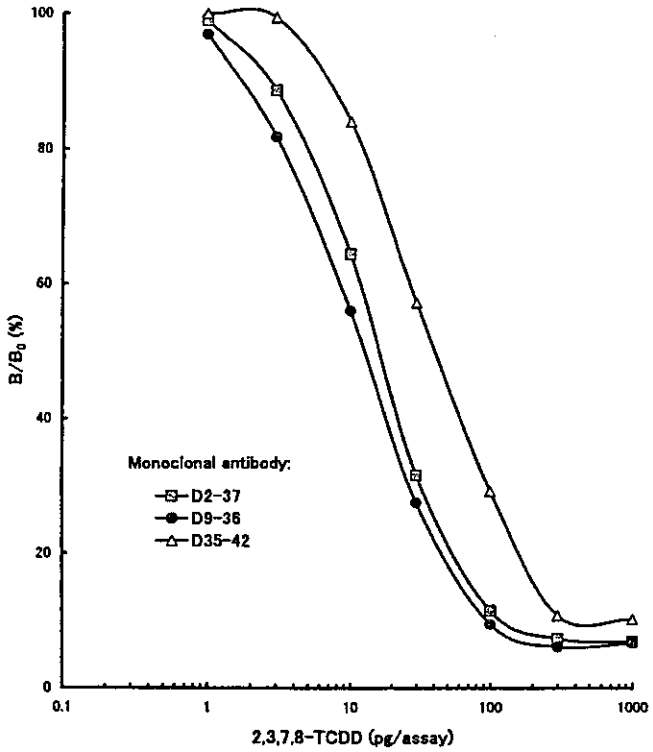

Figure 3. Dose–response curves for 2,3,7,8-TCDD in the ELISA systems using the monoclonal antibody D2-37 (4.0 ng/assay), D9-36 (0.4 ng/assay), or D35-42 (4.0 ng/assay) in combination with the enzyme-labeled hapten Ic (20 ng/assay) at a final Triton X-100 concentration of 0.01%.

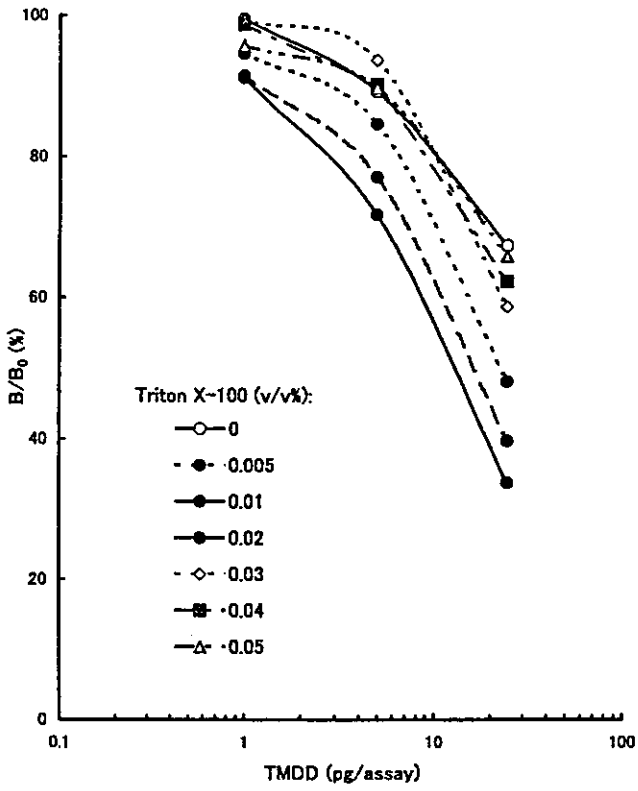

Figure 4. Effect of final concentration of Triton X-100 added to the assay medium on the assay sensitivity. ELISA was performed using the antibody D9-36 (1.0 ng/assay) in combination with the enzyme-labeled hapten Ic (20 ng/assay) at various concentrations of Triton X-100 [0, 0.005, 0.01, 0.02, 0.03, 0.04, or 0.05% (v/v)].

those obtained with 2,3,7,8-TCDD for antibody D9-36 (cross-reactivity of TMDD, 0.87; see Table 1). Therefore, this much less toxic congener is available as the standard compound instead of 2,3,7,8-TCDD, if necessary. Figure 4 also shows that the addition of Triton X-100 clearly increased the assay sensitivity and that the optimum concentration is 0.01%.

The cross-reactivity with various congeners of PCDD/F was then determined by the 50% displacement method[20] with a modification (Table 1), making it convenient to compare it with the toxicity-equivalent factor (TEF). TEF is defined to be the relative toxicity of a congener where the toxicity of 2,3,7,8-TCDD is taken to be 1.0, and TEQ is defined as the mass of a congener multiplied by TEF.[21,22] Namely, the cross-reactivity with each congener was expressed as the reciprocal of the ratio of its amount that is required for 50% displacement of the bound enzyme activity (midpoint), where the midpoint of 2,3,7,8-TCDD was taken to be 1.00 (e.g., the cross-reactivity value of 0.50 means that the congener required twice the amount as 2,3,7,8-TCDD). To establish an ELISA system for monitoring the sum of the toxicity of dioxins in breast milk, the antibody should possess a cross-reaction profile that is similar to the profile of TEF, particularly for the following major congeners detected in milk: 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 2,3,4,7,8-PeCDF. Antibody D2-37 exhibited a poor cross-reactivity (0.19) with 1,2,3,7,8-PeCDD, although this congener exhibits toxicity equal to that of 2,3,7,8-TCDD. Antibody D35-42 reacted with neither 2,3,7,8-tetrachlorodibenzofuran (2,3,7,8-TCDF) nor 2,3,4,7,8-PeCDF, both showing a considerable toxicity (TEF 0.1 and 0.5), although the cross-reactivity with 1,2,3,7,8-PeCDD was quite favorable. On the other hand, antibody D9-36 had a practically acceptable recognition pattern for these congeners, as shown by the cross-reactivity with 2,3,7,8-TCDF (0.14), 1,2,3,7,8-PeCDD (0.48) and 2,3,4,7,8-PeCDF (0.17). The relationship between the cross-reactivity of this antibody and TEF is schematically illustrated in Figure 5. The cross-reactivity of D9-36 with various derivatives of polychlorinated biphenyl (PCB), polybrominated dibenzo-*p*-dioxins and dibenzofrans, and BDE-47 (the polybrominated flame retardant which is most abundant in the environment) was also determined and found to be almost negligible, being in good correlation with the TEF values of these compounds (Table 2).

On the basis of these results, we choose antibody D9-36 for developing the ELISA system in combination with the HRP-labeled hapten Ic. This is a bridge heterologous system[19] because D9-36 is derived from the hapten Ib.

A typical dose–response curve for 2,3,7,8-TCDD obtained using the purified antibody D9-36 that covered the 1–100 pg/assay is shown in Figure 6. The midpoint was 15 pg/assay, and the detection limit was determined to be 2SD below the average B_0 (n = 10) was 1.0 pg/assay.

