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MOLECULAR AND IMMUNOCHEMICAL STUDIES ON MONOCLONAL ANTIBODIES SPECIFIC TO ENVIRONMENTAL CHEMICALS

環境負荷化学物質に特異的なモノクローナル抗体の分子及び免疫化学的研究

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MOLECULAR AND IMMUNOCHEMICAL STUDIES ON MONOCLONAL ANTIBODIES SPECIFIC TO ENVIRONMENTAL CHEMICALS

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CONTENTS

ABBREVIATIONS

AP:	alkylphenol
APEO:	alkylphenol ethoxylate
BSA:	bovine serum albumin
BPA:	bisphenol A
CDR:	complementarity-determining region
EC_{50}:	median effective concentration
ED:	endocrine disruptor
ELISA:	enzyme-linked immunosorbent assay
FR:	framework region
Fv:	variable fragment
GC:	gas chromatography
HPLC:	high-performance liquid chromatography
HRP:	horseradish peroxidase
IC_{50}:	median inhibitory concentration
IgG	immunoglobulin G
KLH:	keyhole limpet hemocyanine
LC:	liquid chromatography
MS:	mass spectrometry
Mab:	monoclonal antibody
NP:	4-nonylphenol
NP1EC:	4-nonylphenol ethoxylate acetic acid
NP10EO:	4-nonylphenol decaethoxylate
OVA:	ovalbumin
Pab:	polyclonal antibody
PBS:	phosphate-buffered saline
RSA:	rabbit serum albumin
scFv:	single-chain Fv
TMB:	3,3′,5,5′-tetramethylbenzidine
VH:	variable heavy chain
VL:	variable light chain

CHAPTER I

GENERAL INTRODUCTION

Biomonitoring for Environmental Chemicals

The compounds stably remained in the environment for a long term and suspected to adversely affect the wildlife and human health such as hormonal balance disruption are called "environmental chemicals," which include dioxins, endocrine disruptors (EDs), pesticide residues and so on. Collecting information on the distribution and fate of these chemicals in the environment is of great significance for risk assessment on wildlife and human health. Currently, monitoring of environmental chemicals has been carried out by instrumental analysis such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-MS (LC-MS) due to the high accuracy and precision. However, these methods are not only time-consuming and very expensive but also require skills and complicated clean-up procedures. In addition, quantitative instrumental analysis provides no information on biological effects necessary to evaluate the potential of such compounds for adverse effects on wildlife and human health. Namely, instrumental analysis is incompatible with a simple determination and assessment of biological effects by environmental chemicals. Because a mixture of environmental chemicals at a trace level may have the potential complex interactions of synergism, antagonism and additivity, a rapid and simple monitoring method is alternatively needed, which can detect the biological effects by these chemicals in extracts of environmental samples such as soil, water and biota. The monitoring methods based on the biological functions can fulfill these requirements as mentioned above. In the biological methods, organisms, cultured cells, enzymes, receptors and antibodies are used as biological materials. The methods provide us with the information about the complex effects by environmental chemicals on wildlife and humans, which are unavailable through an instrumental analysis. However, it is impossible

to quantify and identify the compounds showing the biological effects in environmental samples in lots of biological assays. In this chapter, the outline of the biological monitoring methods, especially immunoassays, for environmental chemicals are described.

Biological Assay Based on Organism and Cultured Cell

Biological assays using organisms and cultured cells to evaluate biological effects of environmental chemicals are generally called "bioassay." The assays are used not only for assessment of acute toxic and genetic tests but also for endocrine disrupting effects.

Organization for Economic Co-operation and Development (OECD) adopted seventeen test guidelines and presented eight draft guidelines (including revised TG202 and TG208) to assess chemicals for the effects on biotic systems as shown in Table 1-1.[1] For example, in the guideline number 202 "Daphnia sp., Acute Immobilization Test and Reproduction Test," immobilization of daphnids by chemicals in a water sample is recorded and the results were calculated as EC_{50} (the effective concentration required for 50% immobilization). Ministry of the Environment of Japan has conducted the ecological risk assessment of chemicals in conformity with the OECD test guidelines. Japanese Industrial Standards (JIS) also standardized some bioassay guidelines as official methods of analysis.

Vitellogenin is a phospholipoglycoprotein precursor of egg yolk and synthesized by the liver in response to estrogens in only mature females. Because vitellogenin is normally undetectable in the blood of male fish, but the vitellogenin levels rise dramatically by exposure to compounds possessing estrogenic activity, a bioassay based on the potential of vitellogenesis used as a biomarker for endocrine disruption is useful for detecting estrogenic contaminants in water samples.[2] Mutatox™ test (Azur Environmental Ltd., Berkshire, UK) is an assay using a special dark strain of luminescent bacteria for the presence of genotoxic chemicals in water samples. Water samples are mixed with a medium in the test kit and incubated for 24 h after

addition of bacteria to the mixture. If genotoxic compounds are in samples and mutations occur, the luminescence will be recovered and checked by luminometer.

Table 1-1. OECD's guidelines for the testing of chemicals

TG Number	Title
201	Alga, Growth Inhibition Test
202	Daphnia sp. Acute Immobilisation Test and Reproduction Test
203	Fish, Acute Toxicity Test
204	Fish, Prolonged Toxicity Test: 14-Day Study
205	Avian Dietary Toxicity Test
206	Avian Reproduction Test
207	Earthworm, Acute Toxicity Tests
208	Terrestrial Plants, Growth Test
209	Activated Sludge, Respiration Inhibition Test
210	Fish, Early-Life Stage Toxicity Test
211	Daphnia magna Reproduction Test
212	Fish, Short-term Toxicity test on Embryo and S ac-Fry Stages
213	Honeybees, Acute Oral Toxicity Test
214	Honeybees, Acute Contact Toxicity Test
215	Fish, Juvenile Growth Test
216	Soil Microorganisms, Nitrogen Transformation Test
217	Soil Microorganisms, Carbon Transformation Test
201	Frechwater Alga and Cyanobacteria, Growth Inhibition Test
202	Daphnia sp., Acute Immobilization Test
208	Terrestrial (Non target)-Plant Test:
208A	Seediling Emergence and Seedling and Seedling Growth
208B	Vegetatice Vigour Test
218	Sediment-Water Chironomid Toxicity Test Using Spiked Sediment
219	Sediment-Water Chironomid Toxicity Test Using Spiked Water
220	Enchytraedae Reproduction Test
221	Lemna sp. Growth Inhibition Test
222	Earthworm Reproduction Test (Eisenia fetida/andrei)

As described, bioassays are reliable methods to evaluate the biological effects of environmental chemicals. However, the assays are not suited for a large-scale screening of chemicals before their release into the environment because they are time-consuming and labor-

intensive to keep animals used for experiment. Besides, there are ethical concerns in some cases such as rodent bioassays.

Biological Assay Based on Enzyme

Because some chemicals affect certain organisms by enzyme inhibition and/or induction, biological assays based on enzymes are useful to detect such chemicals. For example, organophosphorus and carbamate pesticides show acute toxicity by disrupting the neurotransmission based on the inhibition of acetylcholinesterase.[3] Agri-Screen® Ticket (Neogen Co., MI, USA) is a test for detection of these compounds in environmental and food samples based on this principle of cholinesterase inhibition. The results are indicated by color change based on the enzyme reaction whether enough organophosphorus or carbamate insecticide is present or not in tested samples, which inhibits the enzyme activity. This kit is very simple and the results will be obtained in four minutes.

When mammals are exposed with dioxins, these chemicals bind tightly to the aryl hydrocarbon receptor (AhR) and the induction of cytochrome P450 (CYP)1A isozymes (CYP1A1 and 1A2) and their associated ethoxyresorufin-*O*-deethylase (EROD) activity are rapidly and sensitively increased under direct regulation of the AhR in cells.[4,5] The capacity of single compounds or complex mixtures to specifically induce EROD activity after activation of AhR is considered to be a reasonable measure of their toxic potential. While, the toxicity of dioxins including certain polychloricated dibenzo-*p*-dioxins, polychloricated dibonzofurans and polychlorinated biphenyls (PCBs) are different. Toxicity equivalency factor (TEF) is defined for individual dioxin-like compounds as relative toxicity to the most toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and the TEF value of TCDD is equal to one.[6,7] The toxicity of mixture of dioxin-like compounds is evaluated based on toxicity equivalent (TEQ) approach. TEQ is defined as the sum of the concentration of an individual dioxin-like compound in a given mixture multiplied with a corresponding TEF value. Then, some

researchers developed rapid and sensitive enzyme assays for measuring EROD activity as an indicator of CYP1A enzymatic activity with multiwell cell culture plates using primary cultures of chicken embryo hepatocytes and rat hepatoma H4IIE cells[8] based on this mechanistic knowledge,[9,10] because good correlation exists between the TEQ values and their EROD-inducing potency. In the assays, EROD-inducing potencies of single compounds or complex mixtures of dioxins extracted from environmental samples are determined from a dose-response curve prepared with TCDD in order to express the biological potency of the tested samples in TEQs. Li *et al.*[11] evaluated the reliability of an EROD assay by comparing to the data determined by high-resolution GC coupled with high-resolution MS (HRGC-HRMS). TCDD standards or the extracts from chimney soot, fly ash, fish liver and sediment samples were subjected to the assay. The EROD activity was determined by measuring a fluorescent product deethyl-7-ethoxyresorufin by the enzyme reaction in a fluorescence plate reader, and the TEQ values were estimated. The detection limit in this assay was 40 fg TCDD/well in a 96-well plate. Good correlation between the data from both determinations was observed (r^2 = 0.9849, p < 0.05) although the EROD assay results were higher than those from HRGC-HRMS by a factor of 1.1 to 3.4.

Enzyme assays have limitations such as the instability and inavailablility of target enzymes. However, there are possibilities to develop simple and rapid assays for risk assessment of environmental chemicals by using recombinant human enzymes obtained by gene engineering.

Biological Assay Based on Receptor

Receptor assays are based on the binding between chemicals and receptors. Many *in vitro* receptor assays have been developed, especially using AhR and estrogen receptor (ER) specifically binding to dioxins and estrogen-mimicking compounds, respectively. Then, the chemical (ligand)–receptor protein complex induce expression of target genes by

binding to the specific response element on the 5′-upstream of the target genes. Here, some *in vitro* biological assays employing the AhR- and ER-mediated activity are described.

AhR

Dioxins act as a persistent agonist for the AhR, and toxic effects of dioxins are linked to specific AhR-mediated processes. When dioxins enter mammalian cells, they bind to the AhR in the cytosol. Then, AhR is transformed, translocated into the nucleus, and forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (Arnt). The complex binds to a specific DNA enhancer sequence, termed xenobiotics-responsive element (XRE), and acts as transactivator of gene expression, resulting in increasing CYP1A1 gene transcription.[4,5]

A chemical-activated luciferase gene expression (CALUX) assay using AhR was developed by Murk *et al.*[12] A rat hepatoma H4IIE cell line stably transfected with a plasmid containing luciferase reporter gene under transcriptional control of XREs of the mouse CYP1A1 gene was employed. When the cells were exposed with AhR-active compounds like dioxins in samples, luciferase gene was expressed mediated with AhR and dioxin-like chemicals was detected by measuring light intensity as luciferase activity in a luminometer. The assay could detect 0.5 fmol of TCDD. Pauwels *et al.*[13] collected 106 serum and 9 follicular fluid samples from infertile women to assess the exposure to dioxin-like compounds. Samples were analyzed by using GC with electron capture detection (GC-ECD) analysis and the CALUX assay. The TEQ levels determined by CALUX in both matrices were well correlated with the sum of four major PCB congeners (PCB-118, PCB-138, PCB-153 and PCB-180) analyze by GC-ECD ($n = 7$, $r = 0.83$, $p = 0.02$).

An immunoblot assay combined with AhR and anti-AhR antibodies was developed to measure TCDD and its related congeners.[14] Hepatic cytosol was prepared from guinea pigs and treated with dioxins. After that, the transformed AhR was specifically isolated on an affinity

column immobilized with XRE oligonucleotides and eluants were dot-blotted onto a membrane. The blots were detected with anti-AhR antibodies and scanned to calculate a concentration of dioxins with AhR protein amount. Transformation of AhR with dioxins was dose-dependent and detection limit of TCDD was 40 ppt. Ah immunoassay® (Paracelsian, Inc., NY, USA) is a kit for determination of dioxins, which measures the transformed AhR protein with the specific antibodies. Samples mixed with AhR, Arnt and oligonucleotides encoding XRE are added to wells of a 96-well microplate. Dioxins in samples bind to AhR-Arnt dimer and then the complex specifically binds to the surface of the wells via XRE oligonucleotides. After the complexes are captured with specific antibodies and secondary antibodies conjugated to enzyme, chromogenic substrate is added to visualize. The TEQ value of the dioxins in the sample is calculated by comparing with a standard curve for TCDD. The assay is applicable to air, water, soil, food and serum samples, and the detection range of TCDD is 1 ~ 64 pg/well during 6 h of measuring time in an assay.

ER

ER is also a member of the superfamily of transcriptional factors. Estrogens play a major role in vertebrate reproduction. A variety of compounds including natural products, pharmaceuticals, and industrial chemicals have been shown to mimic the natural estrogen 17β-estradiol (E_2) and compete for binding to the ER. Binding of ligands to ER leads to conformational change of the protein, and a homodimer is formed. The complex phosphorylated by protein kinase acts as a transcriptional enhancer by binding to an estrogen responsive element (ERE).[15,16]

Relative binding affinity of estrogen receptor was determined by competitive binding assay with [^{3}H]-E_2.[17] The amount of [^{3}H]-E_2 bound to the receptor was measured by liquid scintillation counter. Garrett *et al.*[18] developed a rapid and sensitive ligand-binding assay combined

with enzyme immunoassay to detect EDs based on human ER (hER) without using radiolabeled E_2. The hER, E_2 and samples were added to E_2–coated wells of a microtiter plate. Because hER was unable to bind to the immobilized E_2 for steric reasons, hER competitively bound to free E_2 and EDs in samples. Then, anti-E_2 antibodies captured free and the immobilized E_2. Comparing with a standard curve for E_2, the relative binding affinity of EDs in samples was calculated.

Estrogen-mimicking chemicals induce the proliferation of cells of the female genital tract. Soto *et al.*[19] developed an E-screen assay to assess the proliferative effect of environmental chemicals using MCF-7 human breast carcinoma cells. This assay measured estrogen-induced increase of the number of the cells. When the cells were exposed with samples containing estrogenic chemicals, the cell number increased more than the cells in the absence of E_2 as reference. Arnold *et al.*[20] developed an yeast estrogen screen (YES) assay using transfected yeast cells harboring hER expression plasmid and reporter plasmid containing two EREs linked to the *lacZ* gene. This assay had some advantages such as the absence of other endogenous receptors and easy handling of recombinant yeast cells. After treatment with E_2 or samples, the β-galactosidase expression was induced. The EC_{50} for transcription with E_2 and diethylstilbestrol were approximately 0.2 nM in this assay.

Murk *et al.*[21] compared the ER-binding assay, YES assay and ER-CALUX assay that was identical to the CALUX assay except for the replacement of AhR with ER, for measuring estrogenic compounds. The detection limits of E_2 by ER-CALUX assay, YES assay and ER-binding assay were 0.5, 10 and 1,000 pM, respectively, with EC_{50} of 6, 100 and 5,000 pM, respectively. In addition, various environmental samples were analyzed by three assays for assessment of estrogenic potency. The results obtained by the ER-CALUX and YES assays correlated well for water samples (surface water, influent and effluent of water treatment plants; $r = 0.82$, $n = 49$) and for solid samples

(particulate matter and sewage sludge; r = 0.93, n = 19). However, the results obtained by the ER-binding assay differed greatly from those obtained by two other assays. It was assumed that both agonists and antagonists gave an estrogenic response in the ER-binding assay, although two reporter gene assays (ER-CALUX and YES) could distinguish from each other.

Receptor assays can be performed simply and readily, and detect all chemicals that act through the receptors in samples, however, further validation is required to become more reliable methods.[22)]

Biological Assay Based on Antibody

Biological assays using antibodies are called "immunoassays" based on the ability of specific molecular recognition of an antigen by antibodies. The first immunoassay was described by Yalow and Berson[23)] for quantification of insulin in serum and labeled with radioisotopes. Later, radiolabeles were replaced by enzymes in immunoassays.[24,25)] On the other hand, immunoassays were applied to the environmental diagnostics from medical diagnostics.[26)] In this section, the principle of immunoassays for small organic compounds and recent reports for environmental chemicals are described.

Antibody Structure

Antibody is a serum glycoprotein of the immunoglobulin class produced by the vertebrate immune system against foreign material of high molecular mass (immunogen). There are five immunoglobulin classes in mammals, which are IgG, IgM, IgA, IgD and IgE determined according to the heavy chain isotypes. IgG is still classified into four subclasses in mice, which are IgG1, IgG2a, IgG2b and IgG3. In addition, there are two classes in light chains, which are κ and λ light chains. IgG is a major immunoglobulin and consists of two identical heavy chains and two identical light chains linked by interchain disulfide bonds. A light chain contains two domains and a heavy chain contains four or five domains. Each

domain is composed of approximately 110 amino acid residues and there are intradomain disulfide bonds in all domains of both chains. The basic structure of an IgG molecule is shown in Fig. 1-1. Both chains are organized into variable and constant domains. Variable domains exhibit high amino acid sequence variability at the amino terminal end and form an antigen-binding site of antibody by the association of both heavy and light chains. The variable domains of both chains are organized into three hypervariable or complementarity-determining regions (CDRs) separated by four framework regions (FRs). The greatest amino acid sequence variation occurs within the CDRs whereas the FRs are more conserved. Variable light chain (VL) gene consists of VL segment and JL (joining) segment. Variable heavy chain (VH) consists of VH segment, DH (diversification) segment and JH segment. There are approximately 200 to 1200 VH segments, 15 DH segments and 4 JH segments in mouse genomes. The assembly of a variable domain gene involves the recombination of one segment from each of these pools in germ-line DNA and this yields 12,000 to 72,000 VH domain repertoire. As for κ chain, there are 200 of Vκ segments and 4 Jκ segments, and this yields 800 Vκ domains.

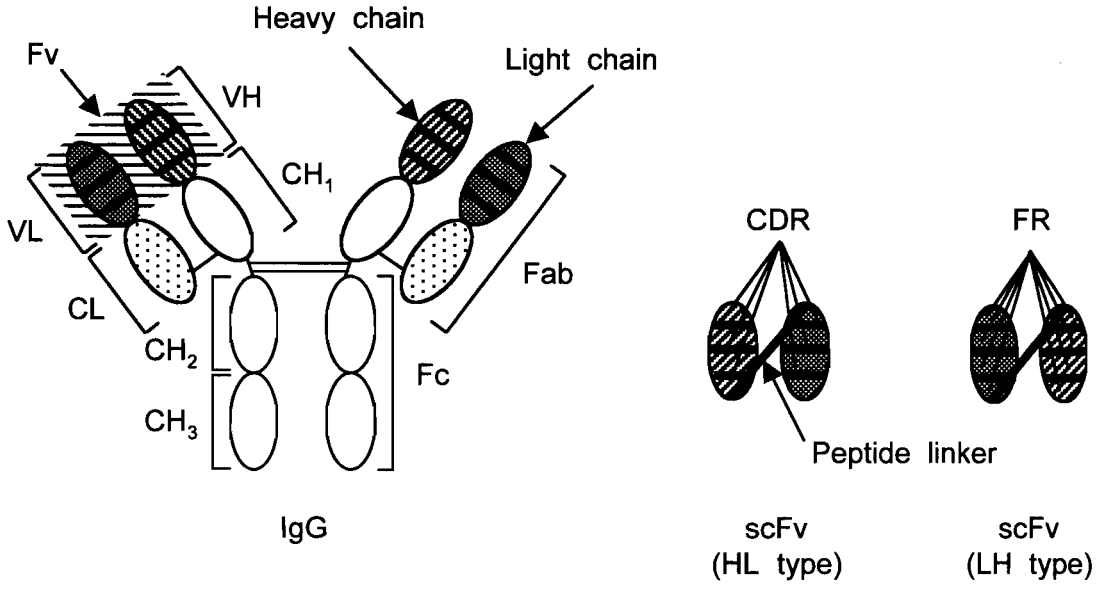

Fig. 1-1. Schematic diagram of IgG and scFv antibodies

Combination of VH and VL makes 1×10^7 to 5×10^7 repertoires. As mentioned above, the enormous diversity of immunoglobulin is derived from a relatively small number of gene segments.[27] Somatic mutation also plays important roles to make the immunoglobulin diversity.

Antibody binds to the antigen with high affinity and selectivity. In the equilibrium reaction between antigen and antibody forming the antigen-antibody complex, the affinity constant K_D is determined by the equation described as below:

$$K_D \text{ (M)} = [\text{antigen}][\text{antibody}]/[\text{antigen-antibody}].$$

Comparison of other interactions between a ligand and a protein are summarized in Table 1-2.[28]

Table 1-2. Some affinity pairs and their dissociation constants

Binding protein	Target	K_D (M)
Antibody	Antigen	$10^{-7} \sim 10^{-11}$
Avidin	Biotin	10^{-15}
Streptavidin	Biotin	10^{-15}
Receptor	Hormone	$10^{-9} \sim 10^{-12}$
Enzyme	Substarate	$10^{-3} \sim 10^{-6}$
Transport protein	Hormone	$10^{-6} \sim 10^{-8}$
Lectin	Glycoconjugate	$10^{-3} \sim 10^{-5}$

Polyclonal antibodies (pabs) are obtained from the serum and comprise a mixture of different antibody populations. Monoclonal antibodies (mabs) consist of a single monospecific antibody population, which are produced in cell culture by a single hybridoma cell derived from the fusion of B-lymphocytes with myeloma cells. The hybridoma cells can be propagated almost indefinitely in culture and will continue to produce the antibody of the lymphocyte parent. Since an individual lymphocyte produces only a single antibody type, all of the antibody molecules produced by a hybridoma cell line derived from a single hybrid cell are identical and have the same binding properties. Therefore, the hybridoma technology guarantees the unlimited

production of mabs with constant characteristics.

Assay Development

Antibodies are produced against high molecular mass substances. However, most environmental chemicals are of low molecular mass and not immunogenic. They have to be coupled to a carrier macromolecule, usually a protein, in order to elicit immune response in the vertebrate immune system.[29] For low molecular mass analytes (haptens) such as pesticides in solution, competitive tests have to be employed, using limiting antibody concentrations. The bound tracer yields a signal that is inversely proportional to the analyte concentration. A typical dose-response curve is of sigmoidal shape when the signal is plotted versus the logarithm of the analyte concentration. A linear range is obtained around the middle of the assay (IC_{50}, concentration of analyte that causes 50% inhibition), which should be used for determinations. Within this working range, the change in absorbance is linearly correlated to the analyte concentration. The most precise measurements are obtained in the region close to the middle of the test.[30]

Many immunochemical analyses are based on pabs due to ease of preparation and low cost. Since the hybridoma technique was developed by Köhler and Milstein,[31] immunoassays based on mabs for environmental chemicals have been reported. Because hybridoma cells can unlimitedly produce antibodies, the assay based on mab guarantees the constant property.

Immunoasssays for Dioxins

Stanker *et al.*[32] prepared five anti-dioxin mabs, and each antibody could detect TCDD in a competitive enzyme-linked immunosorbent assay (ELISA). The most sensitive ELISA was able to detect 0.5 ng of TCDD. Harrison and Carlson[33] developed a test tube immunoassay and a microplate immunoassay for TCDD using one of the five anti-dioxin mabs mentioned above, and detection limits for TCDD were

100 pg/tube and 25 pg/well, respectively. Besides, the cross-reactivity of the tube immunoassay to the dioxin congeners correlated roughly to their TEF values. Therefore, the immunoassay was developed as a TEQ-predicted method, and validated by comparing the data from GC-MS analysis of 43 soil samples. The TEQ calculations resulted in a strong correlation between the predicted immunoassay and GC-MS ($y = 0.99x - 0.53$, $r = 0.988$). This result indicated that the immunoassay could be useful for a TEQ screening method.