Application of ELISA to Milk and Butter Samples. The direct measurement of the dioxins in biological specimens containing considerable amounts of lipids, not only breast milk but also blood specimens, is almost impossible for immunoassays.

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(21) WHO fact sheet no. 225, June, **1999**.
(22) Van den Berg, M.; Peterson, R. E.; Schrenk, D. *Food Addit. Contam.* **2000**, *17*, 347–358.

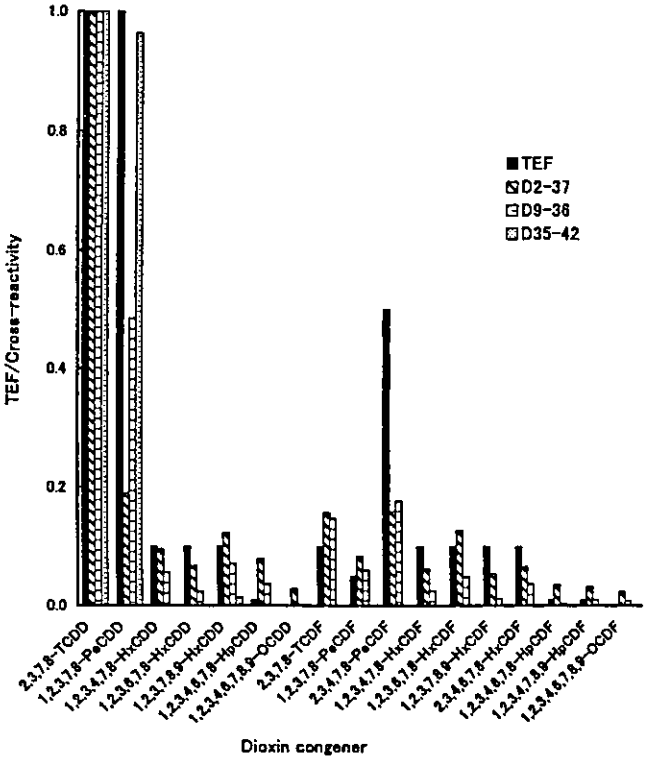

Figure 5. Schematic representation of the relationship between TEQ and the cross-reactivity of the monoclonal antibody D2-37, D9-36, or D35-42 for various dioxin congeners. Cross-reactivity was determined in the ELISA system using the enzyme-labeled hapten Ic (20 ng/assay) at a final Triton X-100 concentration of 0.01%. Abbreviations: HxCDD, hexachlorodibenzo-*p*-dioxin; HpCDD, heptachlorodibenzo-*p*-dioxin; OCDD, octachlorodibenzo-*p*-dioxin; HxCDF, hexachlorodibenzofuran; HpCDF, heptachlorodibenzofuran; OCDF, octachlorodibenzofuran.

Because dioxins are highly lipophilic compounds, it is essential to separate these compounds from the lipids that are abundant in these body fluids. Unless this has been achieved, accompanying lipids would interact with the dioxins by a strong hydrophobic attraction to make a kind of complex and consequently prohibit the dioxins from being captured by the hydrophilic antibody molecules.

Taking into account these difficulties and our goal, that is, the development of an ELISA system for the monitoring of the dioxin-contamination in human breast milk, we investigated a new cleanup procedure that is applicable to the lipid-abundant biological samples. To evaluate the efficiency of removing interfering lipids, we used the milk and butter samples, to which a known amount of toxic PCDD/F congeners (2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, or 2,3,4,7,8-PeCDF) had been added. Initially, these samples were submitted to saponification with potassium hydroxide, followed by the extraction of the dioxins with *n*-hexane. After a thorough washing with sulfuric acid, the *n*-hexane layer was further submitted to solid-phase extraction using the Presep cartridge which allows efficient isolation of the dioxins. As described above, the addition of Triton X-100 to the effluent from the cartridge was essential to afford the reasonable immunoreactivity of the recovered dioxins. The analytical recovery rates (mean ± SD) of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 2,3,4,7,8-PeCDF were 64 ± 3.7, 75 ± 6.7, and 85 ± 8.2, respectively.

Table 2. Cross-Reactivity of Monoclonal Antibody D9-36 with Various PBDD/F Congeners and PBCs in the ELISA[a]

congener[b]	cross-reactivity	TEF
2,3,7,8-TBDD	0.39	
1,2,3,7,8-PeBDD	0.04	
1,2,3,6,7,8-HxBDD	0.003	
1,2,3,4,6,7,8,9-OBDD	0.0007	
2,3,4,7-TBDF	0.16	
2,3,4,7,8-PeBDF	0.11	
3,3′,4,4′-TCB (PCB-77)	0.0008	0.0001
3,4,4′,5-TCB (PCB-81)	0.0009	0.0001
2,3,3′,4,4′- PeCB (PCB-105)	<0.0001	0.0001
2,3,4,4′,5- PeCB (PCB-114)	<0.0001	0.0005
2,3′,4,4′,5- PeCB (PCB-118)	<0.0001	0.0001
2′,3,4,4′,5- PeCB (PCB-123)	<0.0001	0.0001
3,3′,4,4′,5-PeCB (PCB-126)	0.0005	0.1
2,3,3′,4,4′,5-HxCB (PCB-156)	<0.0001	0.0005
2,3,3′,4,4′,5′-HxCB (PCB-157)	<0.0001	0.0005
2,3′,4,4′,5,5′-HxCB (PCB-167)	<0.0001	0.00001
3,3′,4,4′,5,5′-HxCB (PCB-169)	<0.0001	0.01
2,2′,3,3′,4,4′,5-HpCB (PCB-170)	<0.0001	
2,2′,3,4,4′,5,5′-HpCB (PCB-180)	<0.0001	
2,3,3′,4,4′,5,5′-HpCB (PCB-189)	<0.0001	0.0001
2,2′,4,4′-TBDE (BDE-47)	<0.0001	

[a] ELISA conditions are described in Figure 3. [b] Abbreviations: TBDD, tetrabromodibenzo-*p*-dioxin; PeBDD, pentabromodibenzo-*p*-dioxin; HxBDD, hexabromodibenzo-*p*-dioxin; HpCDD, heptabromodibenzo-*p*-dioxin; OBDF, octabromodibenzo-*p*-dioxin; TBDF, tetrabromodibenzofuran; PeBDF, pentabromodibenzofuran; TCB, tetrachlorobiphenyl; PeCB, pentachlorobiphenyl; HxCB, hexachlorobiphenyl; HpCB, heptachlorobiphenyl; TBDE, tetrabromodiphenyl ether.

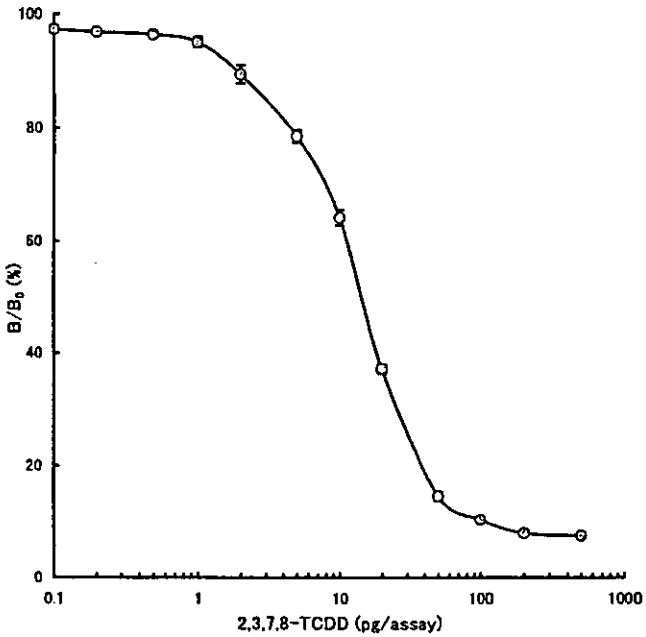

Figure 6. Typical dose–response curve for 2,3,7,8-TCDD in the optimized ELISA system using the monoclonal antibody D9-36 (0.50 ng/assay) in combination with the enzyme-labeled hapten Ic (20 ng/assay) at a final Triton X-100 concentration of 0.01%. Vertical bars denote SD of quintuplicate measurements.

The milk and butter samples containing the various amounts of the dioxin(s) were then extracted by this procedure and submitted to the ELISA. These assay values (2,3,7,8-TCDD equivalent in picograms) were in good correlation ($Y = 0.4182X + 3.5687$, $r = 0.9043$) with the sum of the TEQ (pg) of these

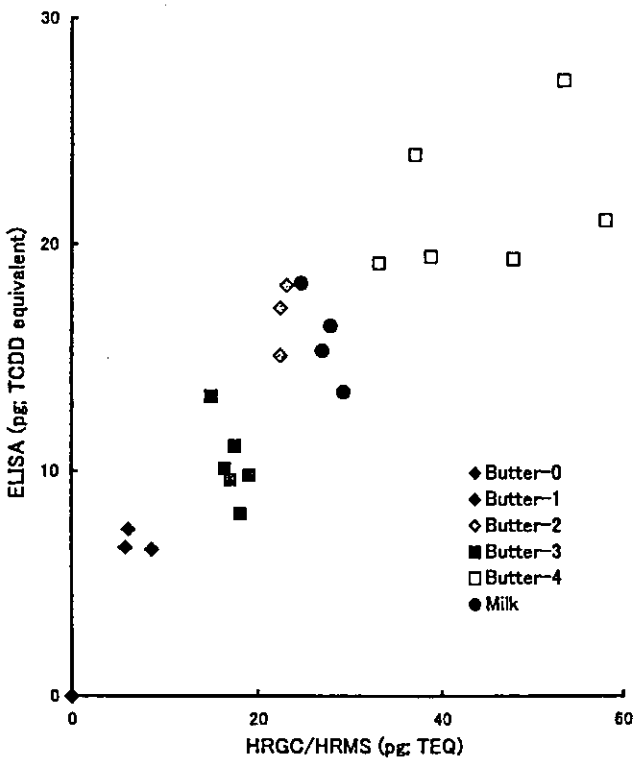

Figure 7. Correlation between the assay values (2,3,7,8-TCDD equivalent) obtained by the ELISA and the values (TEQ) obtained by the HRGC/HRMS method for milk or butter samples to which various amounts of toxic PCDD/F congener(s) were added as follows: milk, 10 mL of ordinal milk + 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 2,3,4,7,8-PeCDF (each 30 pg) (TEQ, 7.5 pg/mL); butter-0, ordinal butter only (TEQ, 0 pg/g); butter-1, the butter 1 g + 2,3,7,8-TCDD (10 pg) (TEQ, 10 pg/g); butter-2, the butter 1 g + 2,3,7,8-TCDD (30 pg) (TEQ, 30 pg/g); butter-3, the butter 1 g + 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 2,3,4,7,8-PeCDF (each 10 pg) (TEQ, 25 pg/g); butter-4, the butter 1 g + 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 2,3,4,7,8-PeCDF (each 30 pg) (TEQ, 75 pg/g).

congeners that had been separately determined by the HRGC/HRMS (Figure 7). Thus, the ELISA values are consistently ~40% of the TEQ values, results which are reasonable when one takes into account the imperfect coincidence between the cross-reactivity of the antibody D9-36 and the TEQ of the three kinds of dioxins (see Figure 5). Satisfactory parallelism was observed for the butter samples between the assay values and the amount of fat extract used in the assay (Table 3), indicating that the interfering materials have practically been removed. Mean analytical recovery rates of the three butter samples (1, 2, 3; Table 3) were 76, 70, and 69%, respectively.

Table 3. Determination of Toxic TCDD/F Congeners Contained in Butter Samples after Purification with the Present Cleanup Method and Serial Dilution

butter sample[a]	fat amount (g/assay)[b]	2,3,7,8-TCDD equivalent[b] pg/assay	pg/g fat
1	0.2	4.01	20.1
	0.4	7.50	18.8
	0.6	10.7	17.8
2	0.2	3.61	18.1
	0.4	7.11	17.8
	0.6	10.1	16.8
3	0.2	3.66	18.3
	0.4	6.79	17.0
	0.6	9.87	16.5

[a] Butter samples containing a known amount of 2,3,7,8-TCDD were prepared and purified in triplicate (for samples 1, 2, and 3) as follows: an ordinal butter (3.0 g) to which 2,3,7,8-TCDD (75 pg) had been added was saponified and extracted with the Presep cartridge according to the procedure described in the Experimental Section. [b] Aliquots of the effluent from the cartridge (0.25, 0.50, and 0.75 mL out from the initial 1.5 mL effluent) [each of which finally corresponds to 0.2, 0.4, and 0.6 g fat (extracted)/assay] were taken separately and mixed with 0.05% Triton X-100 in MeOH (50 μL). After the solvent was evaporated off, the resulting residue was submitted to the ELISA, as described in the Experimental Section.

DISCUSSION

Since Albro and co-workers reported the first immunoassay for dioxins using a polyclonal antibody and a ^{125}I-labeled dioxin analogue,[1] considerable effort has been made to develop a practical dioxin immunoassay system. However, two kinds of problems have been mainly hampering the need to establish a suitable assay system providing enough sensitivity and specificity. One of these problems is the difficulty in producing a practical antibody that group-specifically recognizes the limited kinds of toxic congeners from a family of PCDD/Fs that are composed of 75 kinds of congeners having very similar structures. Another problem has arisen from the highly hydrophobic nature of the dioxins. Because immunoassays rely on the antigen–antibody reaction that proceeds in aqueous media, we have to dissolve the hydrophobic dioxin molecules in the media and preserve their reactivity against the antibody. When an enzyme-aided immunoassay system (containing ELISA) is preferable, such difficulty even increases, because a large difference in the solubility between the highly lipophilic free dioxins and the dioxins labeled with a hydrophilic enzyme will prohibit a desirable competitive reaction against an anti-dioxin antibody.