Sanborn *et al.*[34] synthesized several novel dioxin haptens and these compounds were conjugated to proteins as immunogen for generation of anti-dioxin pabs from rabbits. ELISAs were developed with these antisera and an IC_{50} of 16 ng/ml was observed for 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin (TMDD) which was a close structural analogue to TCDD. TMDD was a useful surrogate for analytical standard of TCDD because this chemical was presumed to be less toxic and more rapidly metabolized[35] and behaved almost identically to TCDD in the ELISA. The assay using TMDD as a standard was optimized and exhibited an IC_{50} value of 240 pg/mL, with working range from 40 to 4800 pg/mL.[36]

Immunoassays for PCBs

Johnson *et al.*[37] developed an ELISA based on pabs for PCBs in environmental samples. The detection limits of the assay for Aroclor 1242 and Aroclor 1248 in soil were 10.5 ng/g and 8.95 ng/g, respectively. Soil samples spiked with Aloclor at 5 mg/kg were extracted by shaking with methanol and the extracts were analyzed in ELISA. The mean recoveries for Aroclor 1248 and Aroclor 1242 were 107% with a relative standard deviation (RSD) of 17% ($n = 8$) and 104% with a RSD of 14% ($n = 5$), respectively. Moreover, 148 environmental samples such as clay, soil, sediment, and paper pulp were analyzed by ELISA and GC-ECD. The samples were extracted by shaking with methanol, Soxhlet extraction or supercritical fluid extraction (SFE) methods. The ELISA results with the extracts by Soxhlet

extraction or SFE methods were in good agreement with GC-ECD analysis. However, the ELISA data with methanol shake extracts did not correlate with the GC-ECD results. PCBs in oily soil were further analyzed by ELISA and GC-ECD. The oily soil samples were extracted using the same methods. Good agreement between the data of GC-ECD and ELISA coupled to SFE was obtained (r^2 = 0.998), although the ELISA results with the methanol shake extracts and the methanolic Soxhlet extracts did not agree with GC-ECD analysis, with r^2 values of 0.0639 and 0.005, respectively.[38] The results suggested that the extraction method had a significant effect on the quality of the ELISA results in determination of PCBs.

A competitive direct ELISA using an anti-coplanar (non-ortho-substituted) PCB mab was developed and the IC_{50} values in the assay were 0.9 ppb for PCB77 and 1.2 ppb for PCB126.[39] The ELISA was highly specific for coplanar PCB congeners and did not recognize non-coplanar congeners, TCDD or dibenzofuran.

Immunoassays for Residual Pesticides

Beasley *et al.*[40] developed several ELISA systems for the organochlorine insecticide DDT and its metabolites DDE, DDD and DDA and the miticide dicofol. Eight analogs of DDT, its metabolites and dicofol were synthesized and used for immunogen or enzyme tracer in ELISA. Several polyclonal antisera were prepared from rabbits immunized with the different immunogens. From the possible combinations of pabs and enzyme tracers, five sensitive ELISAs were developed. A DDA assay was specific to DDA and IC_{50} for DDA was 0.8 ng/mL. A DDT + DDE assay was able to determine the concentrations of the sum of DDT and DDE based on cross-reaction. In this assay, cross-reactivity for DDE was 67% relative to DDT as 100%. A DDT assay and a DDE assay were specific to DDT and DDE, respectively. An IC_{50} of DDT was 13 ng/mL in the DDT assay with 3% of cross-reactivity for DDE, and an IC_{50} of DDE was 9 ng/mL in the DDE assay with 7% of cross-reactivity for DDT. A

dicofol assay was specific to dicofol with IC_{50} of 2 ng/mL. This study showed that the choice of the hapten used for the enzyme tracer remarkably affected the assay specificity. Many immunoasssays for pesticides were reported and reviewed somewhere.[41-44)]

Immunoassays for EDs

Certain natural and synthetic chemicals are assumed to modulate the endocrine system in recent days.[45)] The Environmental Agency of Japan arranged "Strategic Programs on Environmental Endocrine Disruptors '98" (SPEED '98) in May, 1998, and the distribution of EDs in the environment has been vigorously researched.[46)]

Goda *et al.*[47)] developed six ELISA systems using mabs for the quantitative analysis of EDs. An assay for estrogen could determine the total amount of estrone, E_2 and estriol, and detection limit was 0.1 ng/mL for E_2 as a standard. The detection limits of an E_2 assay, a bisphenol A assay and an alkylphenol assay were 0.1 ng/mL, 5 ng/mL, and 70 ng/mL for E_2, bisphenol A and nonylphenol as standards, respectively. The detection limits of an assay for phthalate esters and an assay for chlorophenols were 200 ng/mL and 2 ng/mL for dibutylphthalate and 2,4-chlorophenol as standards, respectively.

Immunoassays Based on Recombinant Antibody

The progress in genetic engineering technology made recombinant antibody available. Single-chain variable fragment (scFv: Fig. 1-1) or recombinant Fab (rFab) is generally used as a recombinant antibody. Recombinant antibody genes were expressed in bacteria,[48)] yeast,[49)] higher plants[50)] and insect cells.[51)] ScFv antibodies has some advantages such as a smaller size than intact an immunoglobulin, rapid and large-scale production in *Escherichia coli* and gene manipulation to improve the property in reactivity, specificity or stability. In this decade many recombinant antibodies specific to environmental chemicals were prepared.

Garrett *et al.*[52] developed an ELISA based on an anti-parathion scFv antibody and applied the assay to the determination of parathion residues in food samples. The immunoglobulin genes were cloned from hybridoma cells producing an anti-parathion mab, and anti-parathion scFv gene was constructed. The anti-parathion scFv antibody produced in *E. coli* cells showed the reactivity to parathion similar to the parent mab in competitive ELISA. The detection limit of ELISA based on scFv was 2.3 ng/well which was comparable to that of 1.6 ng/well with the mab. The scFv antibody also showed the cross-reactivity similar to the mab. The recovery tests using rice and orange peel were each applied to the ELISA assays based on the mab and the scFv antibody. The mean recovery of spiked parathion residues from rice extracts and orange peels was 54 – 101% and 26 - 53% in the assay based on mab, respectively. The results in the assay based on scFv were similar to those based on mab and the mean recovery from rice extracts and orange peels was 54 - 80% and 27 - 45%, respectively. Other recombinant antibodies specific to environmental chemicals were reported and these are listed in Table 1-3.

Objective of This Study

Immunoassays have become popular methods for environmental monitoring because of the simplicity, easy handling and low costs, and are seemed to receive public acceptance due to rather less usage of organic solvents than the instrumental analysis. However, matrix effects often occur in the assay and the assay precision is not so high. Therefore, the efforts to reduce the matrix effects and increase the precision have been required. Besides, more sensitive and specific immunoassays have been needed to determine lower concentration of analyte.

In this study, attempts to produce mabs and recombinant antibodies specific to the insecticide malathion, the industrial chemicals bisphenol A and alkylphenol ethoxylates were done, and ELISA assays

Table 1-3. Summary of the recombinant antibodies specific to environmental chemicals

	Compound	Form	Source	IC_{50}	Reference
Dioxins	TCDD	scFv	Mouse hybridoma cell	10.4 ppb	53
	Coplanar PCB	rFab	Mouse hybridoma cell	10 ppb	54
Herbicide	Atrazine	rFab	Mouse hybridoma cell	ND	55
	Atrazine	scAb	Mouse hybridoma cell	1 nM	56
	Atrazine	scFv	Mouse hybridoma cell	n.d.	57
	Atrazine	scFv	Rabbit spleen cell	n.d.	58
	Atrazine	scFv	Sheep spleen cell	100 ppt	59
	Cyclohexanedione	scFv	Mouse hybridoma cell	3.9 nM	60
	Diuron	rFab	Mouse hybridoma cell	1.6 ng/mL	61
	Mecoprop	scAb	Mouse hybridoma cell	40 nM	62
	Paraquat	scAb	Mouse hybridoma cell	1.5 nM	63
	Paraquat	scFv	Mouse hybridoma cell	n.d.	64
	Picloram	scFv	Mouse hybridoma cell	50 ng/mL	65
	Picloram	scFv	Mouse spleen cell	20 ppb	66
	Terbutryn	scFv	Mouse spleen cell	13.8 ng/mL	67
Insecticide	Chlorpyrifos-ethyl	scFv	Mouse hybridoma cell	500 ng/mL	68
	Parathion	scFv	Mouse hybridoma cell	2.3 ng/well	52
Mycotoxin	Zearalenone	scFv	Mouse hybridoma cell	14 ng/ml	69

based on them were developed for routine monitoring of such chemicals. The analyses of the molecular mechanisms of the antigen-antibody bindings and the interactions within the antibody molecules were also attempted by using recombinant antibodies. Storing knowledge on the reactivity and specificity of the antibodies specific to environmental chemicals would contribute to the improvement of the immunochemical monitoring in agricultural products and the environment.

CHAPTER II

MOLECULAR CHARACTERISTICS OF THE MONOCLONAL AND RECOMBINANT ANTIBODIES SPECIFIC TO THE INSECTICIDE MALATHION

INTRODUCTION

The organophosphorus insecticide malathion is widely in use today for the control of sucking and chewing insects in agricultural and for domestic applications owing to its low mammalian toxicity and selective insecticidal activity.[70] The insecticide is also used for the stored grains as post-harvest treatment.[71] Because of its wide use, particularly for the post-harvest use, it is important to monitor malathion residues in the environment as well as in grains. Generally, GC and/or GC/MS are conventionally used to quantify the amount of residual organophosphorus pesticides.[72] Although these methods are highly accurate and precise, the analytical processes are time-consuming and labor-intensive. On the other hand, ELISAs are convenient for sensitive, rapid, simple and cost-effective assays of pesticide residues. However, no reports were available on immunoassays specific to malathion despite of its wide use in the world, since it was rather difficult to prepare antibodies specific to malathion.

Currently, recombinant antibody technology is enable to manipulate antibody genes for engineering of scFv antibodies consist of VH and VL of an antibody, both of which domains are connected by a flexible peptide linker,[73,74] and for a large-scale production of recombinant antibodies in *E. coli*.[75] ELISAs based on recombinant antibodies against persistent organic pollutants were reported.[54,57,58,60,63,68]

In this paper, we described preparation and characterization of two anti-malathion mabs and of their scFv antibodies produced in recombinant *E. coli* cells for understanding molecular and immunochemical bases of antibodies specific to malathion.

MATERIALS AND METHODS

Chemicals and Biochemicals

Malathion (CAS No. 121-75-5) [*S*-1,2-bis(ethoxycarbonyl)ethyl *O,O*-dimethyl phosphorodithioate] (Fig. 2-1) and the other organophosphorus pesticides used in this study were purchased from Riedel-de Haën AG (Hannover, Germany). The malathion haptens MAL and EIT244 (Fig. 2-1) were synthesized in Otsuka Chemical Co., Ltd. (Tokushima, Japan). [α-^{32}P]dATP was purchased from Amarsham Pharmacia Biotech (Buckinghamshire, UK). Horseradish peroxidase (HRP) was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (MO, USA). Keyhole limpet hemocyanine (KLH) and HRP-conjugated anti-mouse IgG antibody were obtained from Pierce Chemical Co. (IL, USA). Block Ace was obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). DNA restriction endonucleases were purchased from New England Biolabs, Inc. (MA, USA). 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals and organic solvents were purchased from Nakalai Tesque, Inc. (Kyoto, Japan).

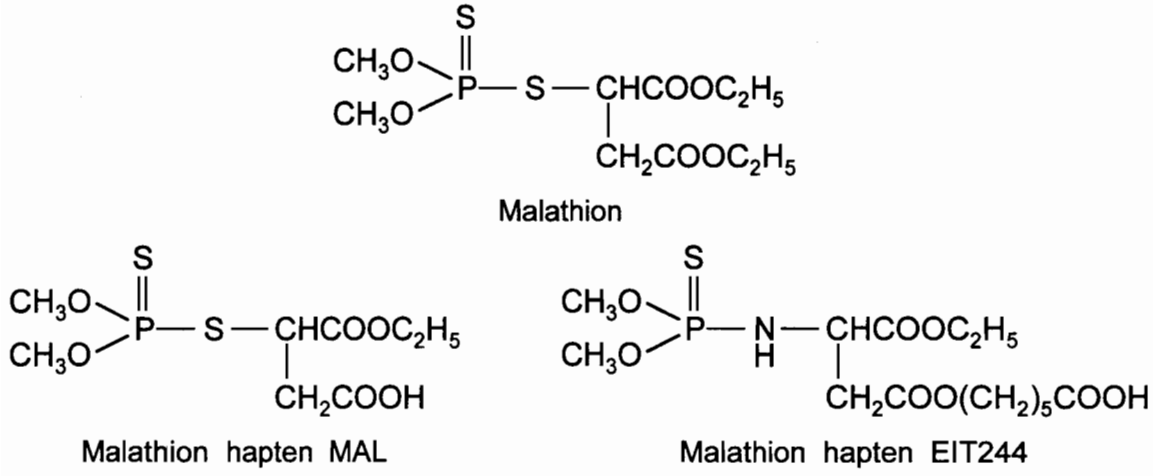

Fig. 2-1. Chemical structures of malathion and its haptenic derivatives MAL and EIT244

Preparation of Malathion Hapten-Protein Conjugates

Conjugation of Hapten and BSA or KLH

The haptens MAL and EIT244 were each covalently coupled to KLH and BSA by the active ester method as described previously.[76] Both MAL-KLH and EIT244-KLH conjugates were used as an immunogen for mice, and EIT244-BSA conjugate was used as a coating antigen in indirect competitive ELISA (ic-ELISA).

Conjugation of Hapten and HRP

The hapten EIT244 was also covalently coupled to HRP. A hundred μl of the hapten (15 μmol) dissolved in DMSO was mixed with 5 μl of *N*-hydroxysuccinimide (20 μmol) and 10 μl of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (20 μmol). After incubation for 1.5 hr at 25°C, the activated hapten was gradually mixed with HRP (10 mg) solution and incubated again for 1.5 hr at 25°C. Then, the mixture was dialyzed against phosphate-buffered saline (PBS: 10 mM phosphate, 0.9% (w/v) NaCl, pH 7.2) for 2 days at 4°C. The resultant EIT244-HRP conjugate was used as a tracer in direct competitive ELISA (dc-ELISA).

Preparation of Mabs Specific to Malathion

Eight-week-old female BALB/c mice were immunized with MAL-KLH or EIT244-KLH as described previously.[77] Titer of antisera from the mice was examined in indirect noncompetitive ELISA (in-ELISA), and their spleen lymphocytes isolated were fused with P3-X63-Ag8.653 myeloma cells[78] according to the method by Köhler and Milstein[31] with some modifications.[79] The hybridomas were screened in ic-ELISA, and the cells producing anti-malathion antibodies were cloned by the limiting dilution method.

Then, the hybridoma cells were injected into male BALB/c mice and ascites fluid were collected as described by Deschamps *et al.*[80] They were fractionated by precipitation with saturated ammonium sulfate and used as anti-malathion mabs in dc-ELISA and ic-ELISA. The isotype of each of mabs was determined with a Mouse-Typer

Isotyping Panel kit (Bio-Rad Laboratories, CA, USA).

Construction and Screening of cDNA Libraries

Approximately 5 μg of poly(A)$^+$ RNA was isolated from the hybridoma cell lines by using a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech). Two λ ZAP II cDNA libraries were constructed with a ZAP-cDNA Synthesis Kit (Stratagene, CA, USA) using the Uni-ZAP XR vector and *E. coli* XL-1 Blue MRF' cells according to the manufacturer's instructions. Both libraries were subsequently screened with the cDNA fragments encoding constant heavy or light chains of mouse IgG as probes, and a plaque hybridization method was carried out. Positive plaques were detected by using an ECL direct nucleic acid labeling and detection systems (Amersham Pharmacia Biotech). Positive phages were converted to the recombinant pBluescript phagemids by *in vivo* excision. The size of an insert of the obtained cDNA clones was determined by digesting with *Eco*RI and *Xho*I, followed by agarose gel electrophoresis.

RNA Gel Blot Analysis

Approximately 1 μg of poly(A)$^+$ RNA isolated from each of the hybridoma cell lines was fractionated by electrophoresis on 1% agarose gel containing formaldehyde, and then stained with ethidium bromide by the standard procedure.[81] Messenger RNA was blotted onto a nylonmembrane and cross-linked to the membrane to hybridize with random primed ^{32}P-labeled cDNA fragments coding for VH or VL of the isolated anti-malathion mabs. Hybridization was performed for 16 hr at 65°C in 5 × SSC, 0.5% sodium dodecyl sulfate (SDS), 5 × Denhardt's solution and 100 μg/ml of denatured salmon sperm DNA. The membranes were washed twice in 2 × SSC containing 0.1% SDS for 20 min at 65°C, once in 1 × SSC containing 0.1% SDS for 15 min and twice in 0.2 × SSC containing 0.1% SDS for 10 min, and subjected to autoradiography.

Construction of Anti-Malathion ScFv Antibody Genes

Based on the nucleotide sequences of the cDNA clones, sixteen synthetic oligonucleotides complementary to N- or C-terminal coding regions of VL or VH of MLT2-23 and MLT40-4 were designed to prepare four scFv genes such as MLT2-23/HL scFv, MLT2-23/LH scFv, MLT40-4/HL scFv and MLT40-4/LH scFv (Table 2-1). In four scFv genes constructed, both VH and VL were connected with a synthetic peptide linker sequence encoding $(Gly_4Ser)_3$. The VH and VL cDNA fragments were amplified in the first PCR with primers H5 and H3, and primers L5 and L3, respectively. Both were assembled in the second PCR. As to the first PCR, the temperature program was 1 min at 95°C, 2 min at 63°C and 2 min at 72°C for 30 cycles. Then, the second PCR was performed without primers by the temperature program of 1 min at 95°C, 2 min at 55°C and 2 min at 72°C for 7 cycles. Then, the primers H5 and L3 were used for construction of HL type of scFv genes and primers L5 and H3 were used for construction of LH type of scFv genes. PCR was performed in the same manner as in the first. After digested with *Sfi*I and *Not*I, the scFv gene was ligated into the phagemid vector pCANTAB5E (Amersham Pharmacia Biotech) and transformed into competent *E. coli* TG1 cells.

Production of ScFv Antibodies in Recombinant *E. coli* Cells

The transformed *E. coli* TG1 cells were infected with the helper phage M13K07 (Amersham Pharmacia Biotech) to produce recombinant phages exhibiting scFv antibodies. The phages were selected by panning against EIT244-BSA according to the manufacturer's instructions. *E. coli* HB2151 cells were infected with the screened phages for preparation of the soluble scFv antibodies, and the clones were grown in 2 × YT medium containing 50 μg/ml of ampicillin and shaken overnight at 28°C. Isopropyl-β-D-thiogalactopyranoside was added to the medium to a final concentration of 1 mM and the cultures were grown for 6 hr at 28°C with shaking. *E. coli* cells were harvested

Table 2-1. The primers used for construction of scFv genes

Primer	Nucleotide sequencea
H5-2HL	5'-TTGGCCCAGCCGGCCCTTCAGGAGTCAGGACCTAGCCT-3'
H3-2HL	5'-**AGAGCCACCTCCGCCTGAACCGCCTCCACC**TGCAGAGACAGTGACCAGAGTCC-3'
L5-2HL	5'-**GGCGGAGGTGGCTCTGGCGGTGGCGGATCG**GATATTGTGATGACCCAAACTCCAC-3'
L3-2HL	5'-TTGCGGCCGCCCGTTTTATTTCCAGCGTGGT-3'
L5-2LH	5'-AAGGCCCAGCCGGCCGATATTGTGATGACCCAAACTCCAC-3'
L3-2LH	5'-**AGAGCCACCTCCGCCTGAACCGCCTCCACC**CCGTTTTATTTCCAGCGTGGT-3'
H5-2LH	5'-**GGCGGAGGTGGCTCTGGCGGTGGCGGATCG**CTTCAGGAGTCAGGACCTAGCCT-3'
H3-2LH	5'-ATGCGGCCGCTGCAGAGACAGTGACCAGAGTCC-3'
H5-4HL	5'-TTGGCCCAGCCGGCCCTTGAAGAGTCTGGAGGAGGCTT-3'
H3-4HL	5'-**AGAGCCACCTCCGCCTGAACCGCCTCCACC**TACAGAGACAGTGACCAGAGTCCCT-3'
L5-4HL	5'-**GGCGGAGGTGGCTCTGGCGGTGGCGGATCG**GACATTGTGATGACCCAGTCTCAA-3'
L3-4HL	5'-TTGCGGCCGCCCGTTTTATTTCCAGCTTGGTC-3'
L5-4LH	5'-AAGGCCCAGCCGGCCGACATTGTGATGACCCAGTCTCAA-3'
L3-4LH	5'-**AGAGCCACCTCCGCCTGAACCGCCTCCACC**CCGTTTTATTTCCAGCTTGGTC-3'
H5-4LH	5'-**GGCGGAGGTGGCTCTGGCGGTGGCGGATCG**CTTGAAGAGTCTGGAGGAGGCTT-3'
H3-4LH	5'-ATGCGGCCGCTACAGAGACAGTGACCAGAGTCCCT-3'

The primers H5 and H3 were complementary to 5'- and 3'-ends of nucleotide sequences of VH, respectively, and the primers L5 and L3 were complementary to 5'- and 3'-ends of nucleotide sequences of VL, respectively. The primers 2HL, 2LH, 4HL and 4LH were used for construction of MLT2-23scFv/HL, MLT2-23scFv/LH, MLT40-4scFv/HL and MLT40-4scFv/LH genes, respectively.
[a]Endonuclease site (*Sfi*I or *Not*I) and a part of a flexible peptide linker sequence encoding $(Gly_4Ser)_3$ were described in underlined and bold characters, respectively.

and resuspended in ice-cold TES buffer (0.2 M Tris-HCl, 0.5 mM ethylenediaminetetraacetic acid, 0.5 M sucrose, pH 8.0). Then, 0.2 × TES buffer was added and voltexed. After placed on ice for 30 min, the suspension was centrifuged for 10 min at 12,000 × g, and the supernatant was directly used for ic-ELISA as anti-malathion scFv antibodies.

DNA Sequence Analysis

Recombinant pBluescript phagemids and recombinant pCANTAB5E phagemids were sequenced in an automated DNA sequencer SQ-5500 (Hitachi, Tokyo, Japan) and ABI 310 (PE Applied Biosystems, CA, USA), respectively. Alignment of sequences was performed with a Genetyx-Mac 7.3 software (Software Development Co., Tokyo, Japan).

ELISA Protocol

Dc-ELISA Based on IgG

Microtiter plates (Maxisorp: Nunc, Roskilde, Denmark) were coated with 100 μl of the mabs MLT2-23 or MLT40-4 (1 μg/ml) in PBS overnight at 4°C, and blocked with 300 μl of Block Ace diluted 1 : 3 in distilled water. Then, 50 μl of standards of malathion or another organophosphorus pesticide used for cross-reactivity test, and 50 μl of EIT244-HRP (1 μg/ml) in PBS were added in triplicate, and incubated for 1 hr at 25°C. After washing with PBS, 100 μl of chromogenic substrate (100 μg/ml of TMB and 0.006% (w/v) of H_2O_2 in 0.1 M acetate buffer, pH 5.5) was added, and incubated for 10 min at 25°C. The enzyme reaction was stopped by adding 100 μl of 1 N sulfuric acid and the absorbance was measured at 450 nm.

Ic-ELISA Based on IgG

Plates were coated with 100 μl of EIT244-BSA (1 μg/ml) in PBS and blocked as described above. Then, 50 μl of malathion standards and 50 μl of mab (1 μg/ml) were added and incubated for 1 hr at 25°C. After washing, 100 μl of anti-mouse IgG conjugated to HRP in PBS containing 10% (v/v) Block Ace (PBS-B) was added and

incubated for 1 hr at 25°C. After washing, the color development and measuring absorbance at 450 nm were performed as described above.

Ic-ELISA Based on ScFv Antibody

The immunoassay was performed as described above, except that 50 μl of a scFv antibody was added in place of a mab and followed by adding 100 μl of anti-E tag antibody (1 μg/ml) (Amersham Pharmacia Biotech) in PBS-B and incubated for 1 hr at 25°C. A scFv antibody preparation was appropriately diluted in PBS to obtain about 1.0 of the absorbance in control wells (B_0) in ELISA.

In-ELISA Based on IgG

The immunoassay was performed as described above, except for adding antiserum in place of a mab, and not adding the malathion standards.

RESULTS

Anti-Malathion Mabs

Following a series of immunization, the titers of antisera from mice were examined in in-ELISA. Spleen cells were prepared from the mice showing a good titer and fused with myeloma cells. Two wells with hybridomas secreting anti-malathion antibodies were found in the fusion experiment using mice immunized with EIT244-KLH. Then, two hybridoma cell lines producing mabs reacting with malathion in ic-ELISA were established by cloning of the hybridoma cells, which were designated as MLT2-23 and MLT40-4. On the other hand, the other immunogens including MAL-KLH failed to raise antibodies against malathion. The hybridoma cell lines were adapted to ascites preparation. Both mabs were each purified and used as anti-malathion mabs in dc-ELISA and ic-ELISA.

Dc-ELISAs by using MLT2-23 and MLT40-4 were each optimized. The standard curves obtained with the respective mabs in the dc-ELISA are shown in Fig. 2-2. An IC_{50} value and detectable range for malathion were 16 ng/ml and 5.3 to 75 ng/ml in the assay

based on MLT2-23, respectively, and those were 40 ng/ml and 7.0 to 190 ng/ml in the assay based on MLT40-4. A variety of organophosphate pesticides were tested for determination of specificity of both mabs in dc-ELISA. The percent cross-reactivity was defined as the IC_{50} value obtained with each of the tested compounds on the basis of comparison with malathion. Based on the results MLT2-23 and MLT40-4 were found to be very specific to malathion (Table 2-2), although MLT2-23 slightly cross-reacted with malaoxon at the degree of 1.6% and with fenitrothion at the degree of 0.5%. MLT40-4 did not cross-react with any organophosphate compounds tested in this study. Isotyping tests indicated that MLT2-23 and MLT40-4 belonged to the isotype IgG2a and IgG1 classes, respectively, with κ light chains.