Recently, Sugawara et al. succeeded in developing an ELISA system that is applicable to biological samples that uses a rabbit polyclonal antibody elicited against a hapten–carrier conjugate in which 2,3,7,8-TCDD had been linked to BSA via the 2-position.[8] This success would be due to the somewhat unusual nature of the antibody used in the assay: namely, the antibody maintained the antigen-binding ability even in an assay medium containing up to 37.5% dimethyl sulfoxide, which worked as a powerful solubilizing agent. This ELISA provided a much higher sensitivity (the midpoint was 12 pg/assay) than those of the previous dioxin immunoassays. It should also be considered that the ELISA system employed the competitive reaction based on the antigen-coated format (and using an enzyme-labeled second antibody as a detector) that could avoid the problem due to the difference in solubility between the labeled and unlabeled haptens mentioned above.

The use of monoclonal antibodies, however, should be desirable, because these antibodies can be constantly supplied by maintaining a well-defined and unique binding property and consequently enable an assay standardization. Although a few attempts have been made to generate monoclonal anti-dioxin antibodies, no one showing practical affinity and specificity to dioxin congeners has been reported so far. The most successful assay based on a monoclonal antibody would be the one reported by Harrison and Carlson.[6] They used a monoclonal antibody showing a practical specificity that had been elicited by Stanker et al.[4] The detection limit of this assay (reported to be 25 pg/

well), however, did not reach a "sub-parts-per-trillion sensitivity" that is required for analysis of dioxins in body fluids.

Taking advantage of the previous experience for producing some monoclonal antibodies against such hydrophobic small molecules as steroids or vitamin D metabolites,[11,12,23,24-26] we arranged a systematic immunization schedule combining two strains of mice and four haptenic derivatives in order to enhance the chance of obtaining a suitable spleen donor showing a stronger humoral response against dioxins. This strategy afforded several candidates as the donor, which allowed us five times the cell fusion experiments, after which we finally succeeded in establishing a hybridoma cell line secreting an antibody D9-36 having a practical recognition profile. Another important factor that led us to the present ELISA system was the use of Triton X-100 for solubilizing dioxins, the usefulness of which has previously been suggested.[1,5] The phenyl group in this detergent might have been a crucial factor for dissolving the dioxins (by forming a somewhat "loose" complex) and further let dioxins be immunoreactive in the aqueous solution. In the presence of the optimized amount of this detergent, excellent assay sensitivity [the midpoint, 15 pg/assay and the detection limit, 1.0 pg (corresponding to 3.1 fmol) per assay for 2,3,7,8-TCDD] was achieved. This sensitivity is comparable to that of the assay reported by Sugawara et al.[8] (see above) and much higher than the previous dioxin immunoassays using monoclonal antibodies. This successful high sensitivity, however, should be due to not only the effective solubilization of the target hapten but also a sufficient binding affinity of the antibody D9-36 to these congeners. Although we have not determined the *Ka* value of the antibody to 2,3,7,8-TCDD because of the unavailability of the corresponding radio-labeled compound, we expect that the value would be in the range of $\sim 10^9$ to 10^{10} (M^{-1}) taking into account our previous studies.[11,12,23,24,26]

It should also be emphasized that the present ELISA is the successful "labeled-antigen format" providing femtomole-range sensitivity that should be difficult to apply to such a hydrophobic target molecule as dioxins. To achieve the maximum sensitivity in this format, the very careful preparation of enzyme-labeled hapten is essential. Namely, in addition to the common consideration of the hapten/enzyme molar ratio[27] and combination of haptens used in the labeling and antibody production,[19,28] exhaustive removal of unreacted hapten-related molecules is required. The usual dialysis or gel chromatography is not effective enough for such hydrophobic haptens (see Experimental Section), because the unreacted haptens are noncovalently adsorbed on hydrophobic portions in enzyme molecules by a strong hydrophobic interaction and comigrate with the desirable enzyme-labeled haptens. We found that washing of the labeling reaction mixture with chloroform effectively removed the excess free dioxins with negligible loss of the enzyme activity [HRP activity after the treatment, 85 ± 3.7% (n = 6) of the initial activity]. This simple procedure should be widely applicable to a workup in the enzyme labeling of various hydrophobic haptens and helpful for improving assay sensitivity. A systematic study of this procedure will be reported elsewhere. One more important factor for obtaining a high sensitivity is the use of the purified monoclonal antibody. When the culture supernatant was used as the source of the antibody, much less sensitivity was obtained. Some components in the supernatant, for example, BSA derived from fetal calf serum supplied to the culture medium, might have become bound to the dioxins with a significant affinity (probably by hydrophobic interactions) and, consequently, might have decreased their reactivity against the antibodies.

We also established a new cleanup procedure that is essential for avoiding the problem inherent in the hydrophobic dioxins (mentioned above). To achieve this, the dioxins in biological fluids must be thoroughly separated from the accompanying lipids before the reaction with antibodies. Even after saponification of the samples, a trace amount of interfering lipids would be extracted together with dioxins, forming a kind of complex by hydrophobic interaction and, consequently, prohibit the access of antibodies. The usual normal-phase and reversed-phase chromatographies were not effective enough for removing such interferences. Fortunately, we found that the Presep cartridge is suitable for separating aromatic dioxin-related compounds from such an interference. Further optimization is now ongoing in our laboratories to improve the analytical recovery of all the target congeners. Affinity chromatography using a solid-phase immobilizing of the present antibody would be another promising way for isolating the dioxins from the lipids, because this method is based on a very specific molecular recognition toward the target compounds.

As shown in Figure 7, the present ELISA provides the assay values as the 2,3,7,8-TCDD equivalent that corresponds to ~40% of the total TEQ values of the important toxic congeners, 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 2,3,4,7,8-PeCDF. Although a new antibody showing perfect group-specificity to these congeners would afford almost the same values as the sum of the TEQ and would be even preferable, a satisfactorily good correlation (r = 0.9043; Figure 7) allows us to evaluate the present ELISA to be useful enough for routine surveillance of dioxin contamination. Very recently, we found that an improved enzyme-labeled antigen, in which the 3-hydrogen (instead of chlorine atom) analogue of the hapten Ic (which is expected to be less toxic than compound Ic itself) is linked to HRP, is also available in the ELISA system without altering the assay performance described so far.

In conclusion, the present "monoclonal ELISA" for dioxins is selective, has a sufficient sensitivity, is feasible, and consequently, does not require skillful operation or an expensive facility. This assay system will be particularly useful for the first screening of a number of breast milk specimens prior to the second examination for each congener by HRGC/HRMS.