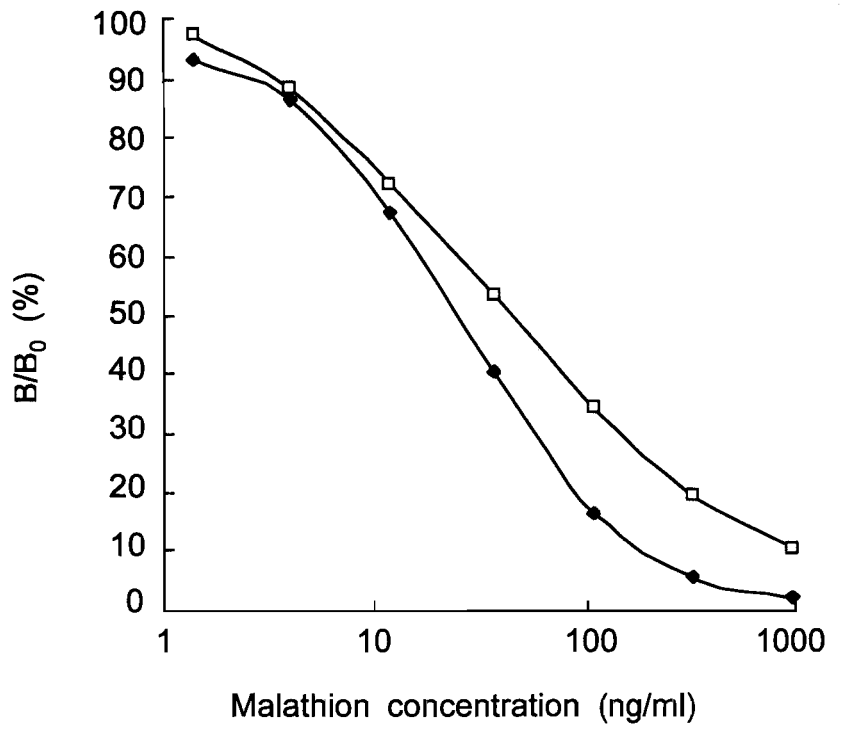

Fig. 2-2. Inhibition curves obtained with the mabs for malathion in dc-ELISA B/B_0 (%) is quantified as: [(absorbance at each concentration of standard) /(absorbance at zero concentration of standard)] ¥ 100. The mab MLT2-23 (ø), the mab MLT40-4 (£).

Table 2-2. Reactivity of the mabs MLT2-23 and MLT40-4 toward organophosphoate pesticides in dc-ELISA

Compound	MLT2-23		MLT40-4	
	IC_{50} value (ng/ml)	Cross-reactivity (%)[a]	IC_{50} value (ng/ml)	Cross-reactivity (%)[a]
Malathion	16	100	40	100
Malaoxon	1000	1.6	>10000	<0.4
Fenitrothion	3200	0.5	>10000	<0.4
Dimethoate	>10000	<0.2	>10000	<0.4
Terbufos	>10000	<0.2	>10000	<0.4
Methamidophos	>10000	<0.2	>10000	<0.4
Acephate	>10000	<0.2	>10000	<0.4
Vamidothion	>10000	<0.2	>10000	<0.4
Dichlorvos	>10000	<0.2	>10000	<0.4
Trichlorfon	>10000	<0.2	>10000	<0.4
Methylparathion	>10000	<0.2	>10000	<0.4
Ethoprophos	>10000	<0.2	>10000	<0.4

[a]Cross-reactivity (%) = [IC_{50} (malathion)/IC_{50} (tested compound)] × 100.

cDNA Cloning

Approximately 10 μg of mRNA was extracted from 3 × 10^7 cells of the hybridoma cell lines producing mab MLT2-23 or MLT40-4, and 5 μg of each of the mRNA fraction was used for construction of two individual cDNA libraries. Then, cDNA library screening was performed with cDNA fragments encoding the constant region of mouse IgG1 or the constant region of mouse κ chain as probes, due to the consistence of the nucleotide sequences of constant region of mouse IgGs. Several positive plaques were picked up and the phages were excised into the recombinant pBluescript phagemids. Since full length sizes of heavy and light chain cDNA clones were known to be about 1600 bp and 1000 bp, respectively, typical cDNA clones containing an appropriate size of inserts determined by restriction digestion were subjected to a DNA sequence analysis. Based on nucleotide sequences, the longest cDNA clones among them were selected and these were designated pG208, pK211, pG408 and pK414, which were identified as heavy chain of MLT2-23, light chain of MLT2-23, heavy chain of MLT40-4 and light chain of MLT40-4, respectively. The nucleotide and deduced amino acid sequences of these cDNA clones corresponding to the variable regions of both mabs are shown in Fig. 2-3 to Fig. 2-6, which are deposited in the GenBank, EMBL and DDBJ databases under the Accession numbers AB097847 (pG208), AB097848 (pK211), AB097849 (pG408) and AB097850 (pK414). The deduced amino acid sequences of variable regions of both mabs were aligned as shown in Fig. 2-7. The overall amino acid identity of VH and VL between both mabs was 40% and 58%, respectively. As for the complementarity-determining regions (CDRs), MLT2-23 VH and VL shared 15% and 31% amino acid sequence identity with those of MLT40-4, respectively. The heavy chain nucleotide sequences of MLT2-23 and MLT40-4 are a match for Kabat subgroup IA and IIIC, respectively.[82)]

```
GAATTCGGCACGAGGGATTTTTGAAGAAAGGGGTTGTAGCCTAAAAGATGATGGTGTTAA   60
                                                  M  M  V  L   -15
GTCTTCTGTACCTGTTGACAGCCCTTCCGGGTATCCTGTCAGAGGTGCAGCTTCAGGAGT  120
S  L  L  Y  L  L  T  A  L  P  G  I  L  S  E  V  Q  L  Q  E     6
CAGGACCTAGCCTCGTGAAACCTTCTCAGACTCTGTCCCTCACCTGTTCTGTCACTGGCG  180
S  G  P  S  L  V  K  P  S  Q  T  L  S  L  T  C  S  V  T  G    26
ACTCCATCACCAGTGGTTACTGGAACTGGATCCGGAAATTCCCAGGGAATAAACTTGAGT  240
D  S  I  T  S  G  Y  W  N  W  I  R  K  F  P  G  N  K  L  E    46
ATATGGGGTACATAAGCTACAGTGGTAGCACTTACTACAATCCATCTCTCAAAAATCGGA  300
Y  M  G  Y  I  S  Y  S  G  S  T  Y  Y  N  P  S  L  K  N  R    66
TCTCCATCACTCGAGACACATCCAGGAACCAGTTCTCCCTGCACCTGAATTCTGTGATTA  360
I  S  I  T  R  D  T  S  R  N  Q  F  S  L  H  L  N  S  V  I    86
CTGAGGACACAGCCACATATTACTGTGCAGGATCCACTATGATTACGACGAGGGCCGGTC  420
T  E  D  T  A  T  Y  Y  C  A  G  S  T  M  I  T  T  R  A  G   106
ACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAGCCAAAACAACAGCCCCATCGGTCT  480
H  W  G  Q  G  T  L  V  T  V  S  A  A  K  T  T  A  P  S  V   126
ATCCACTGGCCCCTGTGTGTGGAGATACAACTGGCTCCTCGGTGACTCTAGGATGCCTGG  540
Y  P  L  A  P  V  C  G  D  T  T  G  S  S  V  T  L  G  C  L   146
TCAAGGGTTATTTCCCTGAGCCAGTGACCTTGACCTGGAACTCTGGATCCCTGTCCAGTG  600
V  K  G  Y  F  P  E  P  V  T  L  T  W  N  S  G  S  L  S  S   166
GTGTGCACACCTTCCCAGCTGTCCTGCAGTCTGACCTCTACACCCTCAGCAGCTCAGTGA  660
G  V  H  T  F  P  A  V  L  Q  S  D  L  Y  T  L  S  S  S  V   186
CTGTAACCTCGAGCACCTGGCCCAGCCAGTCCATCACCTGCAATGTGGCCCACCCGGCAA  720
T  V  T  S  S  T  W  P  S  Q  S  I  T  C  N  V  A  H  P  A   206
GCAGCACCAAGGTGGACAAGAAAATTGAGCCCAGAGGGCCCACAATCAAGCCCTGTCCTC  780
S  S  T  K  V  D  K  K  I  E  P  R  G  P  T  I  K  P  C  P   226
CATGCAAATGCCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAA  840
P  C  K  C  P  A  P  N  L  L  G  G  P  S  V  F  I  F  P  P   246
AGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATG  900
K  I  K  D  V  L  M  I  S  L  S  P  I  V  T  C  V  V  V  D   266
TGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACA  960
V  S  E  D  D  P  D  V  Q  I  S  W  F  V  N  N  V  E  V  H   286
CAGCTCAGACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCC 1020
T  A  Q  T  Q  T  H  R  E  D  Y  N  S  T  L  R  V  V  S  A   306
TCCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACA 1080
L  P  I  Q  H  Q  D  W  M  S  G  K  E  F  K  C  K  V  N  N   326
AAGACCTCCCAGCGCCCATCGAGAGAACCATCTCAAAACCCAAAGGGTCAGTAAGAGCTC 1140
K  D  L  P  A  P  I  E  R  T  I  S  K  P  K  G  S  V  R  A   346
CACAGGTATATGTCTTGCCTCCACCAGAAGAAGAGATGACTAAGAAACAGGTCACTCTGA 1200
P  Q  V  Y  V  L  P  P  P  E  E  E  M  T  K  K  Q  V  T  L   366
CCTGCATGGTCACAGACTTCATGCCTGAAGACATTTACGTGGAGTGGACCAACAACGGGA 1260
T  C  M  V  T  D  F  M  P  E  D  I  Y  V  E  W  T  N  N  G   386
AAACAGAGCTAAACTACAAGAACACTGAACCAGTCCTGGACTCTGATGGTTCTTACTTCA 1320
K  T  E  L  N  Y  K  N  T  E  P  V  L  D  S  D  G  S  Y  F   406
TGTACAGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAAGAAATAGCTACTCCTGTT 1380
M  Y  S  K  L  R  V  E  K  K  N  W  V  E  R  N  S  Y  S  C   426
CAGTGGTCCACGAGGGTCTGCACAATCACCACACGACTAAGAGCTTCTCCCGGACTCCGG 1440
S  V  V  H  E  G  L  H  N  H  H  T  T  K  S  F  S  R  T  P   446
GTAAATGAGCTCAGCACCCACAAAACTCTCAGGTCCAAAGAGACACCCACACTCATCTCC 1500
G  K  *                                                       448
ATGCTTCCCTTGTATAAATAAAGCACCCAGCAATGCCTGGGACCATGTAAAAAAAAAAAA 1560
AAAAAAAAAAAAAAAACTCGAG                                        1582
```

Fig. 2-3. Nucleotide and deduced amino acid sequences of the heavy chain of the mab MLT2-23 (cDNA clone pG208, GenBank accession no. AB097847). The deduced amino acid sequence is shown as 1-letter symbols below the nucleotide sequence. Nucleotides and amino acids are numbered at the right, and amino acid residues are numbered beginning with the N-terminal amino acid (glutaminic acid).

```
GAATTCGGCACGAGGGTCTCCTCAGGTTGCCTCCTCAAAATGAAGTTGCCTGTTAGGCTG  60
                                              M  K  L  P  V  R  L  -13
TTGGTGCTGATGTTCTGGATTCCTGTTTCCAGCAGTGATATTGTGATGACCCAAACTCCA 120
 L  V  L  M  F  W  I  P  V  S  S  S  D  I  V  M  T  Q  T  P    8
CTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGCAGATCTAGTCAGAGC 180
 L  S  L  P  V  S  L  G  D  Q  A  S  I  S  C  R  S  S  Q  S   28
CTTGTACACAGTAATGGAAACACCTATTTACATTGGTACCTGCAGAAGCCAGGCCAGTCT 240
 L  V  H  S  N  G  N  T  Y  L  H  W  Y  L  Q  K  P  G  Q  S   48
CCAAAGCTCCTGATCTACAAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGT 300
 P  K  L  L  I  Y  K  V  S  N  R  F  S  G  V  P  D  R  F  S   68
GGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTAGAGGCTGAGGATCTG 360
 G  S  G  S  G  T  D  F  T  L  K  I  S  R  V  E  A  E  D  L   88
GGACTTTATTTCTGCTCTCAAGCTACACATGTTCCTTTCACGTTCGGAGGGGGGACCACG 420
 G  L  Y  F  C  S  Q  A  T  H  V  P  F  T  F  G  G  G  T  T  108
CTGGAAATAAAACGGGCTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAG 480
 L  E  I  K  R  A  D  A  A  P  T  V  S  I  F  P  P  S  S  E  128
CAGTTAACATCTGGAGGTGCCTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAAAGAC 540
 Q  L  T  S  G  G  A  S  V  V  C  F  L  N  N  F  Y  P  K  D  148
ATCAATGTCAAGTGGAAGATTGATGGCAGTGAACGACAAAATGGCGTCCTGAACAGTTGG 600
 I  N  V  K  W  K  I  D  G  S  E  R  Q  N  G  V  L  N  S  W  168
ACTGATCAGGACAGCAAAGACAGCACCTACAGCATGAGCAGCACCCTCACGTTGACCAAG 660
 T  D  Q  D  S  K  D  S  T  Y  S  M  S  S  T  L  T  L  T  K  188
GACGAGTATGAACGACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAACTTCA 720
 D  E  Y  E  R  H  N  S  Y  T  C  E  A  T  H  K  T  S  T  S  208
CCCATTGTCAAGAGCTTCAACAGGAATGAGTGTTAGAGACAAAGGTCCTGAGACGCCACC 780
 P  I  V  K  S  F  N  R  N  E  C  *                          219
ACCAGCTCCCCAGCTCCATCCTATCTTCCCTTCTAAGGTCTTGGAGGCTTCCCCACAAGC 840
GACCTACCACTGTTGCGGTGCTCCAAACCTCCTCCCCACCTCCTTCTCCTCCTCCTCCCT 900
TTCCTTGGCTTTTATCATGCTAATATTTGCAGAAAATATTCAATAAAGTGAGTCTTTGCA 960
CTTGAAAAAAAAAAAAAAAAAAAAAAAAAAAACTCGAG                       998
```

Fig. 2-4. Nucleotide and deduced amino acid sequences of the light chain of the mab MLT2-23 (cDNA clone pK211, GenBank accession no. AB097848). The deduced amino acid sequence is shown as 1-letter symbols below the nucleotide sequence. Nucleotides and amino acids are numbered at the right, and amino acid residues are numbered beginning with the N-terminal amino acid (asparaginic acid).

```
GAATTCGGCACGAGGCTGGACTCACAAGTCTTTCTCTTCAGTGACAAACACAGAAATAGA   60
ACATTCACCATGTTCTTGGGACTGAGCTGTGTATTCATAGTTTTTCTCTTAAAAGGTGTC  120
          M  F  L  G  L  S  C  V  F  I  V  F  L  L  K  G  V    -3
CAGAGTGAAGTGAAGCTTGAAGAGTCTGGAGGAGGCTTGGTTCAACCTGGAGGATCCATG  180
 Q  S  E  V  K  L  E  E  S  G  G  G  L  V  Q  P  G  G  S  M    18
AAACTCTCCTGTGTTGCCTCTGGATTCACTTTCAGTAACTACTGGATGCTCTGGGTCCGC  240
 K  L  S  C  V  A  S  G  F  T  F  S  N  Y  W  M  L  W  V  R    38
CAGTCTCCAGAGAAGGGGCTTGAATGGATTGCTGAAATTAGATTGAAATCTAATAATTAT  300
 Q  S  P  E  K  G  L  E  W  I  A  E  I  R  L  K  S  N  N  Y    58
GGAGTACATTATGCGGAGTCTGTGAGAGGGAGGTTCATCATCTCAAGGGATGATTCCAGA  360
 G  V  H  Y  A  E  S  V  R  G  R  F  I  I  S  R  D  D  S  R    78
AGTAGTGTCTACCTGCAAATGAACAACTTAAGACCTGAAGATACTGGCATTTATTACTGT  420
 S  S  V  Y  L  Q  M  N  N  L  R  P  E  D  T  G  I  Y  Y  C    98
ACCAGGCCGGGGTATAAGTACGACGGCGCTTACTGGGGCCAAGGGACTCTGGTCACTGTC  480
 T  R  P  G  Y  K  Y  D  G  A  Y  W  G  Q  G  T  L  V  T  V   118
TCTGTAGCCAAAACGACACCCCCATCTGTCTATCCACTGGCCCCTGGATCTGCTGCCCAA  540
 S  V  A  K  T  T  P  P  S  V  Y  P  L  A  P  G  S  A  A  Q   138
ACTAACTCCATGGTGACCCTGGGATGCCTGGTCAAGGGCTATTTCCCTGAGCCAGTGACA  600
 T  N  S  M  V  T  L  G  C  L  V  K  G  Y  F  P  E  P  V  T   158
GTGACCTGGAACTCTGGATCCCTGTCCAGCGGTGTGCACACCTTCCCAGCTGTCCTGCAG  660
 V  T  W  N  S  G  S  L  S  S  G  V  H  T  F  P  A  V  L  Q   178
TCTGACCTCTACACTCTGAGCAGCTCAGTGACTGTCCCCTCCAGCACCTGGCCCAGCGAG  720
 S  D  L  Y  T  L  S  S  S  V  T  V  P  S  S  T  W  P  S  E   198
ACCGTCACCTGCAACGTTGCCCACCCGGCCAGCAGCACCAAGGTGGACAAGAAAATTGTG  780
 T  V  T  C  N  V  A  H  P  A  S  S  T  K  V  D  K  K  I  V   218
CCCAGGGATTGTGGTTGTAAGCCTTGCATATGTACAGTCCCAGAAGTATCATCTGTCTTC  840
 P  R  D  C  G  C  K  P  C  I  C  T  V  P  E  V  S  S  V  F   238
ATCTTCCCCCCAAAGCCCAAGGATGTGCTCACCATTACTCTGACTCCTAAGGTCACGTGT  900
 I  F  P  P  K  P  K  D  V  L  T  I  T  L  T  P  K  V  T  C   258
GTTGTGGTAGACATCAGCAAGGATGATCCCGAGGTCCAGTTCAGCTGGTTTGTAGATGAT  960
 V  V  V  D  I  S  K  D  D  P  E  V  Q  F  S  W  F  V  D  D   278
GTGGAGGTGCACACAGCTCAGACGCAACCCCGGGAGGAGCAGTTCAACAGCACTTTCCGC 1020
 V  E  V  H  T  A  Q  T  Q  P  R  E  E  Q  F  N  S  T  F  R   298
TCAGTCAGTGAACTTCCCATCATGCACCAGGACTGGCTCAATGGCAAGGAGTTCAAATGC 1080
 S  V  S  E  L  P  I  M  H  Q  D  W  L  N  G  K  E  F  K  C   318
AGGGTCAACAGTGCAGCTTTCCCTGCCCCCATCGAGAAAACCATCTCCAAAACCAAAGGC 1140
 R  V  N  S  A  A  F  P  A  P  I  E  K  T  I  S  K  T  K  G   338
AGACCGAAGGCTCCACAGGTGTACACCATTCCACCTCCCAAGGAGCAGATGGCCAAGGAT 1200
 R  P  K  A  P  Q  V  Y  T  I  P  P  P  K  E  Q  M  A  K  D   358
AAAGTCAGTCTGACCTGCATGATAACAGACTTCTTCCCTGAAGACATTACTGTGGAGTGG 1260
 K  V  S  L  T  C  M  I  T  D  F  F  P  E  D  I  T  V  E  W   378
CAGTGGAATGGGCAGCCAGCGGAGAACTACAAGAACACTCAGCCCATCATGGACACAGAT 1320
 Q  W  N  G  Q  P  A  E  N  Y  K  N  T  Q  P  I  M  D  T  D   398
GGCTCTTACTTCGTCTACAGCAAGCTCAATGTGCAGAAGAGCAACTGGGAGGCAGGAAAT 1380
 G  S  Y  F  V  Y  S  K  L  N  V  Q  K  S  N  W  E  A  G  N   418
ACTTTCACCTGCTCTGTGTTACATGAGGGCCTGCACAACCACCATACTGAGAAGAGCCTC 1420
 T  F  T  C  S  V  L  H  E  G  L  H  N  H  H  T  E  K  S  L   438
TCCCACTCTCCTGGTAAATGATCCCAGTGTCCTTGGAGCCCTCTGGTCCTACAGGACTCT 1480
 S  H  S  P  G  K  *                                          444
GACACCTACCTCCACCCCTCCCTGTATAAATAAAGCACCCAGCACTGCCTTGGGACCCTG 1540
CAAAAAAAAAAAAAAAAAAAAAAAAACTCGAG                              1573
```

Fig. 2-5. Nucleotide and deduced amino acid sequences of the heavy chain of the mab MLT40-4 (cDNA clone pG408, GenBank accession no. AB097849). The deduced amino acid sequence is shown as 1-letter symbols below the nucleotide sequence. Nucleotides and amino acids are numbered at the right, and amino acid residues are numbered beginning with the N-terminal amino acid (glutaminic acid).

```
GAATTCGGCACGAGGCTGTGGTTGTCTGGTGTTGATGGAGACATTGTGATGACCCAGTCT  60
 E  F  G  T  R  L  W  L  S  G  V  D  G  D  I  V  M  T  Q  S   7
CAAAAATTCATGTCCACATCAGTAGGAGACAGGGTCAGCGTCACCTGCAAGGCCAGTCAG 120
 Q  K  F  M  S  T  S  V  G  D  R  V  S  V  T  C  K  A  S  Q  27
AATGTGAATAATAATGTAGCCTGGCATCAACAGAAACCAGGTCAATCCCCTAAAGCACTG 180
 N  V  N  N  N  V  A  W  H  Q  Q  K  P  G  Q  S  P  K  A  L  47
TTTTACTCGGCATCCTACCGGTACAGTGGAGTCCCTGATCGCTTCACAGGCAGTGGATAT 240
 F  Y  S  A  S  Y  R  Y  S  G  V  P  D  R  F  T  G  S  G  Y  67
GGGACAGACTTCACTCTCACCATCAGCAATGTGCAGTCTGAAGACCTGGCAGAATATTTC 300
 G  T  D  F  T  L  T  I  S  N  V  Q  S  E  D  L  A  E  Y  F  87
TGTCAGCAATATAACAGCTTTCCGTACACTTTCGGAGGGGGGACCAAGCTGGAAATAAAA 360
 C  Q  Q  Y  N  S  F  P  Y  T  F  G  G  G  T  K  L  E  I  K 107
CGGGCTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATCT 420
 R  A  D  A  A  P  T  V  S  I  F  P  P  S  S  E  Q  L  T  S 127
GGAGGTGCCTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAAAGACATCAATGTCAAG 480
 G  G  A  S  V  V  C  F  L  N  N  F  Y  P  K  D  I  N  V  K 147
TGGAAGATTGATGGCAGTGAACGACAAAATGGCGTCCTGAACAGTTGGACTGATCAGGAC 540
 W  K  I  D  G  S  E  R  Q  N  G  V  L  N  S  W  T  D  Q  D 167
AGCAAAGACAGCACCTACAGCATGAGCAGCACCCTCACGTTGACCAAGGACGAGTATGAA 600
 S  K  D  S  T  Y  S  M  S  S  T  L  T  L  T  K  D  E  Y  E 187
CGACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAACTTCACCCATTGTCAAG 660
 R  H  N  S  Y  T  C  E  A  T  H  K  T  S  T  S  P  I  V  K 207
AGCTTCAACAGGAATGAGTGTTAGAGACAAAGGTCCTGAGACGCCACCACCAGCTCCCCA 720
 S  F  N  R  N  E  C  *                                      214
GCTCCATCCTATCTTCCCTTCTAAGGTCTTGGAGGCTTCCCCACAAGCGACCTACCACTG 780
TTGCGGTGCTCCAAACCTCCTCCCCACCTCCTTCTCCTCCTCCTCCCTTTCCTTGGCTTT 840
TATCATGCTAATATTTGCAGAAAATATTCAATAAAGTGTGTCTTTGCACTTGAAAAAAAA 900
AAAAAAAAAAAAAAAAACTCGAG                                       923
```

Fig. 2-6. Nucleotide and deduced amino acid sequences of the light chain of the mab MLT40-4 (cDNA clone pK414, GenBank accession no. AB097850). The deduced amino acid sequence is shown as 1-letter symbols below the nucleotide sequence. Nucleotides and amino acids are numbered at the right, and amino acid residues are numbered beginning with the N-terminal amino acid (asparaginic acid).

(A)

```
MLT2-23(pG208)    1:LQESGPSLVKPSQTLSLTCSVTGDSITSGYWN-WIRKFPGNKLEYMGYIS  49
MLT40-4(pG408)    1:LEESGGGLVQPGGSMKLSC-VASGFTFSNYWMLWVRQSPEKGLEWIAEIR  49
                    * ***  ** *     * * *       * **  * *  *   **
MLT2-23(pG208)   50:YSGSTY---YNPSLKNRISITRDTSRNQFSLHLNSVITEDTATYYCAGST  96
MLT40-4(pG408)   50:LKSNNYGVHYAESVRGRFIISRDDSRSSVYLQMNNLRPEDTGIYYCTRPG  99
                         *   *  *   * * ** **    *  *    ***  ***
MLT2-23(pG208)   97:MITTRAGHWGQGTLVTVSA                               115
MLT40-4(pG408)  100:-YKYDGAYWGQGTLVTVSV                               117
                          **********
```

(B)

```
MLT2-23(pK211)    1:DIVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYLQKPGQSPK  50
MLT40-4(pK414)    1:DIVMTQSQKFMSTSVGDRVSVTCKASQN-VN-N-N--VAWHQQKPGQSPK  45
                    ******       * **  *  *  **  *  * *    *  ********
MLT2-23(pK211)   51:LLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGLYFCSQATHVP 100
MLT40-4(pK414)   46:ALFYSASYRYSGVPDRFTGSGYGTDFTLTISNVQSEDLAEYFCQQYNSFP  95
                     * *  * * ******* *** ****** ** *  ***  *** *    *
MLT2-23(pK211)  101:FTFGGGTTLEIKR                                     113
MLT40-4(pK414)   96:YTFGGGTKLEIKR                                     108
                     ****** *****
```

Fig. 2-7. Alignment of the deduced amino acid sequences of variable regions of heavy and light chains of MLT2-23 and MLT40-4.
Bold characters indicate CDR. Asterisks denote the amino acid residues that are identical in both sequences. Gaps are inserted to maximize the alignment. (A) VH; (B) VL.

RNA Gel Blot Analysis

In order to determine the origin of the cDNA clones obtained, mRNA isolated from hybridoma cells and that from P3-X63-Ag8.653 myeloma cells, a fusion partner for splenocyte, were subjected to an RNA gel blot analysis. The blot was probed with the cDNA fragments encoding variable region of each of the cDNA clones. As shown in Fig. 2-8, signal was detected only in the hybridoma cell extracts, but not in the myeloma cell mRNA. This result indicated that the four cDNA clones were originated from the mouse spleen cells, but not from myeloma cells. Therefore, pG208 and pK211 were proven to encode heavy and light chains of MLT2-23, respectively, and pG408 and pK414 were heavy and light chains of MLT40-4, respectively.

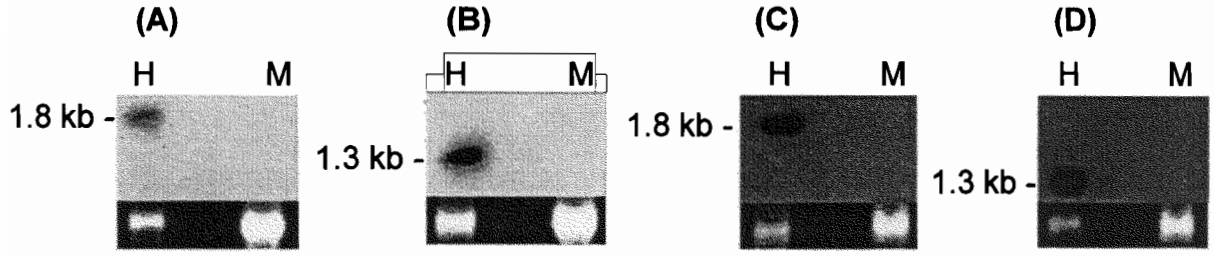

Fig. 2-8. RNA gel blot analysis of mRNA extracted from hybridoma cells and myeloma cells.
One μg of mRNA was applied. H; mRNA extracted from hybridoma cell line MLT2-23 (A, B) and MLT40-4 (C, D). M; mRNA extracted from myeloma cell. Ethidium bromide staining of 28S ribosomal RNA band is shown as an indication of mRNA loading levels. (A), cDNA fragments of variable region of pG208 were used as a probe; (B), cDNA fragments of variable region of pK211 were used as a probe; (C), cDNA fragments of variable region of pG408 were used as a probe; (D), cDNA fragments of variable region of pK414 were used as a probe.

Construction of Four Anti-Malathion ScFv Genes

PCR was performed to construct MLT2-23/HL scFv, MLT2-23/LH scFv, MLT40-4/HL scFv and MLT40-4/LH scFv genes with the primers complementary to each 5′- and 3′-end of VH and VL of both mabs (Table 2-1). At the first PCR cycles, VH and VL cDNA fragments were amplified with the primers (Fig. 2-9A). At the second PCR cycles, both cDNA fragments of the respective mabs were successfully assembled to produce the PCR products of 750 bp, which were confirmed by agarose gel electrophoresis (Fig. 2-9B). The four scFv genes prepared were each inserted into the expression vector pCANTAB5E and transformed into *E. coli* TG1 cells to produce the corresponding fusion protein, scFv-g3p, displayed on the outer coat protein of the recombinant phages.

Reactivity of Anti-Malathion ScFv Antibodies in Ic-ELISA

DNA sequencing verified that there was no nucleotide deletion and substitution among the four expression phagemids constructed. The prepared recombinant phages displaying scFv antibodies which bound to EIT244 were isolated by the panning method against EIT244-BSA and

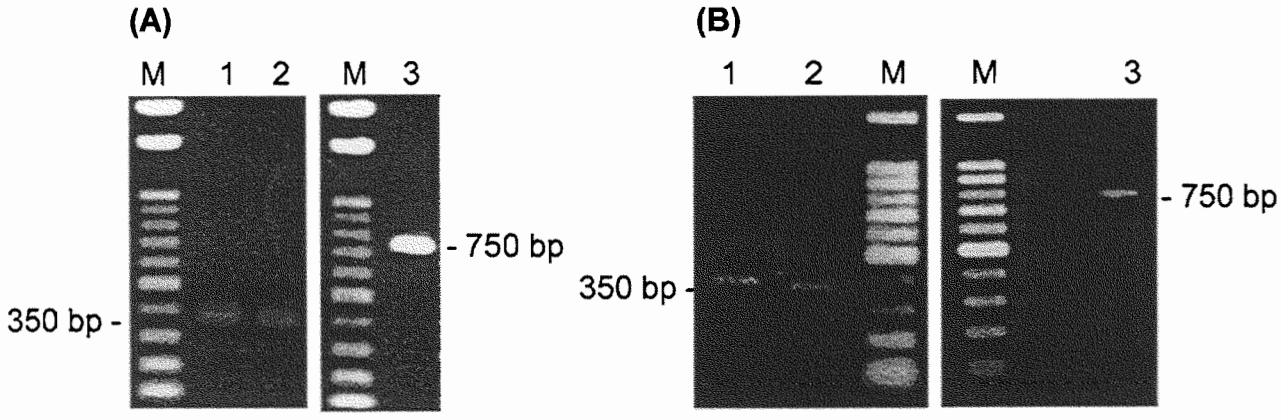

Fig. 2-9. PCR amplification of VH, VL and scFv genes.
(A), MLT2-23. (B), MLT40-4.
M; 100 bp ladder marker, lane 1; cDNA fragments coding for VH domain, lane 2; cDNA fragments coding for VL domain, lane 3; scFv gene.

used for infection to *E. coli* HB2151 cells to produce the corresponding soluble scFv antibodies. Inhibition curves in ic-ELISA with the four scFv antibodies are shown in Fig. 2-10A and 10B. The MLT2-23/HL scFv and MLT2-23/LH scFv were inhibited to bind to the immobilized antigen EIT244-BSA by free malathion in a concentration-dependent manner. The IC_{50} values obtained were 81 and 72 ng/ml in ic-ELISA based on MLT2-23/HL scFv and MLT2-23/LH scFv, respectively. This performance was comparable to that obtained with the parent mab MLT2-23 in ic-ELISA (IC_{50} value = 60 ng/ml). This result indicated that the reactivity of MLT2-23/HL scFv and MLT2-23/LH scFv were similar in ic-ELISA. On the other hand, MLT40-4/HL scFv was prepared at the threshold of the initial attempt, however, a low dilution rate of the scFv antibody preparation was required to obtain sufficient absorbance in ic-ELISA, and an inhibition curve was not good enough to calculate the IC_{50} value for malathion. While the MLT40-4/LH scFv came up to that of the parent mab MLT40-4 and the IC_{50} value with MLT40-4/LH scFv was 150 ng/ml in contrast to that with MLT40-4 mab of 75 ng/ml. The dilution rate of MLT40-4/HL scFv was 1/20 of that of MLT40-4/LH scFv to obtain the absorbance of 1.0 in ic-ELISA. This result indicated that

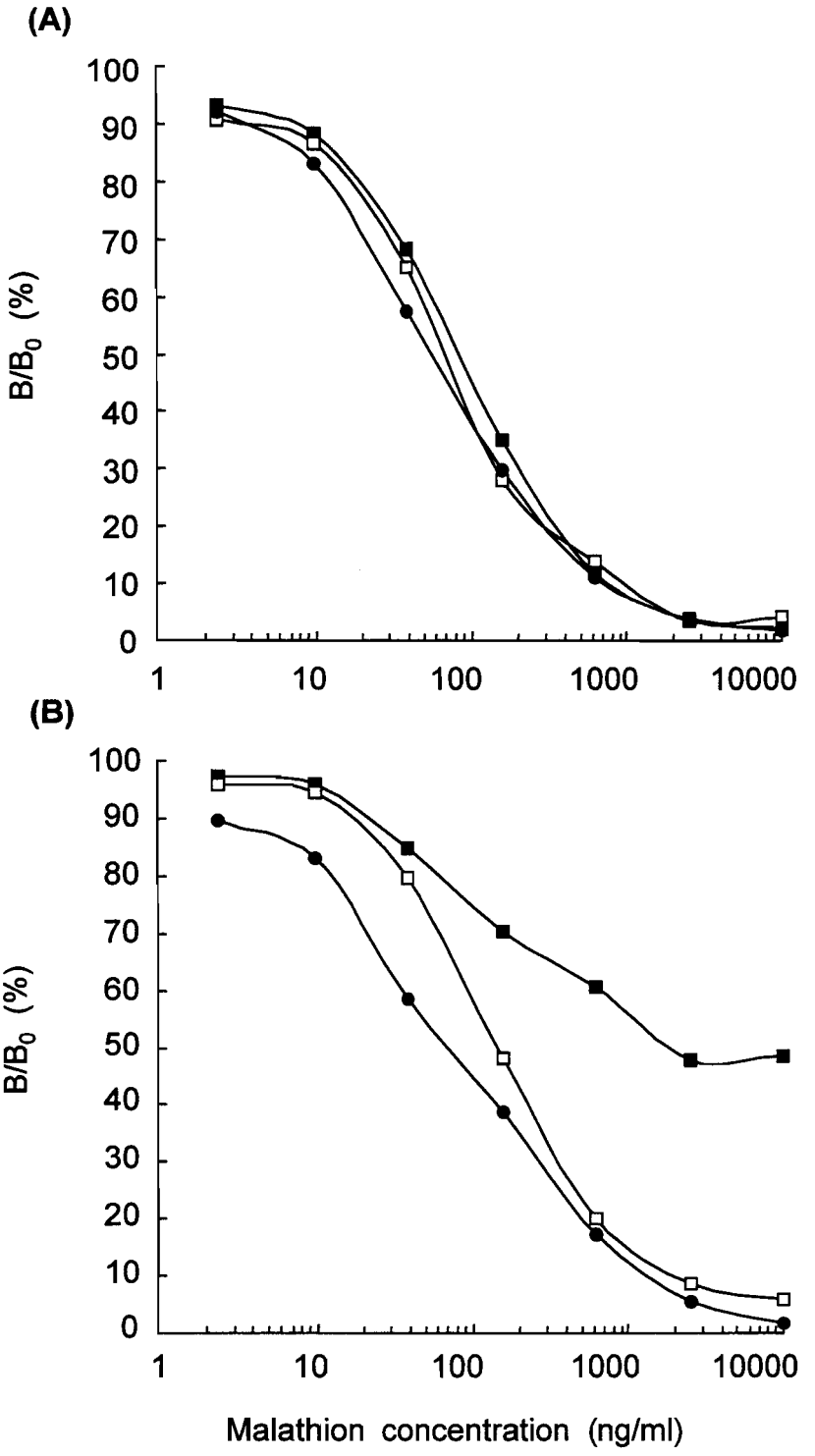

Fig. 2-10. Inhibition curves obtained with the soluble scFv antibodies and the parent mabs for malathion in ic-ELISA.
(A) the purified mab MLT2-23 (●), MLT2-23/HL scFv (■), MLT2-23/LH scFv (□). (B) the purified mab MLT40-4 (●), MLT40-4/HL scFv (■), MLT40-4/LH scFv (□).

MLT40-4/LH scFv reacted to malathion similar to the parent mab, while the reactivity of MLT40-4/HL scFv was very low. It was found that the order of the linkage of both VL and VH domains affected on the reactivity of MLT40-4 scFvs towards malathion in ic-ELISA, although the production of MLT40-4/HL in recombinant *E. coli* cells also seemed to be reduced.

DISCUSSION

Malathion is one of the most widely used insecticides. Since immunoassays were first applied to the pesticide residue analysis in 1971,[26] malathion has always been of importance for immunochemical analysis in the world due to its considerable wide use.[83] However, no reports were available with respect to immunoassays for malathion, because it used to be rather difficult to obtain antibodies specific to malathion. Although the immunoassays for common organophosphorus pesticides were developed by using generic haptens, the IC_{50} for malathion was 700 ng/ml[84] and 6.6 μg/ml.[85] Therefore, the development of a sensitive immunoassay for malathion was a challenging work.

When mice were immunized with the conjugates including MAL-KLH with the P-S-C bond, anti-malathion antibodies with high reactivity were not obtained. Thus, it was considered that the hapten MAL might be easily cleaved at the P-S-C bond in the mouse body, resulting in failure to stimulate the production of antibodies against malathion. Therefore, another malathion hapten EIT244 was synthesized, which contained the amide bond P-NH-C bond rather than the ester bond P-S-C. With the immunogen, two specific anti-malathion mabs were successfully isolated and led to ELISA specific for malathion, detecting malathion at low ppb levels. Since maximum residue limits of malathion in grains, vegetables and so on are equal to or more than 0.1 ppm,[72] the ELISA with the mabs described here can determine the concentration of malathion residues in these samples

with a simple dilution step, and require no complicated concentration procedure like instrumental analyses. Moreover, the ELISA with the mabs can specifically determine the malathion concentration due to considerably low cross-reactivity with the other organophosphorus pesticides. However, the reactivity of the anti-malathion mabs obtained was not very high as compared with that of anti-atrazine antibodies[86,87] or the others.[36,88] Thus, it was considered that the malathion molecule not containing any aromatic rings conjugated to carrier proteins seems to be unsuitable for immunogenicity. Monoclonal antibodies specific to haptens without aromatic rings were rarely reported up to date. Therefore, molecular information on two mabs obtained in this study was considered to be meaningful for understanding molecular basis of antigen-antibody interaction.

We attempted to clone the VH and VL genes directly from the hybridoma cells by RT-PCR. However, the obtained scFvs did not react with malathion in ic-ELISA. It was reported that the myeloma cell line P3-X63-Ag8.653 transcribes to give some non-functional immunoglobulin mRNA in a large quantity although the cells secret no antibodies.[61] Ostermeier *et al.*[89] reported that the aberrant mRNA exceeded the level of the desired immunoglobulin one at times. Then, it was assumed that non-functional immunoglobulin transcript was amplified in the trial. Indeed, the nucleotide sequences of the isolated immunoglobulin genes were found to be identical to the κ pseudogene reported previously.[90] DNA sequence analysis of the obtained cDNA clones pG208, pK211, pG408 and pK414 isolated from the constructed cDNA libraries confirmed to be different from the aberrant cDNA as reported previously.[90] Therefore, these clones obtained were accounted for the desired ones and used for further analysis.

RNA gel blot analysis was performed to clarify the origin of them because there still remained a possibility that they included pseudogenes. The result proved that the four cDNA clones were derived from spleen cells, that is, they encoded heavy and light

chains of MLT2-23 and MLT40-4.

The deduced amino acid sequences of variable regions of both mabs were aligned and compared each other. The variability was confined more to the CDR than to the framework regions. Especially, the highest variability was found in the third CDR of the heavy chain (CDRH3), where both amino acid sequences completely differed from each other, although both mabs showed a similar reactivity for malathion in ELISA. Since the CDRH3 amino acid residues mainly provides more contacts than the other CDR residues,[91] it may cause of difference between both mabs in reactivity with malathion.

We attempted to prepare both HL and LH scFv antibodies for two mabs and assessed by comparing with the parent mabs in ic-ELISA, since the primary structure of the CDR regions was quite different between both mabs. Comparison of both HL and LH scFv antibodies in reactivity toward haptens was not reported yet. Both MLT2-23/HL scFv and MLT2-23/LH scFv similarly reacted towards the immobilized antigen EIT244-BSA as well as malathion in ic-ELISA. Therefore, both MLT2-23/HL and MLT2-23/LH scFv antibodies seemed to behave in a same manner as the parent MLT2-23. This result agreed with that of the reported results.[52,53] It seemed that the order of variable domains had little meaning in so far as MLT2-23 scFvs. Otherwise, the order of the variable domains affected the reactivity of MLT40-4 scFvs for malathion in ic-ELISA. Although MLT40-4/LH scFv reacted towards EIT244-BSA and malathion similarly as with the parent MLT40-4, MLT40-4/HL scFv reduced the reactivity towards both EIT244-BSA and malathion. The disorder of MLT40-4/HL scFv in the reactivity with the antigen may be due to the amino acid residues in CDRH3, which was quite different from that of MLT2-23, although the cross-reactivity of the scFv antibodies towards organophosphorus insecticides was not confirmed on the present study. It was considered that the order of linkage of both variable domains may make the change of conformation of the antigen-binding pocket of MLT40-4/HL scFv, leading to reduce the reactivity to the antigen. It

was reported that the reactivity of scFv antibodies was affected by the order of the variable domains as with the anti-*Salmonella* serogroup B O-antigen mab[92] and the anti-human CD40 mab.[93] The amount of MLT40-4/HL scFv produced in recombinant *E. coli* cells seemed to be lower than MLT40-4/LH scFv, since MLT40-4/HL scFv required over a 1/20 lower dilution rate than that of MLT40-4/LH scFv in ic-ELISA. Tsumoto *et al.*[94] reported that the order of linkage of both variable domains affected the productivity of scFv antibody in recombinant *E. coli* cells. Although it seemed to be difficult to rule out the suitable order of VH and VL regions, it would be useful to replace the variable domains for improvement of affinity or reactivity when scFv antibodies are very inferior to their parent mabs.

The obtained mabs as well as the corresponding scFv antibodies were highly reactive to malathion which has no aromatic moiety in the molecule. Particularly, the recombinant antibody technology was useful for large-scale production in recombinant *E. coli* cells as well as the other heterologous expression systems and improvement of molecular characteristics by modification of the amino acid sequence. Therefore, recombinant antibodies can be exploited in various immunological techniques such as immunoassay, immunosensor and immunoaffinity chromatography for monitoring of environmental chemicals.

CHAPTER III

MOLECULAR AND IMMUNOCHEMICAL CHARACTERISTICS OF MONOCLONAL AND RECOMBINANT ANTIBODIES SPECIFIC TO BISPHENOL A

INTRODUCTION

Expansion of contamination of the environment and agricultural products with persistent organic pollutants (POPs) is a serious global problem. These chemicals include dioxins, pesticide residues and endocrine disruptors such as bisphenol A (BPA). This chemical is a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins which are extensively used as a plastic coating in the food packaging industry as well as in dentistry.[95] In Japan, the production of BPA was about 250,000 tons in 1996.[96] Krishnan[97] reported that BPA was released from flasks made of polycarbonate and showed weak estrogenic property. The binding affinity of the chemical towards the estrogen receptor *in vitro* was reported to be only 1/10,000 potency of 17β-estradiol.[98] However, it was also reported that BPA can be leached from food and beverage containers into food,[99] and leached from the can coating when canned products were heated at a high temperature.[100] In addition, BPA was found in saliva collected from patients who were treated with a BPA diglycidyl ether-based dental sealant.[101] Also, the wastewater containing BPA from its production factories can be the source of contamination in aquatic.[95,102] Environmental Agency of Japan reported that contamination of the environment with BPA was widespread in Japan and the levels of BPA in freshwater and seawater samples ranged from ppt to ppb levels.[103] BPA also shows an acute toxicity in the range of about 1-10 mg/L towards algae, invertebrates and fish.[104] Therefore, monitoring of contamination of the environment and food with BPA seems to be important for risk assessment of the chemical.

At present, GC-MS,[105] high performance liquid chromatography (HPLC)-MS,[106] HPLC with fluorescence detection (HPLC-FLD)[107] and HPLC with electrochemical detection (HPLC-ECD)[108] are used for analysis of BPA. Although these methods are accurate and precise, these are labor-intensive and time-consuming. For rapid assessment of contamination of the environment and food with this chemical, development of a simple analytical method is very important. ELISA is handled readily and rapidly, and also deals with more samples than the instrumental analysis methods. Recently, many ELISA methods have been reported for POPs such as dioxins,[32,36] polychlorinated biphenyls, [37,39] and pesticide residues.[76,77,109]

In this study, we attempted to prepare anti-BPA mabs with high affinity and to develop a highly sensitive ELISA based on mabs. Furthermore, isolation of cDNA clones encoding anti-BPA mabs and expression of these cDNA clones in *E. coli* were also attempted to produce recombinant antibodies. The molecular characteristics of these mabs and corresponding recombinant antibodies were assessed in ELISA.

MATERIALS AND METHODS

Chemicals and Biochemicals

BPA (4,4'-isopropylidenediphenol, CAS Registry No. 80-05-7, Fig. 3-1) was purchased from Kanto Chemical Co. (Tokyo, Japan). BPA-related chemicals used in this study were obtained from Aldrich Chemical Co. (WI, USA) and Tokyo Kasei Kogyo Co. (Tokyo, Japan). Stock solutions were prepared by dissolving 1 mg of each chemical in 1 mL of MeOH. The BPA hapten BPAH (4-{4-[1-(4-hydroxyphenyl)-1-methylethyl]phenoxy}butyric acid, Fig. 3-1) was synthesized in Otsuka Chemical Co. (Tokushima, Japan). Bovine serum albumin (BSA) and rabbit serum albumin (RSA) were purchased from Sigma Chemical Co. (MO, USA). Keyhole limpet hemocyanine (KLH) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody were obtained from Pierce Chemical Co. (IL, USA). Block Ace was obtained from

Dainippon Pharmaceutical Co. (Osaka, Japan). DNA polymerase was purchased from Roche Diagnostics GmbH (Mannheim, Germany). DNA restriction endonucleases were purchased from Takara Shuzo Co. (Shiga, Japan). All other chemicals and organic solvents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

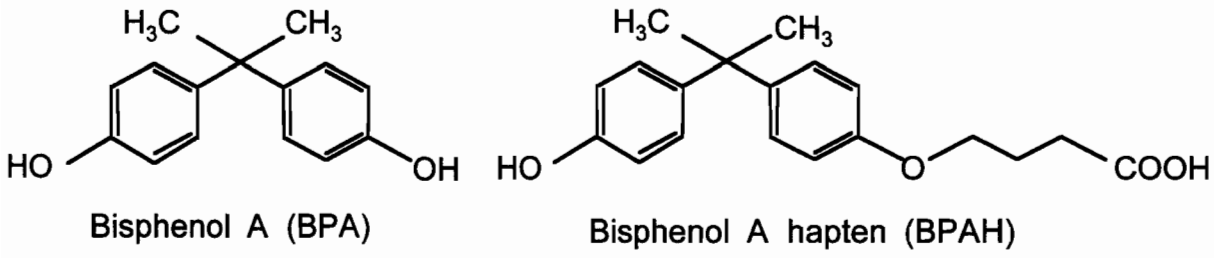

Fig. 3-1. The chemical structures of bisphenol A (BPA) and BPA hapten (BPAH) used in this study

Preparation of Hapten-Protein Conjugates

BPAH was covalently attached to a carrier protein by the active ester method.[110] BPAH (25 μmol), *N*-hydroxysuccinimide (25 μmol) and *N,N'*-dicyclohexylcarbodiimide (25 μmol) were dissolved in 1.0 mL of dimethylformamide and the reaction mixture was stirred in the dark at room temperature overnight. The activated hapten solution was added dropwise to BSA (0.25 μmol), RSA (0.25 μmol) or KLH (0.25 μmol) in 0.1 M borate buffer (pH 9.2) with stirring, and then the mixtures were stirred at room temperature overnight. Unconjugated BPAH was removed by dialysis against distilled water for 2 d at 4°C and the conjugates were lyophilized. The conjugates BPAH-BSA and BPAH-KLH were each used as an immunogen. BPAH-RSA was used as a coating antigen in ELISA.