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食品衛生外部精度管理調査の概要 －大腸菌検査に係る検査方法と調査成績について－

An Outline of External Quality Assessment for Food Hygiene
－ In the Reference to Qualitative Test for Escherichia coli and its Investigation Results －

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はじめに

従来，各地方自治体の衛生研究所（地研協議会主催）や食品衛生指定検査機関（指定検査機関協議会主催）などで独自に外部精度管理調査がなされ，各検査機関の検査精度の向上を推進していたが，食品衛生法の一部改正に伴い，平成9年度からは，全国の食品衛生検査施設（検疫所，地方衛生研究所，政令都市の衛生研究所，食品衛生検査所，食肉衛生検査所，市場衛生検査所，保健所，食品衛生指定検査機関）を対象に統一した外部精度管理調査が開始され財団法人食品薬品安全センターがその役目を果たすこととなった。これまでにも食品衛生にかかわる微生物検査に関する精度管理の重要性は早くから認識されてはいたが，諸外国も含め微生物検査の外部精度管理が実施されるようになったのは最近のことである。大腸菌検査に関する外部精度管理調査は，平成13年度より開始され，マッシュポテト基材（模擬食材）を調査試料として用いた。模擬食材のカテゴリーを平成13年度には「冷凍食品」，平成14年度には「加熱後摂取冷凍食品」と指定し，それぞれに対応する大腸菌の検査法は，各機関で作成されている標準操作手順書（SOP）に従って実施することとした。本誌では過去2年間の大腸菌検査について調査試料の作製，ならびに各機関で採用された検査方法とその検査成績についての概要と，特に今後の外部精度管理調査を進める上での大腸菌検査における注意点について概説する。

Ⅰ　調査試料作製法の検討とその確認試験

1　試験菌株および接種菌液調製

財団法人食品薬品安全センター秦野研究所保存の*Escherichia coli* HIC12011と*Klebsiella oxytoca* HIC12023を試験菌株として用いた。なお，試験菌液の調製に当たっては，調製するごとに試験菌株の生化学的性状を確認し，いずれの菌株も*E. coli*および*K. oxytoca*の性状を示す菌株であることを

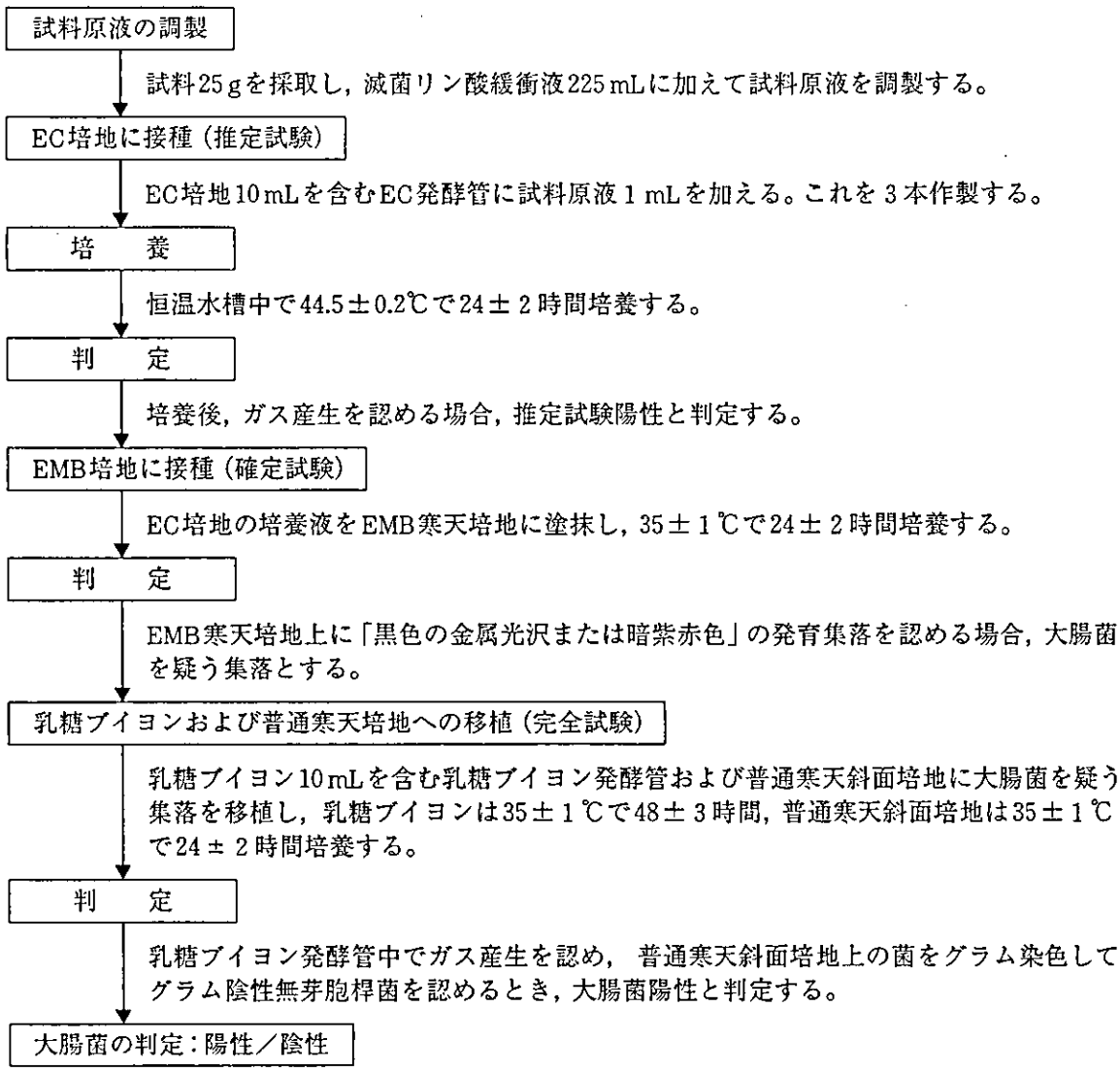

図1　加熱後摂取冷凍食品（凍結直前加熱以外）の大腸菌検査法（公定法）

確認した後，菌液調製に用いた。

いずれの菌株もソイビーン・カゼイン・ダイジェスト培地に移植して32.5±2.5℃で24±2時間培養した後，滅菌生理食塩液に懸濁して十分撹拌，均一化し，最終菌液濃度が約1.0×10^8colony forming units（cfu）/mLとなるように調製したものを試験菌液とした。

2　基材調製と調査試料の作製

市販のマッシュポテト（粉末）を用いて調査試料の基材を調製した。マッシュポテト1容量に対して安定化剤を含む生理食塩液5容量の比で混合し，十分撹拌して基材とした。基材50gを試料瓶に分取して121℃で30分間，高圧蒸気滅菌処理して調査試料用基材とした。調査試料用基材50gに対して試験菌液0.5mL（最終菌液濃度が約1.0×10^6cfu/g）を添加し，試験菌液と基材が均一になるように滅菌撹拌棒で十分撹拌して調査試料を作製した。

3　調査試料中の試験菌の生残菌数

調査試料を5週間低温室（約5℃下）に放置し，試験菌の生残菌数を測定して，試料中の試験菌についてその安定性を確認した。生残菌数測定は，

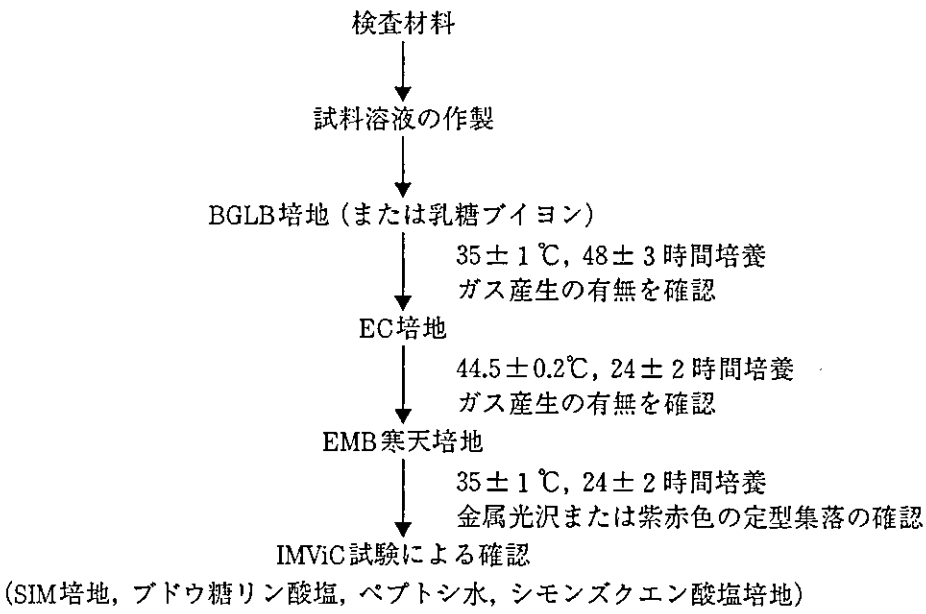

図2　糞便系大腸菌群および大腸菌の検査手順
（食品衛生検査指針）

試験菌接種直後および毎週1回，5週目まで試料瓶よりマッシュポテト10gを無菌的に採取し，滅菌リン酸緩衝液90mLに加えて測定溶液を作製して，標準寒天培地を用いた寒天平板混釈法により生菌数測定を行った。調査試料中の生残菌数を算出して，試験菌の低温保存における安定性を確認した。

4　調査試料からの大腸菌の検出確認

調査試料10gを無菌的に採取し，滅菌リン酸緩衝液90mLに加えて，ストマッカー処理し，得られた溶液を測定溶液とした。

この測定溶液を用いた大腸菌検査は，公定法（食品，添加物等の規格基準：厚生省告示第370号，昭和34年12月28日，図1）に従って実施した。また，公定法以外の方法として，食品衛生検査指針に従ったBGLB法（図2）についても併せて検出確認を実施した。各種確認培地上での発育集落の観察を行い，確認培地上に発育した大腸菌の性状を示す集落については，簡易同定キットを用いてその生化学的性状の確認を行った。なお，対照として使用した*K. oxytoca*についても同様の手順で検出の有無を確認した。この操作を生残菌数測定と同様に低温保存4週目まで大腸菌の検出の有無について毎週確認した。また，EC培地（44.5±0.2℃培養）およびBGLB培地（35±1℃培養）で48時間まで培養した時のガス産生率についても経時的に観察した。

Ⅱ　調査の実施

1　調査試料の配布および検査方法

実施年度の初めに実施項目と配布予定日のお知らせ，ならびに参加の申込みを行い，平成13年度は284施設，平成14年度は267施設が大腸菌検査に参加した。各参加機関に対して検査関連書類および大腸菌検査用調査試料2本／1組をクール宅急便にて郵送し，試験開始前まで冷凍を避けて低温保存を指定した。なお，調査試料は金曜日に期日指定（翌週月曜着）で発送し，週の初めから試験が開始できるように配慮した。また，調査試料は，単独菌（*E. coli*または*K. oxytoca*）接種で作

表1　低温保存によるマッシュポテト基材中での大腸菌（*Escherichia coli* HIC12011）数の推移

試　料	保　存　日　数						
	接種菌数	接種直後	Day 7	Day 14	Day 21	Day 28	Day 35
No.1	6.8×10^6	3.6×10^6	4.0×10^6	2.4×10^6	2.6×10^6	2.3×10^6	1.8×10^6
	6.8×10^4	5.8×10^4	3.6×10^4	4.0×10^4	2.9×10^4	2.1×10^4	1.2×10^4
No.2	6.8×10^6	6.8×10^6	5.6×10^6	2.8×10^6	1.8×10^6	2.5×10^6	1.4×10^6
	6.8×10^4	6.5×10^4	4.6×10^4	3.6×10^4	2.6×10^4	2.5×10^4	1.5×10^4

表中の数値はマッシュポテト基材1gあたりの生菌数を示す。

製し，試料瓶にはナンバーを記載したラベルを貼って区別した（平成13年度，14年度いずれもNo.1は*K. oxytoca*，No.2は*E. coli*とした）。なお，調査試料は，前項に示した方法に従い作製し，作製時と配布後4週間目（低温保存）に調査試料中の生菌数測定，ならびに試験菌の検出確認を行い，調査試料の有効性を確認した。

大腸菌検査の方法は，特に指定せず，模擬食材のカテゴリーを指定することで，各機関が独自に作成しているSOPに従って実施することを明記した。しかしながら，平成14年度実施については，推定試験にEC培地を用いて44.5±0.2℃で培養する方法を採用する時は，24±2時間培養で判定が陰性の場合，念のため培養時間を48±3時間まで延長して最終判定することをお願いした。

2　結果報告と評価方法

調査試料配布後，各機関に速やかに検査を実施されるようお願いし，調査試料の受領日，検査開始および終了日，検査担当者の経験年数，検査経過記録（検査手順の概略などの記載）を記載した書式，検査結果の報告書，ならびに調査試料に関する意見やアンケートなどを指定期日までに結果報告する形式を採用した。検査結果については，調査試料番号（No.1およびNo.2について）ごとに大腸菌の陽性または陰性の別を記載して結果報告することとした。

検査結果の評価については，調査試料該当番号に大腸菌の陽性・陰性の判定が適切になされているかによって行った。しかしながら，検査結果の報告に誤りがあった場合は，採用した試験方法の確認，ならびに試験方法によるものなのか，試料の取り違えなどによるものなのか，または記載ミスによるものなのかなどできるだけ経過記録書を参考に誤りの箇所を確認することとした。なお，調製した調査試料に関する情報，報告された経過記録書，ならびに検査成績に関する資料を評価委員会（外部の専門委員と厚生労働省から構成）に提出して最終評価を行った。参加機関に対する評価結果の報告は，年度ごとに全体の調査結果（食品衛生外部精度管理調査結果報告書）および個別評価結果表（必要に応じてコメントをつけて）を送付した。

Ⅲ　調査試料の確認結果と調査成績

1　調査試料作製および試験菌の検出確認結果

調査試料中の*E. coli*および*K. oxytoca*の生残菌数を測定した結果，試験に用いたいずれの試験菌株も添加直後の生菌数に比べ低温保存5週後では約1 Logの生菌数の減少を認めるが，マッシュポテト基材中で比較的安定した生残菌数で推移した（表1）。