Production of Mabs

Five-week-old BALB/c female mice (Nippon SLC Co., Shizuoka, Japan) were immunized with 100 μg of BPAH-BSA or BPAH-KLH as an immunogen. Their splenocytes were fused with P3-X63-Ag8.653

myeloma cells[78] and screened for their binding ability towards BPA in ELISA as previously described.[111] Isotype of each of mabs was determined with anti-mouse subclass specific antiserum (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions.

Isolation of cDNA Clones Coding for Anti-BPA Variable Regions by 5'-rapid Amplification of cDNA Ends (RACE) Method

Recombinant DNA techniques were according to the standard procedures.[81] Poly A^+ RNA fractions were isolated from 2×10^7 cells of each of hybridoma cell lines producing anti-BPA mabs using a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, NJ, USA). Both purity and amount of RNA were determined by UV spectrophotometry. To synthesize cDNA, 1 mg of a poly A^+ RNA fraction was reverse transcribed using oligo (dT) primer with MMLV reverse transcriptase (Clontech, CA, USA). cDNA fragments encoding variable regions of anti-BPA antibodies were cloned by 5'-RACE method using a SMART-RACE cDNA Amplification Kit (Clontech, CA, USA) with an universal primer mixture in the kit and gene-specific primers (GSPs) for the constant regions of murine IgG1 heavy chain or κ light chain. The GSP sequences were as follows; for IgG1 chain 5'-AGATGGATACAGTTGGTGCAGCATCAGC-3'; for κ chain 5'-ACCGATGGGGCTGTTGTTTTGGC-3'. The amplified cDNA fragments were cloned into pT7Blue T-vector (Novagen, WI, USA) and transformed into *E. coli* JM109 cells. Colony PCR was used to screen positive clones, and plasmids of positive colonies were purified and sequenced.

Nucleotide Sequencing and Analysis of Nucleic Acids

Recombinant plasmids were sequenced in an automated DNA sequencer SQ-5500 (Hitachi, Tokyo, Japan). DNA and deduced amino acid sequences were analyzed and aligned using DNA sequence analysis software Genetyx-Mac 7.3 software (Software Development Co., Tokyo, Japan).

Construction of Expression Plasmids for Antibody Fragment Genes

Four single-chain Fv (scFv) antibody genes were constructed by 2-step PCR as previously described.[112] Briefly, 10 primers were designed to construct scFv antibody genes based on nucleotide sequences of cDNA clones for variable heavy chain (VH) and variable light chain (VL) of four anti-BPA mabs (Table 3-1). The primers contained a flexible peptide linker sequence encoding $(Gly_4Ser)_3$ to assemble VH and VL, or specific sequences to facilitate the insertion of each of scFv genes into the *Nhe*I/*Nco*I site of the expression vector pET-27b (Novagen, WI, USA). VH cDNAs were amplified with the H5-primers and H3-primers, and VL cDNAs were amplified with the L5-primers and L3-primers in the first PCR. Then VH and VL cDNAs were assembled in the second PCR with H5-primers and L3-primers. PCR products were ligated into pT7Blue T-vector and transformed into *E. coli* JM109 cells for DNA sequencing. Four scFv genes were digested with *Nhe*I/*Nco*I and ligated into the *Nhe*I/*Nco*I restricted vector pET-27b, that contained a *pel*B leader sequence[113] for periplasmic secretion of scFv antibody, a hexahistidine tag sequence, and an anti-herpes simplex virus (HSV) tag for detection of scFv antibodies with a mouse anti-HSV mab (Novagen, WI, USA) in ELISA. The resulting expression plasmids (Fig. 3-2) were each transformed into *E. coli* BL21(DE3)pLysS strain cells.

Expression plasmids for VH and VL fragments of four mabs were also constructed by PCR. VH fragment genes were amplified with H5-primers and VH3-primers, and VL fragment genes were with VL5-primers and VL3-primers (Table 3-1). Reaction mixtures were preheated at 94°C for 5 min, followed by 20 cycles of denaturing at 94°C for 30 s, annealing at 63°C for 30 s, and polymerization at 72°C for 30 s, with a final extra 3 min extension. PCR products were ligated into pT7Blue T-vector and transformed into *E. coli* JM109 cells for DNA sequencing. VH and VL genes were recovered by *Nhe*I/*Nco*I and *Bpu1102*I/*Nco*I digestion, respectively, and ligated into the restricted vector pET-27b. Detection of VH and VL fragments in

Table 3-1. The nucleotide sequences of the primers used for construction of recombinant antibodies.

Primer name	Nucleotide sequence
H5-1	5'-CCATGGATGATGTACAGCTTCAGGAGTCAGGAC-3'
H5-2	5'-CCATGGATGACGTGAAGTTCGTGGAGTCTG-3'
H3-1	5'-AGAGCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGACGGTGACTGAGGTT-3'
H3-2	5'-AGAGCCACCTCCGCCTGAACCGCCTCCACCTGCAGAGACAGTGACCAGAGTCC-3'
L5-1	5'-GGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATTGTGCTGACACAGTCTCCT-3'
L5-2	5'-GGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATTGTGCTGACACAGTTTCCT-3'
L5-3	5'-GGCGGAGGTGGCTCTGGCGGTGGCGGATCGGATGTTGTGATGACCCAAACTCC-3'
L3-1	5'-GCTAGCCCGTTTGATTTCCAGCTTGGT-3'
L3-2	5'-GCTAGCCCGTTTCAGCTCCAGCTTG-3'
L3-3	5'-GCTAGCCCGTTTTATTTCCAACTTTGTCC-3'
VL5-1	5'-CCATGGATGACATTGTGCTGACACAGTCTCCT-3'
VL5-2	5'-CCATGGATGACATTGTGCTGACACAGTTTCCT-3'
VL5-3	5'-CCATGGATGATGTTGTGATGACCCAAACTCC-3'
VL3-1	5'-GCTCAGCTTATTTATCATCATCATCTTTATAATCCCGTTTGATTTCCAGCTTGGT-3'
VL3-2	5'-GCTCAGCTTATTTATCATCATCATCTTTATAATCCCGTTTCAGCTCCAGCTTG-3'
VL3-3	5'-GCTCAGCTTATTTATCATCATCATCTTTATAATCCCGTTTTATTTCCAACTTTGTCC-3'
VH5-1	5'-GCTAGCTGAGGAGACGGTGACTGAGGTT-3'
VH5-2	5'-GCTAGCTGCAGAGACAGTGACCAGAGTCC-3'

The combination of the primers was used for construction of scFv, VH and VL fragments as follow. BBA-2187scFv, H5-1, H3-1, L5-1 and L3-1; BBA-2617scFv, H5-1, H3-1, L5-2 and L3-1; BKE-3430scFv, H5-2, H3-2, L5-3 and L3-2; BTE-3456scFv, H5-1, H3-1, L5-1 and L3-3; BBA-2187scFv, H5-1, H3-1, L5-1 and L3-1; VL of BBA-2187, VL5-1 and VL3-1; VL of BBA-2617, VL5-2 and VL3-1; VL of BKE-3430, VL5-3 and VL3-2; VL of BTE-3456, VL5-1 and VL3-3; VH of BBA-2187, H5-1 and VH3-1; VH of BBA-2617, H5-1 and VH3-1; VH of BKE-3430, H5-2 and VH3-2; VH of BTE-3456, H5-1 and VH3-1.

ELISA was performed with a mouse anti-HSV mab and a mouse anti-FLAG mab (Sigma, MO, USA), respectively. These expression plasmids (Fig. 3-2) were each transformed into *E. coli* BL21(DE3)pLysS strain cells.

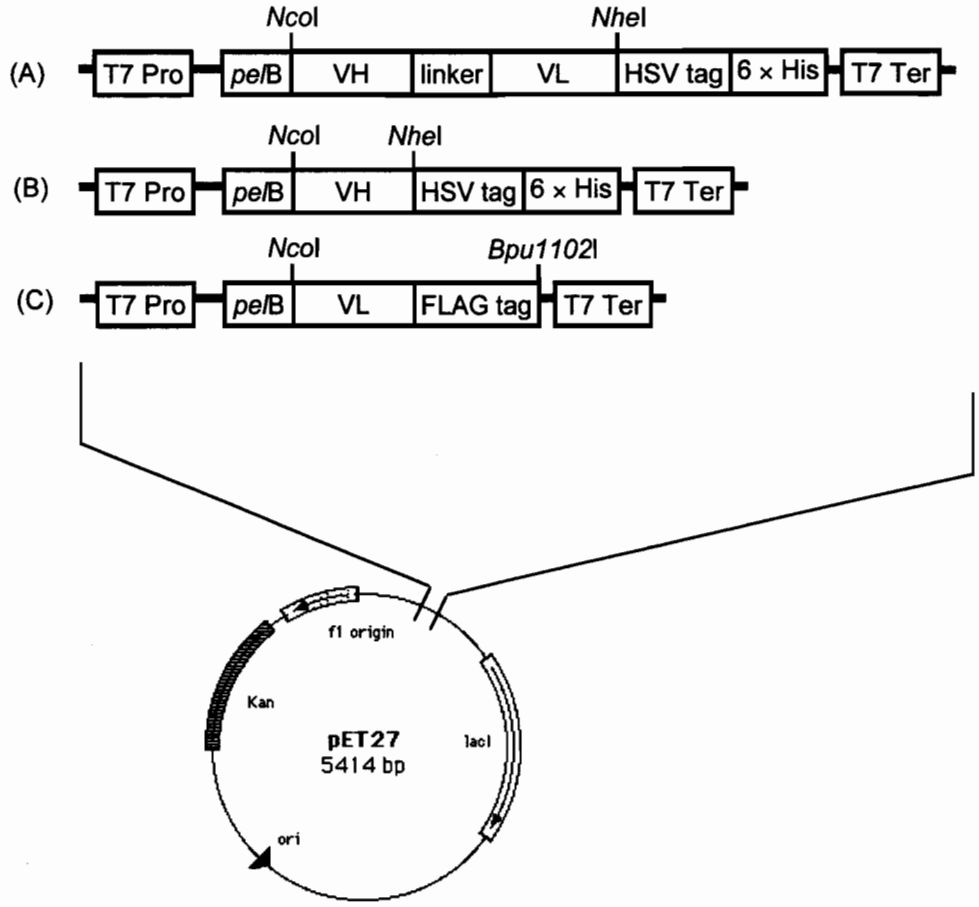

Fig. 3-2. The schematic diagrams of the expression plasmids for the anti-BPA scFv antibodies, the VH and VL fragments of anti-BPA mabs
(A) The plasmids for the scFv antibody genes to be expressed in recombinant *E. coli* cells are represented. (B) The plasmids for cDNAs encoding the VH fragments to be expressed in recombinant *E. coli* cells are represented. (C) The plasmids for cDNAs encoding the VL fragments to be expressed in recombinant *E. coli* cells are represented. Abbreviations: T7 Pro, T7 promoter; *pel*B, *pel*B leader sequence; HSV tag, an epitope tag for detection with an anti-HSV mab; 6 x His, a hexahistidine tag; FLAG tag, an epitope tag for detection with an anti-FLAG mab; T7 Ter, T7 terminator.

Production of Recombinant Antibody Fragments in *E. coli*

Periplasmic extraction was performed as described by Strachan *et al.*[114] with some modifications. Transformed *E. coli* BL21(DE3)pLysS cells were grown at 23°C in 250 mL of 2 × YT medium containing 25 μg/mL kanamycin and 33 μg/mL chrolamphenicol, and induced by adding isopropyl-β-D-thiogalactopyranoside to the culture medium to a final concentration of 1 mM. After induction period of 4 h at 23°C, cells were harvested and washed with 250 mL of ice-cold TES buffer (0.2 M Tris-HCl, 0.5 mM EDTA, 0.5 M sucrose, pH 8.0). After centrifugation, cells were resuspended with 25 mL of ice-cold 5 mM $MgSO_4$. After standing on ice for 30 min, cells were centrifuged by 10,000 × *g* at 4°C for 10 min. The supernatant was recovered and used for ELISA assay as a scFv antibody.

ELISA with Mabs

ELISA assays were performed as previously described[112] with some modifications. Wells of microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) was each coated with 100 μL of BPAH-RSA (0.1 μg/mL) in phosphate-buffered saline (PBS; 10 mM phosphate, 0.9% (w/v) NaCl, pH 7.2) overnight at 4°C, and blocked with 300 μL of Block Ace diluted 1 : 3 in distilled water. Fifty μL of standards of BPA or BPA-related compounds used in a cross-reactivity test and 50 μL of a mab were added into wells in triplicate, and reacted for 1 h at 25°C. After washing with PBS, 100 μL of anti-mouse IgG conjugated to HRP diluted in PBS containing 10% Block Ace (PBS-B) was added and incubated for 1 h at 25°C. After washing, 100 μL of chromogenic substrate (100 μg/mL 3,3',5,5'-tetramethylbenzidine and 0.006% (w/v) H_2O_2 in 0.1 M acetate buffer, pH 5.5) was added and incubated for 10 min at 25°C. The enzyme reaction was stopped by adding 100 μL of 1 N sulfuric acid and the A_{450} was measured in a microplate reader (Corona Electric Co., Ibaraki, Japan).

ELISA with ScFv Antibodies

ELISA assay was performed as described above except for addition of 50 μL of a scFv antibody in place of a mab, followed by washing and adding 100 μL of anti-HSV mab (0.1 μg/mL) in 2 × PBS containing 10% Block Ace reacted for 1 h at 25°C.

ELISA with VH and VL Fragments

Wells were coated with 200 μL of BPAH-RSA (0.1 μg/mL) and blocked. A hundred μL of standards of BPA, 50 μL of VL fragment and 50 μL of VH fragment were added and reacted for 1 h at 25°C. After washing, 200 μL of anti-FLAG mab (1 μg/mL) in PBS-B was added and incubated for 1 h at 25°C. After washing, 200 μL of HRP-labeled anti-mouse IgG antibody in PBS-B was added and incubated for 1 h at 25°C. After washing, 200 μL of chromogenic substrate was added and incubated for 10 min at 25°C. The enzyme reaction was stopped with sulfuric acid, and the A_{450} was measured.

Noncompetitive ELISA with Antisera

ELISA assay was performed as described above in ELISA with mabs except for addition of 50 μL of antiserum in place of a mab.

RESULTS

Preparation of Anti-BPA Mabs and Development of ELISA for BPA

The titer of antisera from mice immunized with BPAH-KLH and BPAH-BSA was tested in noncompetitive ELISA. Mice exhibiting a good antibody titer in antisera were used for cell fusion. Culture supernatants of hybridoma cells from fusion experiments were assayed in ELISA and selected hybridoma cells producing anti-BPA antibodies were cloned. Four hybridoma cell lines producing anti-BPA mabs were established. These mabs were named as BBA-2187, BBA-2617, BKE-3430 and BTE-3456. The culture supernatant of these cell lines was used as a mab in ELISA without further purification. Based on the

immunoglobulin-isotyping experiments, all of four anti-BPA mabs were found to have IgG1 heavy chains and κ light chains. Thus, these anti-BPA mabs were in the class of IgG.

Assay conditions in ELISA (buffer pH, reaction temperature, reaction time, dilution rate of mab and concentration of coating antigen) were examined with the mab BBA-2187 which showed the highest activity among four mabs towards BPA. The sensitivity of an ELISA assay performed at 25°C for BPA in competitive reaction was similar to that at 37°C. While, the sensitivity of an assay at 4°C was superior to two former assays (data not shown). However, reaction temperature in competitive step was optimized at 25°C since a competition step at 4°C took 2 h and a bit adverse effort due to keeping reagents at 4°C for a longer time. The pH of an assay buffer was also examined in the range between pH 4 and 9. Except for decrease of sensitivity at pH 9, the assay sensitivity was not significantly different between pH 4 and pH 8 (data not shown). The optimized conditions were described in the materials and methods.

The standard curves with each of four mabs for BPA are shown in Fig. 3-3. The assays were performed at least three times to provide sufficient reproducibility. Data were calculated using at least three sets of assays obtained on different days. IC_{50} values were determined as the concentration of the compound required to reduce the absorbance of control (zero concentration of analyte) to half. The IC_{50} values of BBA-2187, BBA-2617, BKE-3430 and BTE-3456 for BPA were 0.59 ng/mL, 1.9 ng/mL, 1.5 ng/mL and 1.6 ng/mL, respectively. Thus, it was found that the ELISA with BBA-2187 was the most sensitive towards BPA among four anti-BPA mabs. The detection limit of BPA in the ELISA with BBA-2187 was 0.13 ng/ml as IC_{20}.

cDNA Cloning of VH and VL of Four Anti-BPA Mabs

Four hybridoma cell lines producing anti-BPA mabs BBA-2187, BBA-2617, BKE-3430 and BTE-3456 were used as sources of anti-BPA immunoglobulin genes. Two mg of mRNA extracted from 2×10^7

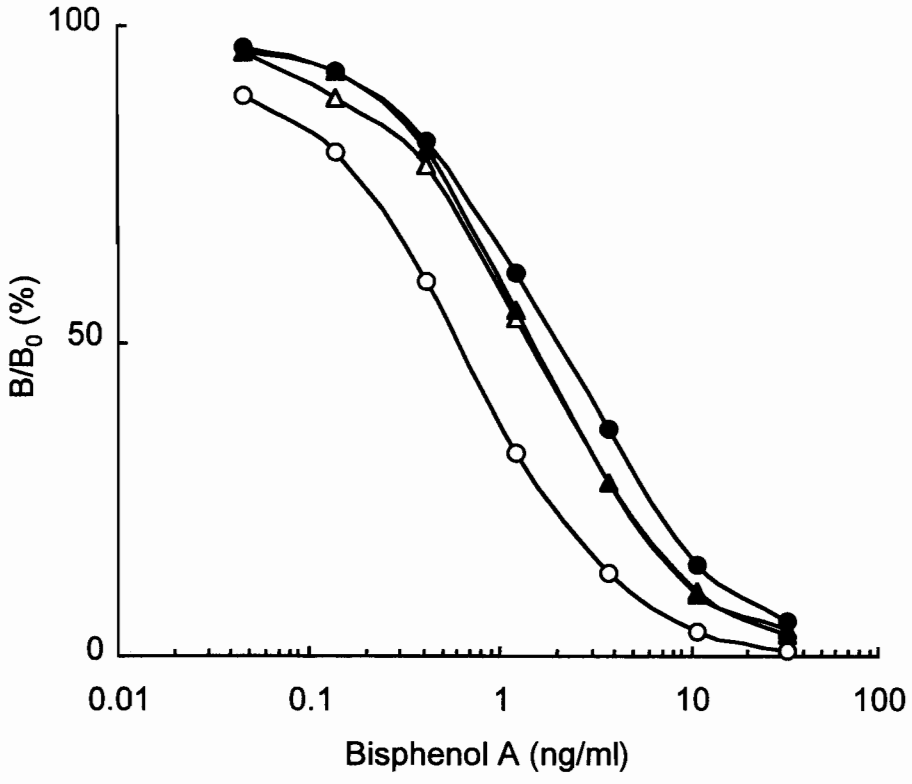

Fig. 3-3. The standard curves for BPA in ELISA based on the anti-BPA mabs
The values of absorbance were converted to B/B_0 (%) according to the following formula: B/B_0 (%) = $(A/A_0) \times 100$, where A is a value of absorbance for the standard and A_0 is a value of the absorbance for the control. (○), BBA-2187; (●), BBA-2617; (△), BKE-3430; (▲), BTE-3456.

cells of each hybridoma cell line was used for the first-strand cDNA synthesis. To isolate immunoglobulin cDNAs, 5'-RACE method was employed because DNA sequences of the immunoglobulin constant region are consistent. Then, two GSPs complementary to constant regions of IgG1 and κ chains were each designed for 5'-RACE method. An expected size of cDNA fragments (approximately 750 bp) were amplified with each of GSPs (data not shown). DNA sequencing revealed that all of inserts were murine immunoglobulin genes. The cloned VH cDNA sequences of BBA-2187, BBA-2617, and BTE-3456 contained 357 bp encoding 119 amino acids, and that of BKE-3430 contained 366 bp encoding 122 amino acids as shown in Fig. 3-4 to Fig. 3-7. The nucleotide sequences of cDNA clones coding for the

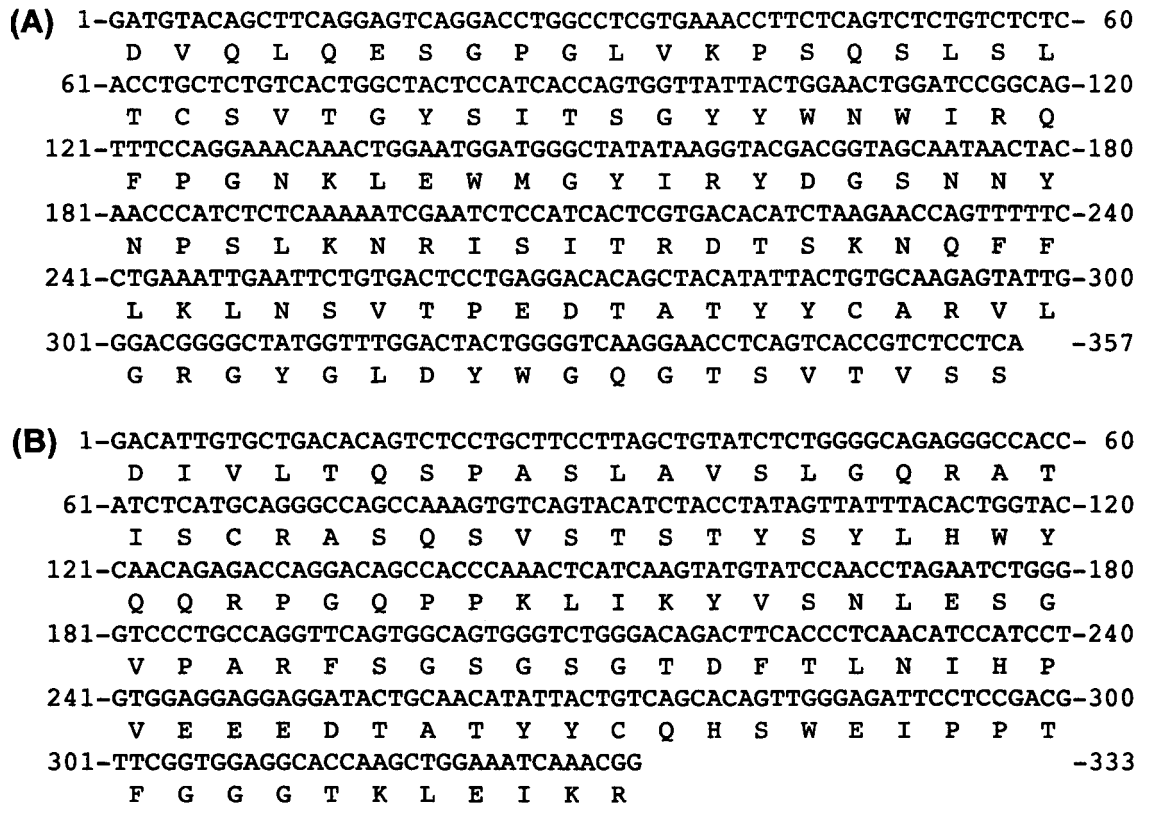

Fig. 3-4. Nucleotide and deduced amino acid sequences of the mab BBA-2187 (A), VH; (B), VL.

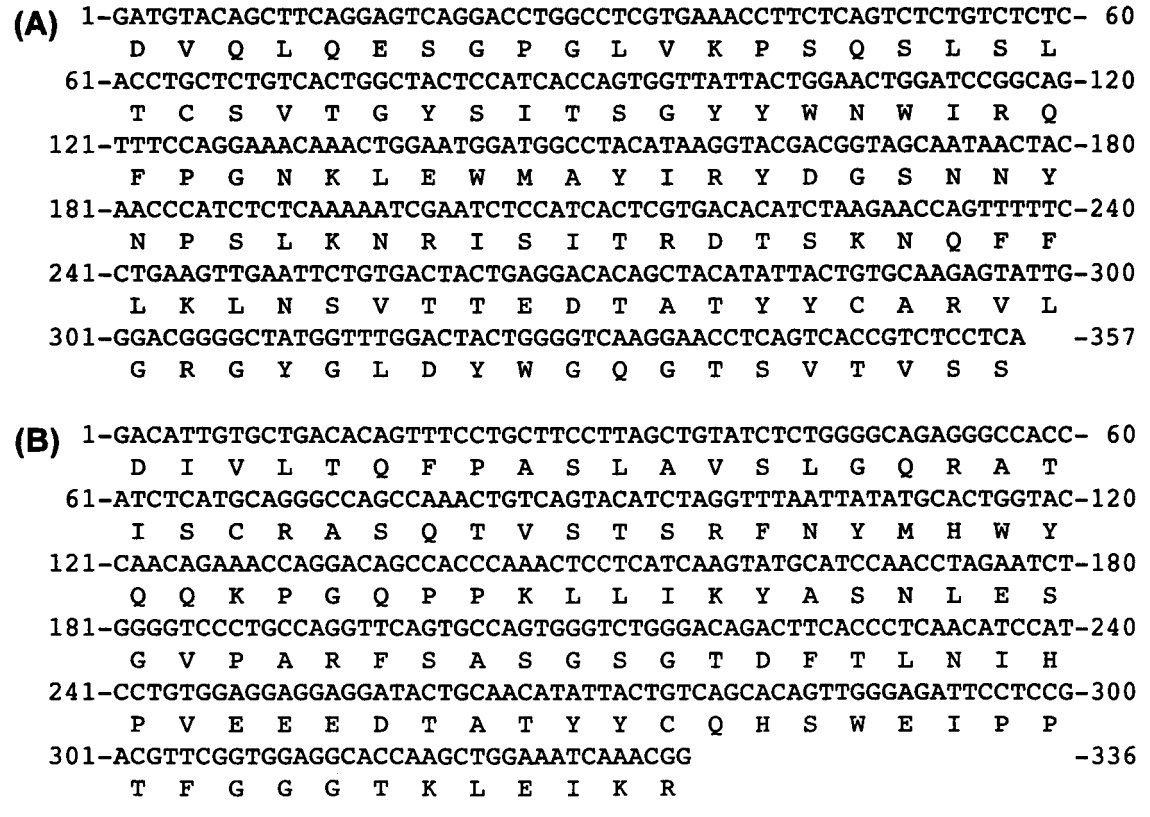

Fig. 3-5. Nucleotide and deduced amino acid sequences of the mab BBA-2617. (A), VH; (B), VL.

```
(A)1-GACGTGAAGTTCGTGGAGTCTGGGGGAGGCTTAGTGAAGCTTGGAGGGTCCCTGAAACTC -60
     D  V  K  F  V  E  S  G  G  G  L  V  K  L  G  G  S  L  K  L
 61-TCATGTGCAGCCTCTGGATTCACTTTCAGAAACTATTACATGTCTTGGGTTCGCCAGACT-120
     S  C  A  A  S  G  F  T  F  R  N  Y  Y  M  S  W  V  R  Q  T
121-CCAGAGAAGAGGCTGGAGTTGGTCGCAGGCATTAATACCAATGGTGGTTTCACCTACTAT-180
     P  E  K  R  L  E  L  V  A  G  I  N  T  N  G  G  F  T  Y  Y
181-CCAGACACTGTGAAGGGCCGATTCACCATCTCCAGAGACAATGCCAAGAACACCCTGTAC-240
     P  D  T  V  K  G  R  F  T  I  S  R  D  N  A  K  N  T  L  Y
241-CTGCAAATGAGCAGTCTGAAGTCTGAGGACACGGCCTTCTACTATTGTGCAAGACCGGAG-300
     L  Q  M  S  S  L  K  S  E  D  T  A  F  Y  Y  C  A  R  P  E
301-TTTGATACTTCCTACGTAGCCTGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTC-360
     F  D  T  S  Y  V  A  W  F  A  Y  W  G  Q  G  T  L  V  T  V
361-TCTGCA                                                    -366
     S  A

(B)1-GATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCC- 60
     D  V  V  M  T  Q  T  P  L  S  L  P  V  S  L  G  D  Q  A  S
 61-ATCTCTTGCAGATCTAGTCAGAGCCTTGTACACAGTAATGGAAACACCTATTTACATTGG-120
     I  S  C  R  S  S  Q  S  L  V  H  S  N  G  N  T  Y  L  H  W
121-TACCTGCAGAAGCCAGGCCAGTCTCCAAAGCTCCTGATCTACAAACTTTCCAACCGATTT-180
     Y  L  Q  K  P  G  Q  S  P  K  L  L  I  Y  K  L  S  N  R  F
181-TCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCATACTCAAGATC-240
     S  G  V  P  D  R  F  S  G  S  G  S  G  T  D  F  I  L  K  I
241-AGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAAAGTACACATGTTCCG-300
     S  R  V  E  A  E  D  L  G  V  Y  F  C  S  Q  S  T  H  V  P
301-CTGACGTTCGGTTCTGGGACCAAGCTGGAGCTGAAACGG                   -339
     L  T  F  G  S  G  T  K  L  E  L  K  R
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Fig. 3-6. Nucleotide and deduced amino acid sequences of the mab BKE-3430 (A), VH; (B), VL.

```
(A)1-GATGTACAGCTTCAGGAGTCAGGACCTGGCCTCGTGAAACCTTCTCACTCTCTGTCTCTC- 60
     D  V  Q  L  Q  E  S  G  P  G  L  V  K  P  S  H  S  L  S  L
 61-ACCTGCTCTGTCACTGGGTATTCCATCACCAGTGGTTATTACTGGAACTGGATCCGGCAG-120
     T  C  S  V  T  G  Y  S  I  T  S  G  Y  Y  W  N  W  I  R  Q
121-TTTCCAGGAAACAAACTGGAATGGATGGGCTACATAAACTACGACGGCAGCAATAACTAC-180
     F  P  G  N  K  L  E  W  M  G  Y  I  N  Y  D  G  S  N  N  Y
181-AACCCATCTCTCAAAAATCGAATCTCCATCACTCGTGACACATCAAAGAACCAGTTTTTC-240
     N  P  S  L  K  N  R  I  S  I  T  R  D  T  S  K  N  Q  F  F
241-CTGAAGTTGACTTCTGTGACTACTGAGGACACAGCTACATATTACTGTGCACGAGTCTAT-300
     L  K  L  T  S  V  T  T  E  D  T  A  T  Y  Y  C  A  R  V  Y
301-AGTTACTACGATGGTCTGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA   -357
     S  Y  Y  D  G  L  D  Y  W  G  Q  G  T  S  V  T  V  S  S

(B)1-GACATTGTGCTGACACAGTCTCCTGCTTCCTTAGCTGTGTCTCTGGGGCAGAGGGCCACC- 60
     D  I  V  L  T  Q  S  P  A  S  L  A  V  S  L  G  Q  R  A  T
 61-ATCTCATGCAGGGCCAGCAAAAGTGTCAGTATATCTGGCAATAGTCATATGCACTGGTAC-120
     I  S  C  R  A  S  K  S  V  S  I  S  G  N  S  H  M  H  W  Y
121-CAACAGAGACCAGGACAGGCACCCAAACTCCTCATCTATCTTGCATCCAACCTAGAATCT-180
     Q  Q  R  P  G  Q  A  P  K  L  L  I  Y  L  A  S  N  L  E  S
181-GGGGTCCCTGCCAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACCCTCAACATCCAT-240
     G  V  P  A  R  F  S  G  S  G  S  G  T  D  F  T  L  N  I  H
241-CCTGTGGAGGAGGAGGATGCTGCAACCTATTACTGTCAACACAGTAGGGAACTTCCTCCC-300
     P  V  E  E  E  D  A  A  T  Y  Y  C  Q  H  S  R  E  L  P  P
301-ACGTTCGGCTCGGGGACAAAGTTGGAAATAAAACGG                     -336
     T  F  G  S  G  T  K  L  E  I  K  R
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Fig. 3-7. Nucleotide and deduced amino acid sequences of the mab BTE-3456 (A), VH; (B), VL.

VH domains of BBA-2187, BBA-2617, BKE-3430 and BTE-3456 were registered at GenBank and their accession numbers were AB097934, AB097935, AB097936 and AB097937, respectively. Similarly, the cloned VL cDNA sequences of BBA-2187, BBA-2617, BKE-3430 and BTE-3456 contained 333 bp, 336 bp, 339 bp and 336 bp encoding 111 amino acids, 112 amino acids, 113 amino acids and 112 amino acids, respectively, as shown in Fig. 3-4 to Fig. 3-7. The nucleotide sequences of cDNA clones coding for the VL domains of BBA-2187, BBA-2617, BKE-3430 and BTE-3456 were also registered at GenBank and their accession numbers were AB097938, AB097939, AB097940 and AB097941, respectively.

The amino acid sequences of the variable regions of four mabs were compared. It was found that the heavy chain of BBA-2187, BBA-2617 and BTE-3456 was a member of the family XIX subgroup IB and possessed a κ light chain from family I subgroup II, whereas the heavy chain of BKE-3430 belonged to the family VIII subgroup IIA and possessed a κ light chain from family II subgroup I.[82] The alignment of the VH and VL sequences is shown in Fig. 3-8. It was found that the amino acid sequences of variable regions of BBA-2187, BBA-2617 and BTE-3456 were very similar each other, except for BKE-3430. The identity in the amino acid sequence of the VH of BBA-2187 was 98% for BBA-2617, 92% for BTE-3456 and 45% for BKE-3430, and in that of the VL of BBA-2187 was 91% for BBA-2617, 86% for BTE-3456 and 63% for BKE-3430. In general, almost all of the mouse immunoglobulins registered in database have 16 amino acid residues in the framework region (FR) 2. However, the region of VL of BBA-2187 had 15 amino acid residues. Therefore, it was found that BBA-2187 had a unique VL domain.

Anti-BPA Recombinant ScFv Antibodies

The scFv genes were constructed for four anti-BPA mabs by PCR using primers complementary to each 5′-end and 3′-end of the variable regions of four mabs. Each of the primers contained either a

(A)

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BBA-2187:   1 DVQLQESGPGLVKPSQSLSLTCSVTGYSITSGYYWNWIRQFPGNKLEWMG  50
BBA-2617:   1 DVQLQESGPGLVKPSQSLSLTCSVTGYSITSGYYWNWIRQFPGNKLEWMA  50
BTE-3456:   1 DVQLQESGPGLVKPSHSLSLTCSVTGYSITSGYYWNWIRQFPGNKLEWMG  50
BKE-3430:   1 DVKFVESGGGLVKLGGSLKLSCAASGFTFRN-YYMSWVRQTPEKRLELVA  50
              **   *** ****   ** * *   *      **  * ** *   **

BBA-2187:  51 YIRYDGSNNYNPSL-KNRISITRDTSKNQFFLKLNSVTPEDTATYYCARV  99
BBA-2617:  51 YIRYDGSNNYNPSL-KNRISITRDTSKNQFFLKLNSVTTEDTATYYCARV  99
BTE-3456:  51 YINYDGSNNYNPSL-KNRISITRDTSKNQFFLKLTSVTTEDTATYYCARV  99
BKE-3430:  51 GINTNGGFTYYPDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCARP 100
               *   *   * *   * *  * **  **   *   *   **** *****

BBA-2187: 100 LGRG-YG--LDYWGQGTSVTVSS                           119
BBA-2617: 100 LGRG-YG--LDYWGQGTSVTVSS                           119
BTE-3456: 100 Y--SYYDG-LDYWGQGTSVTVSS                           119
BKE-3430: 101 EFDTSYVAWFAYWGQGTLVTVSA                           122
                    ****** ****
```

(B)

```
BBA-2187:   1 DIVLTQSPASLAVSLGQRATISCRASQSVSTS-TYSYLHWYQQRPGQPPK   49
BBA-2617:   1 DIVLTQFPASLAVSLGQRATISCRASQTVSTS-RFNYMHWYQQKPGQPPK   49
BTE-3456:   1 DIVLTQSPASLAVSLGQRATISCRASKSVSIS-GNSHMHWYQQRPGQAPK   49
BKE-3430:   1 DVVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYLQKPGQSPK   50
              * * ** * ** ****  * **** *     *      *** * *** **

BBA-2187:  50 L-IKY-VSNLESGVPARFSGSGSGTDFTLNIHPVEEEDTATYYCQHSWEI   97
BBA-2617:  50 LLIKY-ASNLESGVPARFSASGSGTDFTLNIHPVEEEDTATYYCQHSWEI   98
BTE-3456:  50 LLI-YLASNLESGVPARFSGSGSGTDFTLNIHPVEEEDAATYYCQHSREL   98
BKE-3430:  51 LLI-YKLSNRFSGVPDRFSGSGSGTDFILKISRVEAEDLGVYFCSQSTHV   99
              * * *  **  **** *** ******* * *  ** **   * *  *

BBA-2187:  98 PPTFGGGTKLEIKR                                     111
BBA-2617:  99 PPTFGGGTKLEIKR                                     112
BTE-3456:  99 PPTFGSGTKLEIKR                                     112
BKE-3430: 100 PLTFGSGTKLELKR                                     113
              * *** ***** **
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Fig. 3-8. Alignment of the amino acid sequences of the variable regions of four anti-BPA scFv antibodies

The alignment of the deduced amino acid sequences of VH and VL cDNA of four anti-BPA mabs are represented. Bold characters represent the complementarity-determining regions. Asterisks denote the amino acid residues identical in all sequences. Gaps are inserted to maximize the alignment. (A) VH; (B) VL.

specific restriction enzyme site for directional cloning or a synthetic peptide linker sequence. In the first round, VH and VL cDNAs were amplified and about 400 bp of PCR products were obtained. Assembly of both VH and VL cDNA fragments was performed in the second PCR. About 750 bp of resulting products were cloned into pT7Blue T-vector and transformed into *E. coli* cells. The recombinant plasmids verified to contain neither nucleotide deletion nor substitution by DNA sequencing were digested with *Nhe*I/*Nco*I restriction enzymes and cloned into pET-27b vector, forming pBBA-2187scFv, pBBA-2617scFv, pBKE-3430scFv, and pBTE-3456scFv. Four expression plasmids constructed were each transformed into *E. coli* BL21(DE3)pLysS cells. Recombinant *E. coli* cells were cultured in 2 × YT medium containing kanamycin and chloramphenicol, and scFv antibodies were each extracted by the osmotic shock. The periplasmic fractions were each directly subjected to an ELISA for their ability to bind to free BPA.

Standard curves for BPA with the scFv antibodies are shown in Fig. 3-9. The reactivity of scFv antibodies towards BPA was similar to that of the corresponding parent mabs. The IC_{50} values with BBA-2187scFv, BBA-2617scFv, BKE-3430scFv and BTE-3456scFv were 0.32 ng/mL, 0.81 ng/mL, 0.63 ng/mL and 0.93 ng/mL, respectively. The results showed that the IC_{50} values with the scFv antibodies were comparable to those with the corresponding parent mabs. The BBA-2187scFv was the most reactive among four anti-BPA scFv antibodies as the same with the mabs.

Then, BBA-2187scFv was further purified from periplasmic extracts (Fig. 3-10) and both of crude scFv antibody and purified scFv antibody were used in an ELISA. The purified BBA-2187scFv showed the reactivity similar to the crude scFv antibody (Fig. 3-11). The assay results indicated that some proteins in the periplasmic extracts did not affect the reactivity of BBA-2187scFv antibody towards BPA in ELISA. Therefore, crude BBA-2187scFv was used in ELISA for further studies.

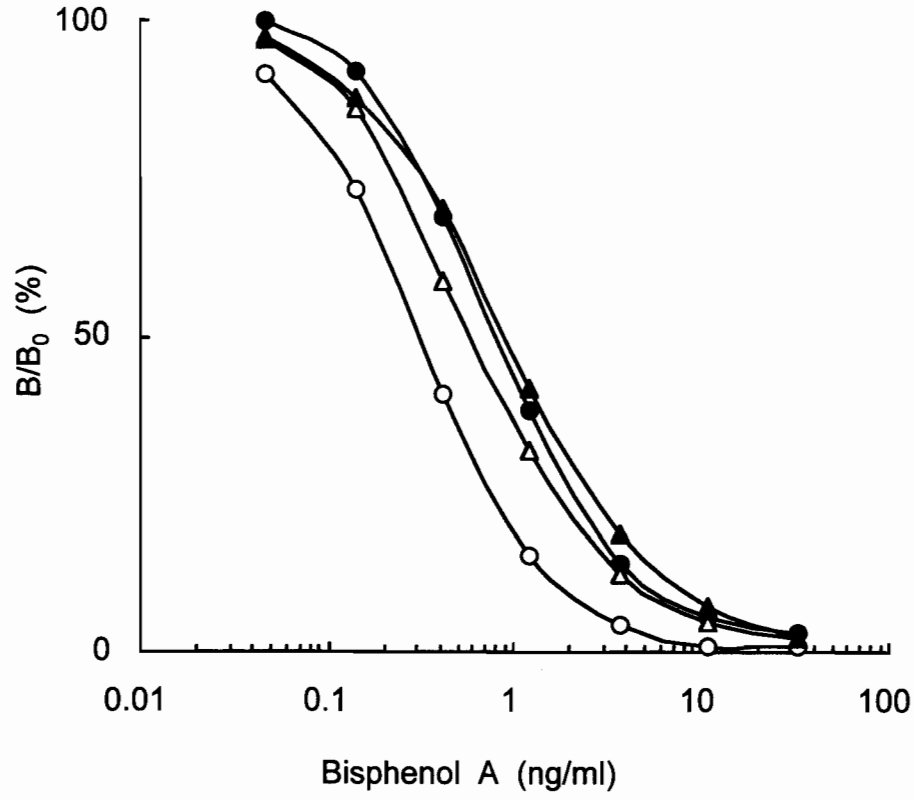


Fig. 3-9. The standard curves for BPA in ELISA based on the anti-BPA scFv antibodies.
The values of absorbance were converted to B/B_0 (%) according to the following formula: B/B_0 (%) = (A/A_0) × 100, where A is a value of absorbance for the standard and A_0 is a value of the absorbance for the control. (○), BBA-2187scFv; (●), BBA-2617scFv; (△), BKE-3430scFv; (▲), BTE-3456 scFv.

Cross-Reactivity

The specificity of BBA-2187 and BBA-2187scFv was determined towards structurally related compounds in ELISA for further characterization. The percent cross-reactivity was calculated as a ratio of the IC_{50} of BPA to that of a tested compound. The IC_{50} values and the percent cross-reactivity for BPA-related compounds are listed in Table 3-2. Both antibodies slightly reacted with bisphenol E and bisphenol F, but did not bind to any other compounds used in this study to a degree of more than 1%. The results indicated that both the mab BBA-2187 and BBA-2187scFv were very specific towards BPA and that the cross-reactivity of BBA-2187scFv for the related compounds was similar to that of the mab BBA-2187.

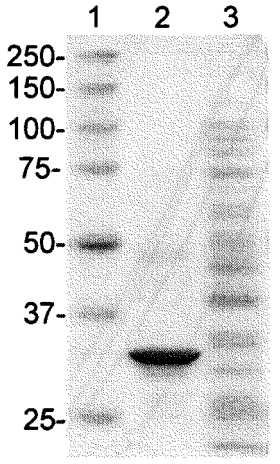


Fig. 3-10. SDS-PAGE of the purified BBA-2187 scFv fragment
Lane 1; Molecular weight marker, lane 2; scFv fragment purified with saturated ammonium sulfate precipitation and a nickel chelate column, lane 3; Periplasmic extract. Staining was done with Coomasie Blue.

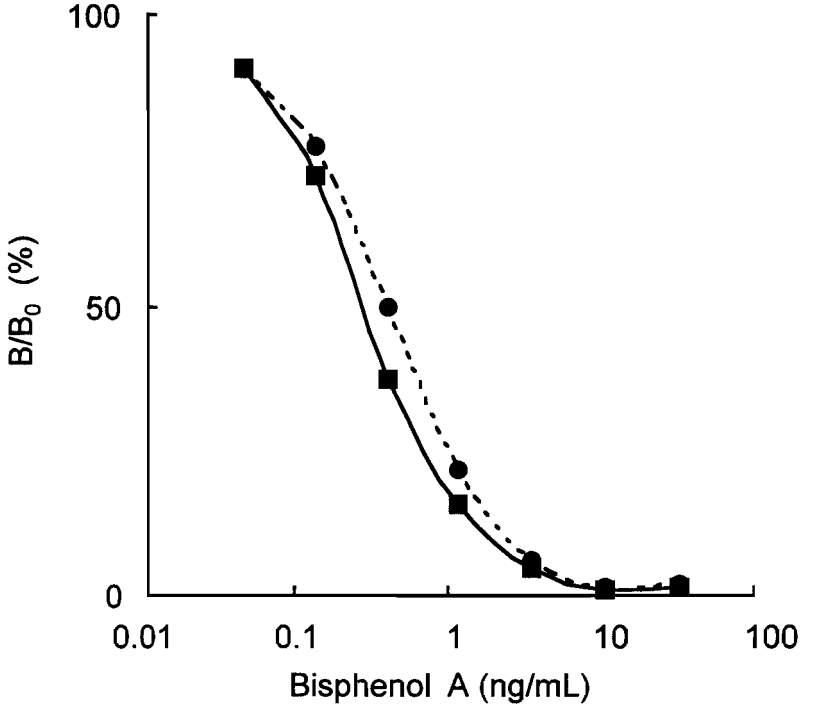
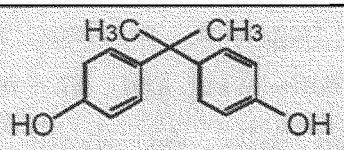
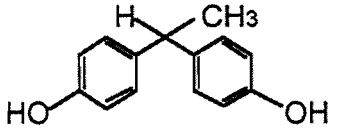
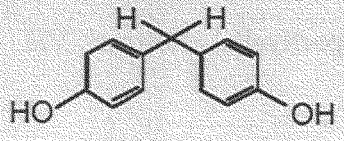
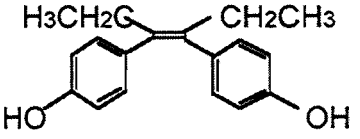
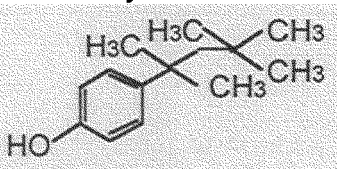
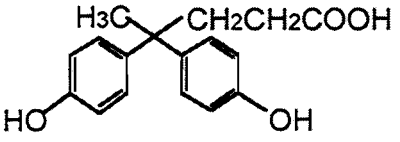


Fig. 3-11. The standard curves for BPA in ELISA based on the purified BBA-2187scFv
The values of absorbance were converted to B/B_0 (%) according to the following formula: B/B_0 (%) = (A/A_0) × 100, where A is a value of absorbance for the standard and A_0 is a value of the absorbance for the control. (●), the purified BBA-2187scFv; (■), the crude BBA-2187scFv.

Table 3-2. The cross-reactivity of the mab BBA-2187 and BBA-2187scFv with BPA-related compounds in ELISA.

Compound	Mab BBA-2187		BBA-2187scFv	
	IC_{50} (ng/mL)	CR (%)[a]	IC_{50} (ng/mL)	CR (%)[a]
 Bisphenol A	0.32	100	0.59	100
 Bisphenol E	5.0	6.5	7.8	7.6
 Bisphenol F	35	0.9	51	1.2
 Diethylstilbestrol	89	0.4	300	0.2
 4-(*tert* -Octyl)phenol	110	0.3	220	0.3
 Diphenolic acid	>320	<0.1	>590	<0.1
Bisphenol S	>320	<0.1	470	0.1
Bisphenol AP	>320	<0.1	>590	<0.1
Bisphenol A dimethacrylate	>320	<0.1	>590	<0.1
4-Octylphenol	110	0.3	470	0.1
4-n-Buthylphenol	>320	<0.1	>590	<0.1
4-*tert*- Butylphenol	>320	<0.1	>590	<0.1
4-*sec*- Butylphenol	>320	<0.1	>590	<0.1
3-Methyl-4-nitrophenol	>320	<0.1	>590	<0.1
2,2-Bis(4-glycidyloxyphenyl)propane	>320	<0.1	>590	<0.1
p,p'- DDT	>320	<0.1	>590	<0.1
Dicofol	>320	<0.1	>590	<0.1

[a]CR (%) was calculated by the formula as below. CR (%) = (IC_{50} of BPA)/(IC_{50} of tested compound) × 100.