EC培地で44.5±0.2℃で24±2時間培養した後，*E. coli*の発育確認とガス産生を観察した結

表２　EC培地（44.5±0.2℃培養）での大腸菌（*Escherichia coli* HIC12011）数の経時変化とガス産生率

接種菌数	培養時間					
	接種直後	3時間	6時間	24時間	30時間	48時間
10^1レベル (1.8×10^1)	＜10	＜10	1.6×10^3	6.2×10^7	1.2×10^8	3.1×10^7
ガス産生	ND*1	ND	ND	15*2	25	60
10^3レベル (1.8×10^3)	2.0×10^2	8.7×10^3	7.6×10^4	5.7×10^7	6.0×10^7	5.7×10^7
ガス産生	ND	ND	ND	20	30	60

＊１：not detected　＊２：ダーラム管全体に占めるガス産生率（%）を示す

表３　BGLB培地（35±1℃培養）での大腸菌（*Escherichia coli* HIC12011）数の経時変化とガス産生率

接種菌数	培養時間					
	接種直後	3時間	6時間	24時間	30時間	48時間
10^1レベル (1.8×10^1)	＜10	2.1×10^2	2.1×10^3	1.2×10^8	7.5×10^7	6.2×10^7
ガス産生	ND*1	ND	ND	30*2	40	80
10^3レベル (1.8×10^3)	1.1×10^2	3.6×10^3	9.9×10^4	1.0×10^8	8.3×10^7	7.0×10^7
ガス産生	ND	ND	ND	30	40	60

＊１：not detected　＊２：ダーラム管全体に占めるガス産生率（%）を示す

表４　EC培地およびBGLB培地中でのガス産生率を指標とした低温保存調査試料からの大腸菌（*Escherichia coli* HIC12011）の発育確認結果

増殖培地		初発菌数*1	低温保存日数（約5℃下放置）									
			Day 0		Day 7		Day 14		Day 21		Day 28	
			24h*2	48h	24h	48h	24h	48h	24h	48h	24h	48h
EC培地	No.1	3.5×10^6	15*3	60	10	60	5	40	5	30	10	50
44.5±0.2℃培養	No.2	4.8×10^6	20	60	15	60	15	50	15	60	10	50
BGLB培地	No.1	3.5×10^6	30	70	30	60	25	60	30	55	20	70
35±1℃培養	No.2	4.8×10^6	30	70	30	50	25	60	35	60	15	40

＊１：初発菌数はマッシュポテト１gあたりの生菌数を示す
＊２：培養時間を示す
＊３：ダーラム管全体に占めるガス産生率（%）を示す

果，推定試験においてガス産生の確認は十分可能であったが（表２，３），低温保存時間に依存してガス産生能は低下する傾向が見られた（表４）。また，マッシュポテト基材中の接種菌数によっては推定試験の判定結果に影響を及ぼすと推測され（表２），約10^6cfu/g程度の生菌数が適切であると判断した（表４）。対照菌である*K. oxytoca*は，この条件下で発育しないことを確認した。調査試料は，試料作製から発送，試験開始までの時間的経過を考慮しても，大腸菌の検出は可能であると

推察した。また，EC培地で44.5±0.2℃で24±2時間培養により十分判定ができない場合でも48±3時間まで培養時間を延長すると判定がより明確であった。これらの結果より，マッシュポテトを基材とした調査試料は，模擬食材として食品衛生外部精度管理調査に使用可能であると判断した。

2　参加施設で採用された検査方法および検査結果

大腸菌検査の外部精度管理調査を実施した検査機関は，平成13年度が281施設，平成14年度が258施設であり，これらの施設を対象に調査結果をまとめた。

調査試料について模擬食材のカテゴリーを指定した結果，平成13年度は公定法（図1）を採用した検査機関が166施設，公定法と公定法以外の方法を併用した検査機関が30施設，平成14年度は公定法および公定法に一部準拠した方法（EC発酵管で44.5±0.2℃培養）を採用した検査機関が212施設であった。また，公定法以外の方法のみを採用して実施した検査機関が，平成13年度は85施設，平成14年度は46施設であった。平成13年度は，公定法の判定基準で大腸菌の検出ができなかった施設においては，公定法および公定法以外の方法を併用して検査成績を報告した施設が見受けられたが，公定法以外の方法のみで実施した検査機関は，公定法の判定基準での大腸菌の検出の可否については明らかではなかった。なお，報告された公定法以外の検査方法について代表的な例を表5に示した。また，大腸菌の同定に簡易同定キットを採用して最終判定を行った検査機関は，平成13年度は129施設，平成14年度は123施設であり，最終判定をIMViC試験で行った検査機関は，平成13年度は61施設，平成14年度は65施設であった。なお，用いられた簡易同定キットは，アピ（API20E）同定キット，バイオテスト1号，IDテストEB20，BBL CRYSTAL E/NF，エンテオグラム，エンテロチューブⅡ，VITECなどが採用されていた。また，EC発酵管で44.5±0.2℃培養した場合，培養時間が24時間では判定が困難なことも考えられたため（表4　平成13年度実施の経過記録書より），平成14年度については，EC培地での培養時間を必要に応じて延長するようにあらかじめお願いした。その結果，平成14年度では，公定法を採用した機関の中で48時間まで培養時間を延長して得られた結果を報告した検査機関は27施設あった。

大腸菌を含む調査試料の検査結果を陰性（検出せず）として報告した検査機関は，平成13年度は7施設あり，この内の5施設は公定法以外の検査方法を併用して正しい回答を得ていたが，報告書には公定法での結果を記載していた。また，平成14年度は2施設が誤った回答を示していたが，いずれの施設も経過記録書中では試験菌が正しく検出されており，誤回答は記載ミスによるものと判断した。

Ⅳ　考　　察

過去2年間の実施では，マッシュポテト基材を用いた調査試料を模擬食材とし，模擬食品のカテゴリーを指定することで食品衛生外部精度管理調査を実施した。また，模擬食材は，いずれも冷凍食品（または加熱後摂取冷凍食品）をそのカテゴリーとして検査するよう指定した。さらに，大腸菌検査は，日常実施している検査手順（標準操作手順書SOP）に従って実施することとし，添付資料には検査方法などを指定せず，各機関のSOPに従って実施することを記載した。

加熱後摂取冷凍食品を指定した場合，大腸菌の検査方法は，EC培地3本法（図1）が通常選択されることとなる（公定法）。平成13年度実施の調査結果では，公定法で実施した結果，試料中に

表５－１　大腸菌検査において採用された公定法以外の検査方法（第一段階で液体培地による増菌培養を実施する）

	第一段階培養	第二段階培養	第三段階培養	第四段階培養	判　定
1	BGLB培地 35±1℃ 48±3時間培養	EC培地 44.5±0.2℃ 24±2時間培養	EMB寒天培地 35±1℃ 24±2時間培養	LB培地 35±1℃ 48±3時間培養	簡易同定キット IMViC試験
				－	簡易同定キット IMViC試験
2	LB培地 35±1℃ 48±3時間培養	EC培地 44.5±0.2℃ 24±2時間培養	EMB寒天培地 35±1℃ 24±2時間培養	－	IMViC試験 簡易同定キット
		－		－	簡易同定キット
3	普通ブイヨン 35±1℃ 48±3時間培養	マッコンキー寒天培地 DHL寒天培地 35±1℃ 24±2時間培養	普通寒天培地 35±1℃ 24±2時間培養	－	IMViC試験 簡易同定キット
4	トリプトソイ液体培地 35±1℃ 48±3時間培養	DHL寒天培地 EMB寒天培地 MLCB寒天培地 35±1℃ 24±2時間培養	－	－	簡易同定キット