The Reactivity of Fv Fragments towards BPA in ELISA

The reactivity of the VH and VL fragments of four anti-BPA mabs for BPA was assessed in ELISA. Both VH and VL fragments were each produced in recombinant *E. coli* cells. As shown in Fig. 3-12, both VH and VL fragments of BBA-2187 cooperatively reacted with BPA in ELISA, although the assay sensitivity in an ELISA for BPA was inferior to that of BBA-2187scFv assembling both VH and VL fragments with a linker. The slope and linear range of the standard curve with a Fv fragment of BBA-2187 were sharper and narrower than those with BBA-2187scFv, respectively. Neither VH nor

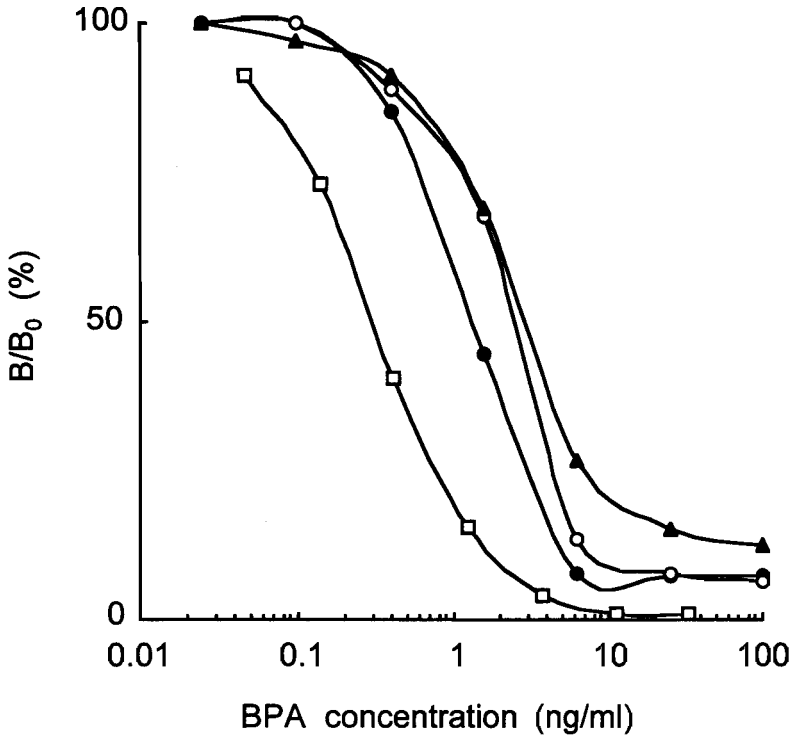


Fig. 3-12. The standard curves for BPA in ELISA based on the combination of the VH and VL fragments of the anti-BPA mabs
The values of absorbance were converted to B/B_0 (%) according to the following formula: B/B_0 (%) = $(A/A_0) \times 100$, where A is a value of absorbance for the standard and A_0 is a value of the absorbance for the control. (●), a combination between VH of BBA-2187 and VL of BBA-2187; (○), a combination between VH of BBA-2187 and VL of BBA-2617; (▲), a combination between VH of BBA-2187 and VL of BTE-3456; (□), BBA-2187scFv.

VL fragment alone bound to free BPA in ELISA (data not shown). Then, heterologous combination of Fv fragments was examined in ELISA. The VH fragment of BBA-2187 bound to BPA together with the VL fragment of BBA-2617 and the VL fragment of BTE-3456 in ELISA. While, the VL fragment of BBA-2187 could not bind to BPA together with each of the VH fragments of the other three mabs in ELISA (data not shown). Neither VH nor VL fragment of BBA-2187 bound to BPA as with the VL and VH fragments of BKE-3430, respectively. The results indicated that it was possible to replace the VL of BBA-2187 with either the VL of BBA-2617 or the VL of BTE-3456 for association with the VH of BBA-2187, although the VL of BBA-2187 was inreplaceable with the other VL fragments for association with the VH of BBA-2187.

DISCUSSION

We attempted to develop a highly sensitive ELISA based on mabs for BPA which has been widely detected at ppb to ppt levels in environmental water samples. The optimized ELISA based on BBA-2187 was the most sensitive among four anti-BPA mabs and the quantitation limit was 0.13 ng/ml for BPA. The ELISA assay established here was as sensitive as those of HPLC-ECD (quantitaion limit; 0.2 ng/mL),[115] HPLC-FLD (detection limit; 0.9 ng/mL)[116] and LC-MS (detection limit; 0.1 ng/mL).[117] Therefore, the ELISA developed in this study seemed to be practically useful for detection of BPA in environmental water samples at actual low levels. This ELISA was more sensitive than the other ELISAs for BPA reported.[47,118-120] The ELISA developed in this study was also more specific to BPA than the other ELISAs reported. For example, the cross-reactivity with bisphenol E was 7.6% in the present ELISA, whereas the cross-reactivity with bisphenol E was ranged from 18% to 144% in the other ELISAs reported.[47,118,120] Recently, the ELISA kit based on BBA-2187 is commercially available.[121]

In order to characterize these mabs further, the anti-BPA scFv antibodies corresponding to the mabs were each produced by the culture of the recombinant *E. coli* cells. The ELISA based on the anti-BPA scFv antibodies gave the IC_{50} values for BPA similar to those based on the corresponding mabs. As would be expected, BBA-2187scFv was the most sensitive towards BPA among the four anti-BPA scFv antibodies in ELISA. The alignment of the amino acid sequences in the variable regions of four anti-BPA mabs revealed that there was a high degree of sequence identity among three antibodies, except for BKE-3430. Particularly, it was found that the most sensitive BBA-2187 had an unique VL domain which consisted of 15 amino acid residues in FR2, although the domain generally contains 16 amino acid residues in this region. This unique structural feature of the VL may reflect the specific association with the VH, resulted in the highest reactivity to BPA in ELISA.

The ELISA based on the scFv antibodies took more time than that based on the mabs because of the request of an additional reaction step. This problem will be resolved by using the scFv antibodies fused with an enzyme or another marker protein. On the other hand, the production of the scFv antibodies by the culture of the recombinant *E. coli* cells was even more efficient than that of the mabs. Therefore, the scFv antibodies seemed to be reliable for practical application to ELISA and immunoaffinity columns.

The cross-reactivity of BBA-2187scFv with BPA-related compounds was similar to that of the parent mab BBA-2187. The scFv antibody specific to the insecticide parathion also showed the cross-reactivity similar to the parent mab with structurally related compounds in ELISA.[52] Both BBA-2187 and BBA-2187scFv slightly cross-reacted to bisphenol E and bisphenol F, both of which have only one (bisphenol E) and two (bisphenol F) of the methylene group deleted as compared with that of BPA. Since the cross-reactivity of both antibodies with bisphenol E was higher than that to bisphenol F. Thus, these methylene chains may be important for the antigen-

antibody interaction. Also, both antibodies did not cross-react with 4-*tert*-butylphenol, indicating that both antibodies may recognize both hydroxyphenyl groups in BPA. Based on the results of cross-reactivity tested, BBA-2187 was found to recognize specifically the whole molecule of BPA.

It has been known that the VH and VL fragments of an antibody associated each other in the presence of an antigen under certain conditions.[122,123] When the VH and VL fragments of BBA-2187 were separately prepared and subjected to ELISA together on co-incubation with BPA, both VH and VL fragments seemed to associate noncovalently each other via the antigen BPA in ELISA, although the reactivity was lower than that with BBA-2187scFv. Thus, it was suggested that the VH and VL of BBA-2187 interacted specifically each other and that the peptide linker of BBA-2187scFv allowed both VH and VL to be more stably maintained. When heterologous combinations of the VH and VL fragments based on BBA-2187 were also examined, the VH fragment of BBA-2187 associated with each of the VL fragments of BBA-2617 and BTE-3456 to react with BPA, although the reactivity of these combinations was lower than the homologous combination. On the other hand, the VL fragment of BBA-2187 assembled specifically with the VH fragment of BBA-2187, but not with the VH fragments of the other anti-BPA antibodies. The results suggested that the conformation of the VL fragment of BBA-2187 may be different from the others. The FR2 in the VL appeared to be the key factor for the specific assembly between VH and VL of BBA-2187, since the region had an unique sequence as mentioned above. The VH and VL fragments of BKE-3430 did not associate with the VL and VH fragments of BBA-2187, respectively, probably due to relatively low identity of the amino acid sequences in the variable regions between both mabs.

CHAPTER IV

MOLECULAR AND IMMUNOCHEMICAL CHARACTERIZATION OF RECOMBINANT ANTIBODIES SPECIFIC TO ALKYLPHENOL ETHOXYLATES

INTRODUCTION

Alkylphenol ethoxylates (APEOs: Fig. 4-1) are non-ionic surfactants and used widely as industrial cleaners, emulsifiers and solubilizers in pharmaceutical and agrochemical formulations for many years due to their favorable physicochemical characteristics. The global production of APEOs was about 500,000 tons per year in 1997.[124] The most commonly used APEO is 4-nonylphenol ethoxylate (NPEO) which is produced by addition of ethylene oxide to a mixture of branched 4-nonylphenol (NP) isomers. NP is manufactured by Friedel-Crafts reaction of a technical mixture of nonene with phenol.[125] Released into the environment or entered into sewage treatment, APEOs are biodegraded and resulted in shortening of the ethoxy chain and/or carboxylation at the terminal ethoxy unit (alkylphenoxy carboxylates: APECs), ultimately transformed to alkylphenols (APs: Fig. 4-1) through the pathways as shown in Fig. 4-2. The hydrophobicity and toxicity to fish of the biodegraded products increased as an ethoxy chain length decreased.[126,127] It has been also reported that the metabolites of APEOs, 4-nonylphenol diethoxylates (NP2EO), 4-nonylphenoxyacetic acid (NP1EC) and NP, showed estrogenic activities.[128,129]

Because of massive use of APEOs and ubiquitous occurrence of their metabolites in the environment, monitoring of APEOs, APECs and APs is of great importance. Quantification of these compounds is usually performed using a HPLC with an UV detector,[130] HPLC with a fluorescence detector,[131] liquid chromatography-mass spectrometry[132] and gas chromatography-mass spectrometry.[133] However, simultaneous extraction and determination of these compounds are rather difficult, since APs

and APEOs are less polar and carboxylic degradation products are polar.[134] On the other hand, ELISA for detergents has been reported.[135,136] Goda *et al.*[47] prepared two anti-APEO monoclonal antibodies (mabs) MOF3-139 and AP-14 raised against APEO hapten (Fig. 4-1) conjugated to a carrier protein used as immunogen. Both MOF3-139 and AP-14 were isolated by screening with APEO hapten and AP hapten (Fig. 4-1), respectively. Then, an ELISA based on MOF3-139 was developed for APEOs and another ELISA based on AP-14 was for APEOs and APs.

Recently, recombinant antibodies for certain environmental chemicals have been produced and used for ELISA.[60,68,137] These recombinant antibodies were easily produced by cultivation of recombinant *E. coli* cells. This technology is also a powerful tool to analyze the property

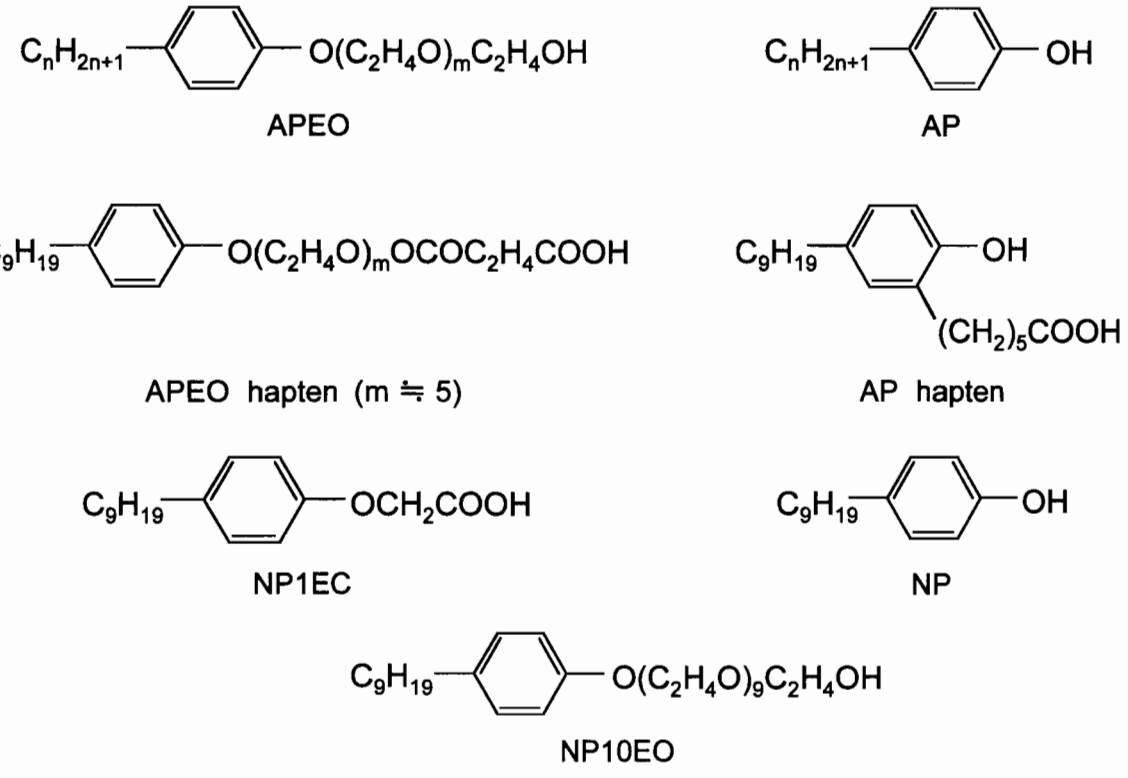


Fig. 4-1. Chemical structures of alkylphenol ethoxylate, alkylphenol, nonylphenolic compounds and haptens used in this study
APEO, alkylphenol ethoxylate; AP, alkylphenol; NP1EC, nonylphenoxyacetic acid; NP, nonylphenol; NP10EO, nonylphenol decaethoxylate.

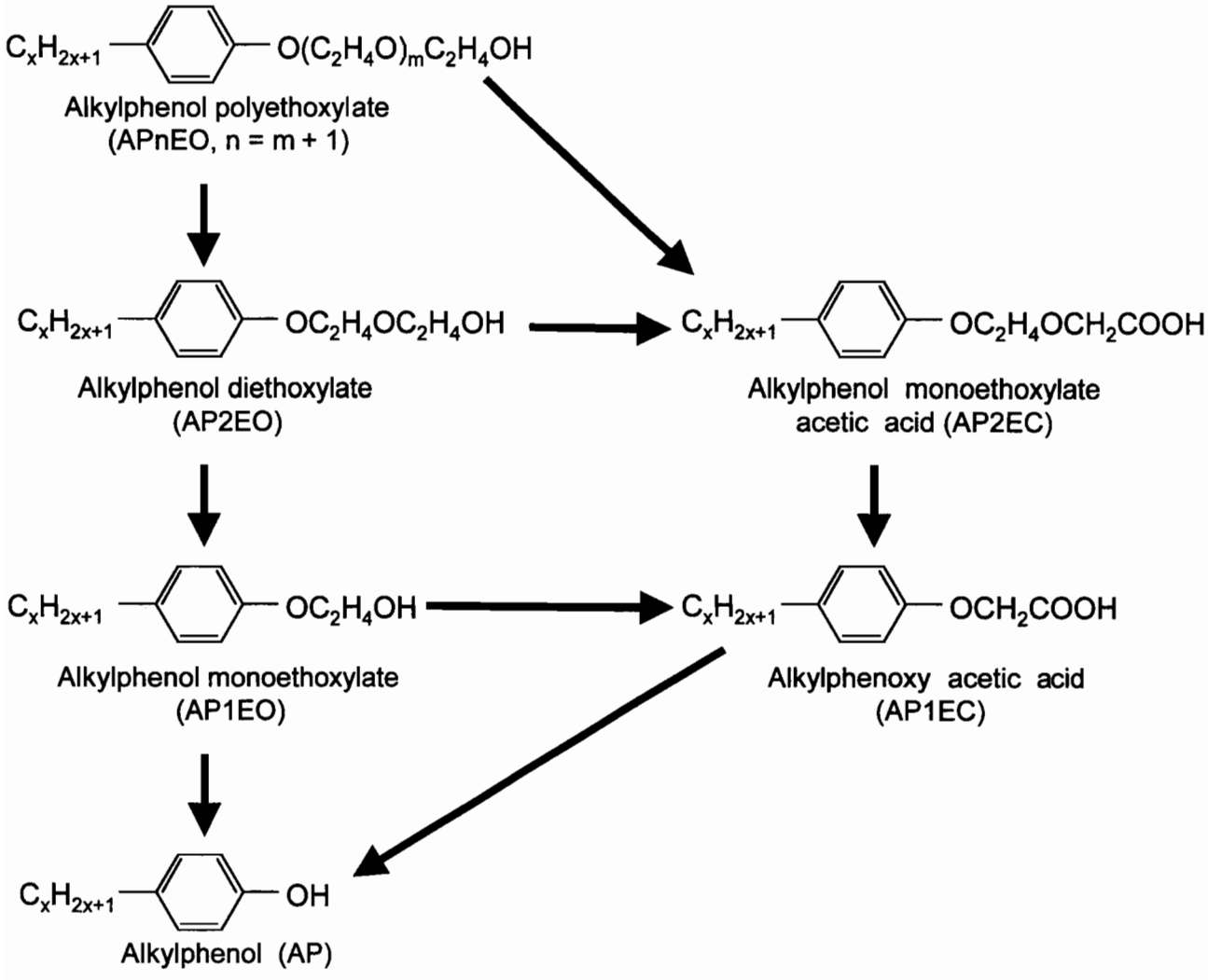


Fig. 4-2. Biodegradation pathways for alkylphenol ethoxylates

of antibodies by the combined use of site-directed mutagenesis. In this report, scFv antibodies of two anti-APEO mabs MOF3-139 and AP-14 were produced in recombinant *E. coli* cells and characterized in ELISA. Based on the comparison of the primary structures between both mabs, various mutants of both scFv antibodies were produced and characterized for understanding molecular basis on reactivity and specificity of these scFv antibodies.

MATERIALS AND METHODS

Chemicals and Biochemicals

4-Nonylphenol (NP: Fig. 4-1) and 4-*n*-nonylphenol (*n*-NP) were purchased from Kanto Chemicals Co. (Tokyo, Japan). NP monoethoxylate (NP1EO), NP triethoxylate (NP3EO), NP hexaethoxylate (NP6EO), NP decaethoxylate (NP10EO: Fig. 4-1), NP pentadecaethoxylate (NP15EO), nonylphenoxy acetic acid (NP1EC: Fig. 4-1), NP monoethoxylate acetic acid (NP2EC) and NP triethoxylate acetic acid (NP4EC) were obtained from Hayashi Pure Chemicals Ind. Co. (Osaka, Japan). Stock solutions were prepared in MeOH. The APEO hapten conjugated to ovalbumin (APEO-OVA), two mabs MOF3-139 and AP-14 were prepared in the previous study.[47] Block Ace was purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). Restriction endonucleases were purchased from New England Biolabs, Inc. (MA, USA). Mouse anti-herpes simplex virus (HSV) tag mab was obtained from Novagen, Inc. (WI, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were purchased from ICN Biomedicals Inc. (OH, USA). All other chemicals and solvents were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Isolation of cDNA Clones Coding for Immunoglobulin Variable Regions by 5'-RACE Method

Recombinant DNA techniques were according to the standard procedures.[81] Two hybridoma cell lines producing the mabs MOF3-139 specific to APEOs and AP-14 cross-reacting with APEOs and APs were each grown in DMEM supplemented with 190 U/mL of penicillin, 190 μg/mL of streptomycin and 10% (v/v) heat-inactivated fetal bovine serum (JRH Biosciences, KS, USA) in a 5% (v/v) CO_2-humidified incubator at 37°C. Isotype of both mabs was determined with culture supernatant by using anti-mouse subclass-specific antisera (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions. Hybridoma cells were pelleted by centrifugation and used for mRNA

extraction. mRNA fractions were isolated by the use of a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) from 2 × 10^7 cells of each hybridoma cell line. One mg of mRNA was reverse transcribed using oligo (dT) primers with MMLV reverse transcriptase (BD Biosciences Clontech, CA, USA). cDNA fragments coding for the variable regions of both mabs were isolated by 5'-RACE method by using a SMART-RACE cDNA Amplification Kit (BD Biosciences Clontech) according to the manufacturer's instructions. Three gene-specific primers (GSPs) were designed to be complementary to nucleotide sequences of the constant regions of mouse immunoglobulin. The GSP sequences were as follows: 5'-GACAGATGGGGGTGTCGTTTTGGC-3' for IgG1 chain, 5'-GGAGGAACCAGTTGTATCTCCACACC-3' for IgG2b chain, 5'-AGATGGATACAGTTGGTGCAGCATCAGC-3' for κ chain. Amplified cDNA fragments were cloned into pT7Blue T-vector (Novagen) and transformed into competent *E. coli* JM109 cells for DNA sequencing.

Nucleotide Sequencing and Analysis of Nucleic Acids

Inserts of recombinant pT7Blue plasmids were sequenced by the dideoxy method[138] in an automated DNA sequencer SQ-5500 (Hitachi, Ltd., Tokyo, Japan) for determination of nucleotide sequences and confirming no nucleotide deletion and substitution of constructed scFv antibody genes. DNA and deduced amino acid sequences were analyzed and aligned using DNA sequence analysis software Genetyx-Mac 7.3 (Software Development Co., Tokyo, Japan).

Construction of Expression Plasmids and Production of ScFv Antibodies in *E. coli* Cells

The variable heavy chain (VH) and variable light chain (VL) of both mabs were covalently linked with a peptide linker $(Gly_4Ser)_3$ consisting of 15 amino acid residues.[139] In this study, two types of scFv antibodies were prepared, in which the order of variable domains was replaced (i.e., VH-linker-VL and VL-linker-VH) as shown

in Fig. 4-3. Construction of scFv genes was achieved by splicing by overlap extension (SOE)-PCR as described previously.[112] Briefly, 12 internal overlapping oligonucleotides were designed based on cDNA nucleotide sequences corresponding to the variable domains of both mabs, which contained a part of a linker sequence or a specific sequence for *Nhe*I or *Nco*I (Table 4-1) to facilitate the insertion of PCR products in the *Nhe*I/*Nco*I site of the expression vector pET-27b. After PCR reaction, the resulting products were cloned into pT7Blue T-vector and transformed into competent *E. coli* JM109 cells. That the scFv antibody genes had no nucleotide deletion and substitution was confirmed by DNA sequencing as described above. Plasmids containing

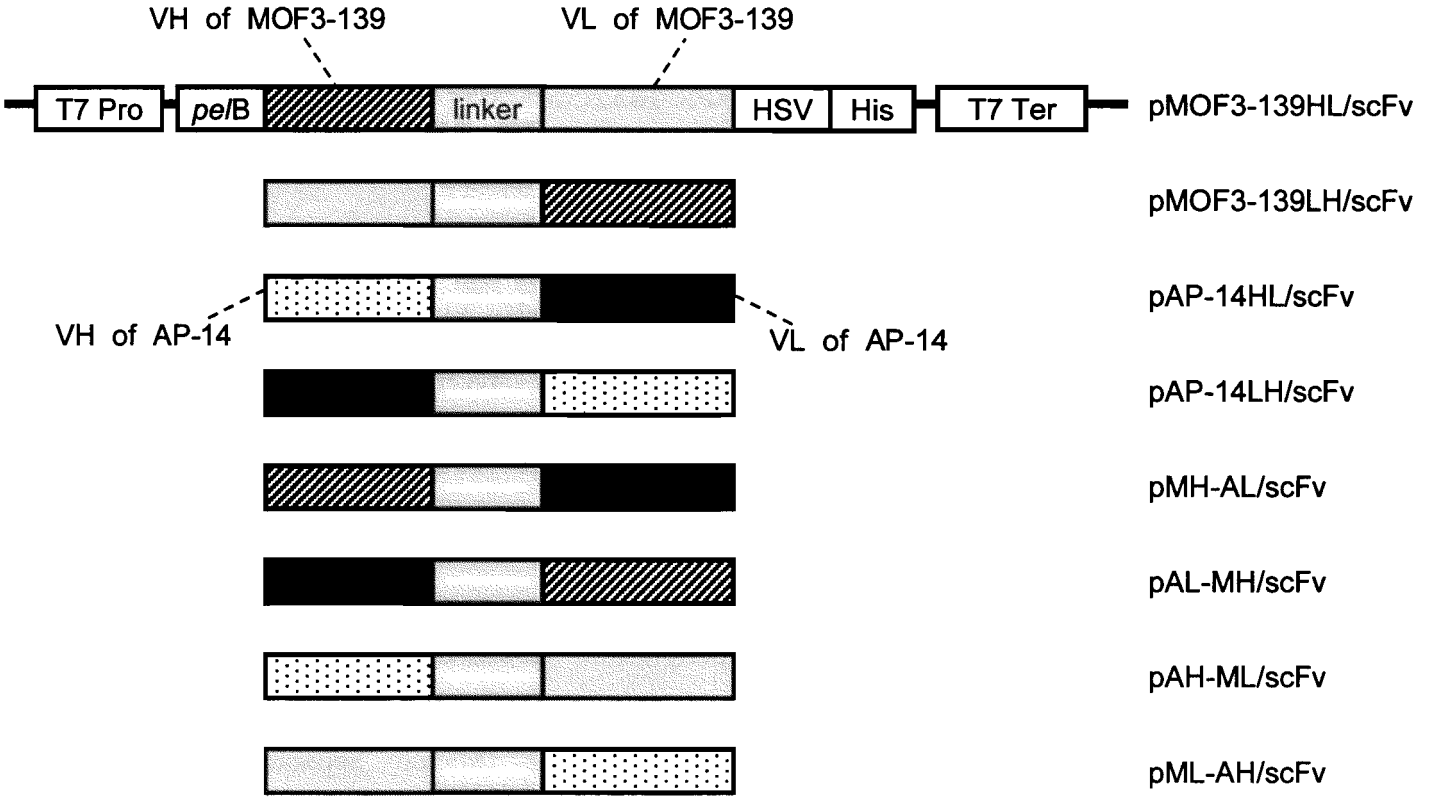


Fig. 4-3. The schematic diagrams of the expression plasmids for both HL and LH types of wild-type and hybrid scFv antibody genes
The plasmids for the scFv antibody genes to be expressed in recombinant *E. coli* cells are represented. Abbreviations: T7 Pro, T7 promoter; *pel*B, *pel*B leader sequence; HSV tag, an epitope tag for detection with an anti-HSV mab; His, a hexahistidine tag; T7 Ter, T7 terminator.

a scFv antibody gene were digested with *Nhe*I and *Nco*I and cloned into similarly digested expression vector pET-27b (Novagen). The resulting expression plasmids were transformed into competent *E. coli* BL21(DE3)pLysS cells. Production of scFv antibodies in recombinant *E. coli* cells was done as described previously.[140]

Table 4-1. The nucleic acid sequences of the primers used for construction of scFv antibody genes.