表５－２　大腸菌検査において採用された公定法以外の検査方法（第一段階で液体培地による増菌培養を実施しない）

	第一段階培養	第二段階培養	第三段階培養	判　定
1	デソキシコレート寒天培地 35±1℃，48±3時間培養	EMB寒天培地，35±1℃， 24±2時間培養	LB培地，35±1℃， 48±3時間培養	IMViC試験
		トリプトソイ培地， 35±1℃，24±2時間培養	－	簡易同定キット
2	標準寒天培地，35±1℃， 48±3時間培養	EMB寒天培地，35±1℃， 24±2時間培養	EC培地 44.5±0.2℃，24±2時間培養	簡易同定キット

大腸菌が存在することを確認しているにもかかわらず大腸菌が検出されないと結果報告した機関も少なくなかったが，最終的にはそれらの機関の多くが，表５に示したような公定法以外の方法を併用して検査結果を記載していた。一方，公定法以外の方法では大腸菌が検出されているが，結果報告には公定法で実施した結果を採用して「大腸菌は検出せず」と記載して提出した機関もあった。

これまで実施した大腸菌検査の各機関の回答と経過記録の内容について吟味すると，外部精度管理調査（日常の検査においても）を行ううえでさまざまな問題点が含まれていると考えられる。すなわち，１）調査試料の不備，２）輸送における調査試料中の試験菌数の減少，３）推定試験で実施されるEC培地の培養温度（44.5±0.2℃）による試験菌の発育遅延または死滅，４）EC培地での培養時間（24±2時間）の不足，５）推定試験（EC培地の培養）におけるガス産生による判定基準，６）調製したEC培地の培地性能，７）調査試料の低温保存期間中に生じた大腸菌の細胞損傷などが，それぞれ大腸菌検査における陰性判定の主な要因と考えられる。

調査試料の調製は，マッシュポテト基材に試験菌を接種して作製しているため，実際の食材中で

起こる汚染大腸菌の分布と模擬食材に分散させた試験菌の分布を考えた時，第1にそのマトリックスへの分布様相に大きな違いが考えられる。今回使用した調査試料中には，大腸菌は比較的均一に分散し，基材中で少なくとも4週間は生菌数に大きな変動を生じないことは確認されている。また，調査試料中に添加されている安定化剤やマッシュポテト自体がEC培地中での大腸菌の増殖に影響を及ぼすような結果は認められていない（未発表成績)。さらに，EC培地で不検出の参加機関における調査試料中の生菌数測定成績を見ると，発送時の調製菌数とほぼ同レベルの数値が記されている。これらのことから輸送中での試験菌数の減少はほとんどないものと判断される。

日常検査の対象となる食材の保存条件を考えると，汚染大腸菌の存在する環境条件はさまざまであり，冷凍や低温環境下に長期間保存されることは良く見受けられる。このような保存条件下においては大腸菌が保存期間中に細胞損傷を起す可能性が十分考えられ，日常の検査も含め，保存期間中に大腸菌に何らかの細胞損傷が生じた場合，推定試験にEC培地を用いて培養する公定法では培養温度（44.5±0.2℃）や培地の選択性から大腸菌の発育遅延や死滅が起こりうる可能性が容易に想像される。特に，発育遅延が生じた場合には，規定される培養時間（24±2時間）内にガス産生を指標とした結果判定に影響を及ぼすことになる。したがって，推定試験でのEC培地の培養条件（培養温度および培養時間）は，検査の判定結果に大きな違いを導く重要な要因となりうる可能性が高い。調査結果の一例として，公定法に従って推定試験を実施した結果，24時間培養では大腸菌の判定が陰性（ガス産生を認めない）であったが，さらにこの培地を翌日まで放置するとガス産生が確認できたので同定を実施した事例もある。

以上の事象を考えあわせると，糞便性大腸菌に対する選択性を高めるためにあえて高めの培養温度（EC培地，44.5±0.2℃培養）に設定されている推定試験条件下では，調査試料の保存条件や試料受領後の検査開始時期の違いによって試験菌の発育障害とガス産生能の低下が懸念される。したがって，平成14年度実施の大腸菌検査に当たって公定法による推定試験を実施する場合，基準となる24±2時間培養で判定が不確かな時は，培養時間を48±3時間までさらに延長して判定するよう記載した（評価の段階では，各機関で使用されているSOPから逸脱することは考慮した)。その結果，公定法を用いて実施した検査成績を報告した施設が前年に比べて増加した。また，24±2時間で判定可能であった機関は185施設，48±3時間まで延長して判定した機関は27施設であった。今回の調査結果からのみではEC培地の培地性能に関するメーカー間の相違について明確な結果は得られていない。

食品衛生外部精度管理調査試料の作製に当たっては，標準菌としてどのような由来または性状の

試験菌株を用いるか，基材中・保存期間中または輸送中の試験菌の安定性をどのように確保するか，輸送中の調査試料の変質をどのように避けるか，検査方法の違いによる試験菌の検出の有無などについて予備検討を重ねながら安定した調査試料の配布に心がけており，日頃から菌株の選択，輸送法，保管法などに関する検討を重ねて，改良策を講じてきている。参加機関からも1）調査試料の発送日に関する要望，2）調査試料の形態（実際の食材を用いた調査試料）に関する要望，3）調査試料の配布量に関する要望，4）検査手順に関する要望，5）検査成績の評価法に関するさまざまな意見が寄せられている。

現在，調査試料は，週の初めから試験が開始できるように週末に期日指定（月曜日着）で発送するように変更し，試験菌の安定性や基材の変質を避けるため，調製から発送までの期間の短縮と冷蔵便による発送に変更するなど対応している。試料形態や配布量についても，実際の食材を基材とした調査試料の調製に関する検討を進めており，輸送による調査試料の安定性への影響を確認した後，配布量も考慮した調査試料の提供が可能と考える。

おわりに

大腸菌にかかわる食品衛生外部精度管理調査は，開始されて3年目を迎えるが，過去2年間の調査結果を総括すると模擬食材のカテゴリーの指定によって選択された検査方法と検査方法のもつ特異性を十分把握して検査を実施しなければ，外部精度管理調査の結果評価に大きな影響をもたらす要因となりうることが示唆される。また，これらのことは日常検査においても起こりうる問題と考えられる。したがって，適切な検査の遂行と検査結果の評価を行うために，試験菌株の選択，調査試料の形態とその作製方法，調査試料の輸送方法，検査方法の選択，検査結果の評価方法などについてさらに詳細な検討を加える必要があり，今後取り組まなければならない課題が多いと考えている。

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