Primer	Nucleotide sequence (5'-3')
MH-F	CCATGGATCAGGTCCAGCTCCAGCAGTCT
MH-R	AGAGCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGACGGTGACTGAGGTT
VL-F	GGCGGAGGTGGCTCTGGCGGTGGCGGATCGGATATTGTGATGACGCAGGCTG
VL-R	GCTAGCCCGTTTTATTTCCAGCTTGGTC
AH-F	CCATGGATCAGGTTCAGCTGCAGCAGTCT
AH-R	AGAGCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGACTGTGAGAGTGGTGC
VL-F/LH	CCATGGATGATATTGTGATGACGCAGGCTG
VL-R/LH	AGAGCCACCTCCGCCTGAACCGCCTCCACCCCGTTTTATTTCCAGCTTGGTC
MH-F/LH	GGCGGAGGTGGCTCTGGCGGTGGCGGATCGCAGGTCCAGCTCCAGCAGTCT
MH-R/LH	GCTAGCTGAGGAGACGGTGACTGAGGTT
AH-F/LH	GGCGGAGGTGGCTCTGGCGGTGGCGGATCGCAGGTCCAGCTCCAGCAGTCT
AH-R/LH	GCTAGCTGAGGAGACTGTGAGAGTGGTGC

The combination of the primers used for construction of scFv antibody genes was as follows. MOF3-139HL/scFv, MH-F, MH-R, VL-F and VL-R; AP-14HL/scFv, AH-F, AH-R, VL-F and VL-R; MOF3-139LH/scFv, VL-F/LH, VL-R/LH, MH-F/LH and MH-R/LH; AP-14LH/scFv, VL-F/LH, VL-R/LH, AH-F/LH and AH-R/LH.

Immunoblot Analysis

Equal amounts (10 μg) of periplasmic proteins were separated in a 12% SDS-polyacrylamide gel as described previously.[141] Following transfer to a polyvinylidene fluoride membrane (Micron Separations Inc., MA, USA), blots were blocked in Block Ace and probed with an anti-HSV mab (0.2 μg/mL). Immunoreactive proteins were visualized using anti-mouse IgG antibody conjugated to alkaline phosphatase (Pierce Chemical Co., IL, USA) at a dilution of 1 : 1000. Staining solution contained 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and nitro blue tetrazolium chloride.

Site-Directed Mutagenesis

Site-directed mutagenesis was carried out by SOE-PCR using a pair of mutagenic primers complementary to a scFv gene sequence except for positions of desired mutations. Then, appropriate base changes were introduced into a scFv gene, and nucleotide sequences were ensured by DNA sequencing as mentioned above.

ELISA Protocol

An ELISA was basically performed as described previously.[140] Wells of microtiter plates (Maxisorp: Nunc, Roskilde, Denmark) were coated with 100 μL of APEO-OVA (5 μg/mL) overnight at 4°C and blocked with 300 μL of 4 fold-diluted Block Ace in distilled water. Fifty μL of standard solutions and an equal volume of a scFv antibody were added to each of wells and reacted for 1 h at 25°C. After washing with phosphate-buffered saline (PBS: 10 mM phosphate, 0.9% (w/v) NaCl, pH 7.2), 100 μL of an anti-HSV mab (0.1 μg/mL) in 2 × PBS containing 10% Block Ace was added and incubated for 1 h at 25°C. After washing, 100 μL of goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Pierce Chemical Co., IL, USA) diluted 1 : 2,000 in PBS containing 10% Block Ace was added and incubated for 1 h at 25°C. After washing, 100 μL of chromogenic substrate (100 μg/mL 3,3′,5,5′-tetramethylbenzidine and 0.006% (w/v) H_2O_2 in 0.1 M acetate buffer, pH 5.5) was added and incubated for 10 min at 25°C. The enzyme reaction was stopped by adding 100 μL of 1 N sulfuric acid and A_{450} was measured in a microplate reader (Corona Electric Co., Ibaraki, Japan).

Assay was also performed for mabs as described above, except for addition of 50 μL of a mab in place of a scFv antibody and the addition of the anti-HSV mab was excluded, as reported previously.[140]

RESULTS

cDNA Clones Encoding VH and VL Domains

Two hybridoma cell lines producing the mabs AP-14 and MOF3-139 were used for mRNA preparation. Approxymately 3 μg of mRNA fraction extracted from each of the hybridoma cell lines was subjected to synthesis of the first-strand cDNA. An immunoglobulin-isotyping experiment revealed that AP-14 and MOF3-139 had IgG2b and IgG1 heavy chains, respectively, with κ light chains. Therefore, specific amplification of cDNA fragments encoding the VL domains of both mabs, the VH domain of AP-14 and the VH domain of MOF3-139 was performed by 5'-RACE method using a GSP for κ chain, a GSP for IgG2b chain and a GSP for IgG1 chain, respectively. An expected size of PCR products (approximately 600 bp) was obtained with each GSP. The products were cloned into pT7Blue T-vector and transformed into *E. coli* cells. Each of cDNA clones coding for the VH and VL domains of both mabs was determined by DNA sequencing. The nucleic acid and deduced amino acid sequences of the VH and VL domains of AP-14 and MOF3-139 are shown in Fig. 4-4 and Fig. 4-5, respectively. The cDNA clones encoding the VH and VL domains of AP-14 and MOF3-139 are deposited in the GenBank databases under the accession no. AB100164 (VL domain of AP-14), AB100165 (VL domain of MOF3-139), AB100166 (VH domain of AP-14) and AB100167 (VH domain of MOF3-139). The cDNA clones coding for the VH and VL domains of AP-14 consisted of 363 bp encoding 121 amino acids and 339 bp encoding 113 amino acids, respectively. Similarly, the cDNA clones coding for the VH and VL domains of MOF3-139 consisted of 375 bp encoding 125 amino acids and 339 bp encoding 113 amino acids, respectively.

The amino acid sequences of the VH and VL domains of two mabs were compared as shown in Fig. 4-6. The identity of the VH and VL domains between both mabs was 55 % and 95 %, respectively. There were only 5 amino acid residues different from

each other in the VL domains. On the other hand, more differences were found in primary sequences of the VH domains, especially in the complementarity-determining region 1 (CDR1), CDR2, CDR3 and the framework region 1 (FR1) and FR3. The length of CDR3 in the VH domain of MOF3-139 was four amino acids shorter than that of AP-14. These might be related to the difference in the reactivity between both antibodies.

(A)

```
  1-CAGGTTCAGCTGCAGCAGTCTGGAGCTGAGCTGATGAAGCCTGGGGCCTCAGTGAAGATA- 60
     Q  V  Q  L  Q  Q  S  G  A  E  L  M  K  P  G  A  S  V  K  I
 61-TCCTGCAAGGTTACTGGCTACACATTCAGGAGCTACTGGATAGAGTGGGTAAAGCAGAGG-120
     S  C  K  V  T  G  Y  T  F  R  S  Y  W  I  E  W  V  K  Q  R
121-CCTGGACATGGCCTTGAGTGGATTGGAGAGATTTTAGTTGGAAGTGGTAGTACTAAGTAT-180
     P  G  H  G  L  E  W  I  G  E  I  L  V  G  S  G  S  T  K  Y
181-AATGAGAAGTTCAAGGGCAAGGCCACAATCACTGCACAGACATCCTCCAATACAGTATAC-240
     N  E  K  F  K  G  K  A  T  I  T  A  Q  T  S  S  N  T  V  Y
241-ATGCAACTCAGCAGCCTGACATCTGAGGACTCTGCCGTCTATTACTGTGCAAGACCTTTC-300
     M  Q  L  S  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R  P  F
301-TTCGGTAGTAGGTACGACTACTCTGACTTCTGGGGCCAAGGCACCACTCTCACAGTCTCC-360
     F  G  S  R  Y  D  Y  S  D  F  W  G  Q  G  T  T  L  T  V  S
361-TCA
     S
```

(B)

```
  1-GATATTGTGATGACGCAGGCTGCATTCTCCAATCCAGTCACTCTTGGATCATCAGCTTCC- 60
     D  I  V  M  T  Q  A  A  F  S  N  P  V  T  L  G  S  S  A  S
 61-ATCTCCTGCAGGTCTAGTAAGAGTCTCCTACATAGAAATGGCATCACTTATTTGTATCAG-120
     I  S  C  R  S  S  K  S  L  L  H  R  N  G  I  T  Y  L  Y  W
121-TTGGTATCTGCAGAAGCCAGGCCAGTCTCCTCAGGTCCTGATTTATCAGATGTCCAACCT-180
     Y  L  Q  K  P  G  Q  S  P  Q  V  L  I  Y  Q  M  S  N  L  A
181-TGCCGAGTCCCAGACAGGTTCAGTAGCAGTGGGTCAGGAACTGATTTCACACTGAGAATC-240
     S  G  V  P  D  R  F  S  S  S  G  S  G  T  D  F  T  L  R  I
241-AGCAGAGTGGAGGCTGAGGATGTGGGTGTTTATTACTGTGCTCAAAATCTAGAACTTCCG-300
     S  R  V  E  A  E  D  V  G  V  Y  Y  C  A  Q  N  L  E  L  P
301-TACACGTTCGGAGGGGGGACCAAGCTGGAAATAAAACGG
     Y  T  F  G  G  G  T  K  L  E  I  K  R
```

Fig. 4-4. Nucleotide and deduced amino acid sequences of the variable domains of the mab MOF3-139
(A), VH domain of MOF3-139 (GenBank accession no. AB100167); (B), VL domain of MOF3-139 (GenBank accession no. AB100165).

(A)

```
  1-CAGGTCCAGCTCCAGCAGTCTGCAGCTGAACTGGCAAGACCTGGGGCCTCAGTGAAGATG- 60
     Q  V  Q  L  Q  Q  S  A  A  E  L  A  R  P  G  A  S  V  K  M
 61-TCCTGCAAGGCTTCTGGCTATAGATTTACTAGGTACACGATGCACTGGGTAAAACAGAGG- 90
     S  C  K  A  S  G  Y  R  F  T  R  Y  T  M  H  W  V  K  Q  R
121-CCTGGACAGGGTCTGGAATGGATTGGAAACATTAATCCTAGCAGTGGAGATACTGAGTAC-180
     P  G  Q  G  L  E  W  I  G  N  I  N  P  S  S  G  D  T  E  Y
181-AATCAGAAGTTCAAGGACAAGACCACATTGACTGCAGACAAATCCTCCACCACAGCCTAC-240
     N  Q  K  F  K  D  K  T  T  L  T  A  D  K  S  S  T  T  A  Y
241-ATGCAGCTGAGCAGCCTGACATCTGAGGACTCTGCGGTCTATTACTGTGCAACCCCAATT-300
     M  Q  L  S  S  L  T  S  E  D  S  A  V  Y  Y  C  A  T  P  I
301-TTTTACTACGGTAGTAGGTACGTGAGGTATGAAATGGACTACTGGGGTCAAGGAACCTCA-360
     F  Y  Y  G  S  R  Y  V  R  Y  E  M  D  Y  W  G  Q  G  T  S
361-GTCACCGTCTCCTCA
     V  T  V  S  S
```

(B)

```
  1-GATATTGTGATGACGCAGGCTGCATTCTCCAATCCAGTCACTCTTGGAACATCAACTTCC- 60
     D  I  V  M  T  Q  A  A  F  S  N  P  V  T  L  G  T  S  T  S
 61-ATCTCCTGCAGGTCTACTAAGAGTCTCCTACATAGTAATGGCATCACTTATTTGTATTGG-120
     I  S  C  R  S  T  K  S  L  L  H  S  N  G  I  T  Y  L  Y  W
121-TATCTGCAGAAGCCAGGCCAGTCTCCTCAGCTCCTGATTTATCAGATGTCCAACCTTGCC-180
     Y  L  Q  K  P  G  Q  S  P  Q  L  L  I  Y  Q  M  S  N  L  A
181-TCAGGAGTCCCAGACAGGTTCAGTAGCAGTGGGTCAGGAACTGATTTCACACTGAGAATC-240
     S  G  V  P  D  R  F  S  S  S  G  S  G  T  D  F  T  L  R  I
241-AGCAGAGTGGAGGCTGAGGATGTGGGTGTTTATTACTGTGCTCAAAATCTAGAACTTCCG-300
     S  R  V  E  A  E  D  V  G  V  Y  Y  C  A  Q  N  L  E  L  P
301-TACACGTTCGGAGGGGGGACCAAGCTGGAAATAAAACGG
     Y  T  F  G  G  G  T  K  L  E  I  K  R
```

Fig. 4-5. Nucleotide and deduced amino acid sequences of the variable region of the mab AP-14
(A), VH domain of AP-14 (GenBank accession no. AB100166); (B), VL domain of AP-14 (GenBank accession no. AB100164).

Production and Characterization of ScFv Antibodies

Twelve primers were designed for construction of four scFv antibody genes based on the information on the nucleotide sequences of the cDNA clones. The scFv antibody genes were generated in SOE-PCR process. In the first PCR, cDNA fragments encoding the VH and VL domains were each amplified, which contained a complementary sequence encoding the peptide linker. These were spliced together in the second PCR and ligated into the expression vector pET-27b. A *pel*B signal sequence[113] placed immediately upstream of the

(A)

```
                          FR1                CDR1          FR2           CDR2
AP-14        1 QVQLQQSAAELARPGASVKMSCKASGYRFTRYTMHWVKQRPGQGLEWIGNINPSSGDTEY  60
MOF3-139     1 QVQLQQSGAELMKPGASVKISCKVTGYTFRSYWIEWVKQRPGHGLEWIGEILVGSGSTKY  60
               ******* ***  ****** ***  ** *  *   ******* ****** *   ** * *

                              FR3                              CDR3        FR4
AP-14       61 NQKFKDKTTLTADKSSTTAYMQLSSLTSEDSAVYYCATPIFYYGSRYVRYEMDYWGQGTS 120
MOF3-139    61 NEKFKGKATITAQTSSNTVYMQLSSLTSEDSAVYYCARP--FFGSRY-DYS-DFWGQGTT 116
               * *** * * **  ** * ******************* *    ****  *  * *****

AP-14      121 VTVSS                                                        125
MOF3-139   117 LTVSS                                                        121
                ****
```

(B)

```
                        FR1                CDR1            FR2       CDR2
AP-14        1 DIVMTQAAFSNPVTLGTSTSISCRSTKSLLHSNGITYLYWYLQKPGQSPQLLIYQMSNLA  60
MOF3-139     1 DIVMTQAAFSNPVTLGSSASISCRSSKSLLHRNGITYLYWYLQKPGQSPQVLIYQMSNLA  60
               *************** * ****** ***** ****************** *********

                              FR3                    CDR3      FR4
AP-14       61 SGVPDRFSSSGSGTDFTLRISRVEAEDVGVYYCAQNLELPYTFGGGTKLEIKR        113
MOF3-139    61 SGVPDRFSSSGSGTDFTLRISRVEAEDVGVYYCAQNLELPYTFGGGTKLEIKR        113
               ************************************************
```

Fig. 4-6. Alignment of the amino acid sequences of AP-14HL/scFv and MOF3-139HL/scFv
Bold characters indicate CDRs. Asterisks denote the amino acid residues that are identical in both sequences. Gaps are inserted to maximize the alignment.

cloning site in the vector facilitated secretion of a scFv antibody into periplasmic space of bacteria to achieve a functional soluble protein. The resulting expression plasmids are shown in Fig. 4-3. The expression plasmids for the scFv antibody genes were each transformed into *E. coli* cells and their periplasm was extracted and used as a scFv antibody. The VH-linker-VL type (HL type) of two scFv antibodies derived from MOF3-139 and AP-14 was produced in recombinant *E. coli* cells and named as MOF-139HL/scFv and AP-14HL/scFv, respectively. In the same way, the VL-linker-VH type (LH type) of two scFv antibodies derived from both mabs was named as MOF-139LH/scFv and AP-14LH/scFv, respectively. The scFv antibodies produced were analyzed by electrophoresis followed by immunoblotting using the anti-HSV mab, showing migration of the produced scFv antibodies at the expected size of about 30 kDa as shown in Fig. 4-7.

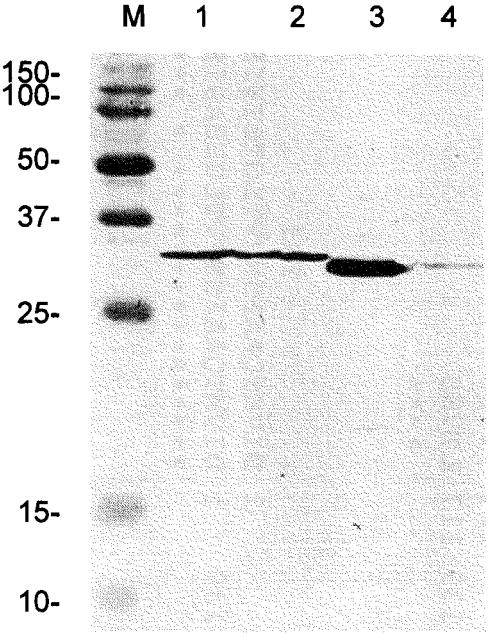


Fig. 4-7. Immunoblots of the scFv antibodies produced in *E. coli* cells
The scFv antibody genes were expressed in *E. coli* BL21(DE3)pLysS strain cells. Membrane preparation was immunoblotted with an anti-HSV mab. Lane 1; AP-14HL/scFv, lane 2; AP-14LH/scFv, lane 3; MOF3-139HL/scFv, lane 4; MOF3-139LH/scFv, M; molecular marker.

The reactivity of four scFv antibodies and two parent mabs was compared in ELISA. The standard curves of these antibodies for NP10EO are shown in Fig. 4-8. The IC_{50} values of the parent mab MOF3-139 and the corresponding MOF3-139HL/scFv were 27 ng/mL and 33 ng/mL for NP10EO, respectively. Similarly, the IC_{50} values of AP-14 and AP-14HL/scFv were 41 ng/mL and 48 ng/mL for NP10EO, respectively. Based on the results, the reactivity of both scFv antibodies was found to be similar to that of the corresponding mabs in ELISA. The reactivity of HL and LH types of scFv antibodies was also compared each other. The IC_{50} values of MOF3-139LH/scFv and AP-14LH/scFv for NP10EO were 32 ng/mL and 67

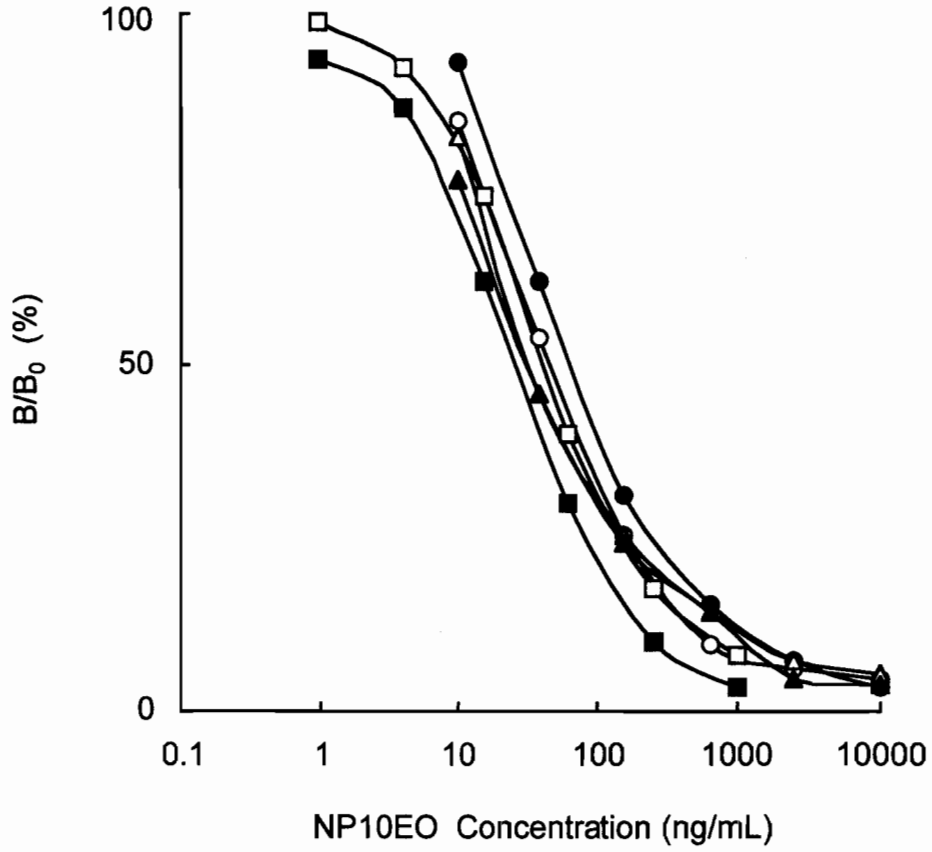


Fig. 4-8. Standard curves obtained with the mabs and their scFv antibodies for NP10EO in ELISA
B/B_0 (%) is quantified as: (absorbance at each concentration of standard)/(absorbance at zero concentration of standard) × 100. AP-14, (□); MOF3-139 mab, (■); AP-14HL/scFv, (○); AP-14LH/scFv, (●); MOF3-139HL/scFv, (△); MOF3-139LH/scFv, (▲).

ng/mL, respectively. The results indicated that the order of linkage of both VH and VL domains via a linker in both scFv antibodies showed little effect on the reactivity of the scFv antibodies towards NP10EO in ELISA.

Reactivity of Hybrid ScFv Antibodies

The expression plasmids for the HL type of two hybrid scFv antibody genes VH(AP-14)-linker-VL(MOF3-139) and VH(MOF3-139)-linker-VL(AP-14), and the LH type of two hybrid scFv antibodies VL(AP-14)-linker-VH(MOF3-139) and VL(MOF3-139)-linker-VH(AP-14) were constructed and named as AH-ML/scFv, MH-AL/scFv, AL-MH/scFv and ML-AH/scFv, respectively (Fig. 4-3). These were each transformed into *E. coli* cells. The hybrid scFv antibodies produced were each extracted and used for ELISA assay. The standard curves of four hybrid scFv antibodies towards NP10EO are shown in Fig. 4-9. The IC_{50} values of MH-AL/scFv, AL-MH/scFv, AH-ML/scFv and ML-AH/scFv were 25 ng/mL, 44 ng/mL, 91 ng/mL and 111 ng/mL, respectively. Two scFv antibodies of MH-AL/scFv and AL-MH/scFv showed the reactivity similar to the wild-type MOF3-139HL/scFv and AP-14HL/scFv towards NP10EO, although the reactivity of the other two scFv antibodies of AH-ML/scFv and ML-AH/scFv was inferior to that of the wild-type scFv antibodies, probably due to the combination of the VH domain of AP-14 and the VL domain of MOF3-139, but not due to the order of both domains.

Cross-Reactivity of the Mabs, Wild-Type and Hybrid ScFv Antibodies

The cross-reactivity of the wild-type MOF3-139HL/scFv and AP-14HL/scFv, two hybrid AH-ML/scFv and MH-AL/scFv, and two mabs was examined for various nonylphenol-like compounds in ELISA. The results are listed in Table 4-2. Both mabs MOF3-139 and AP-14 showed similar cross-reactivity each other with nonylphenol ethoxylates and nonylphenoxy carboxylates except for NP1EC and NP. Thus, AP-14 cross-reacted with NP1EC and NP, although MOF3-139 did not react

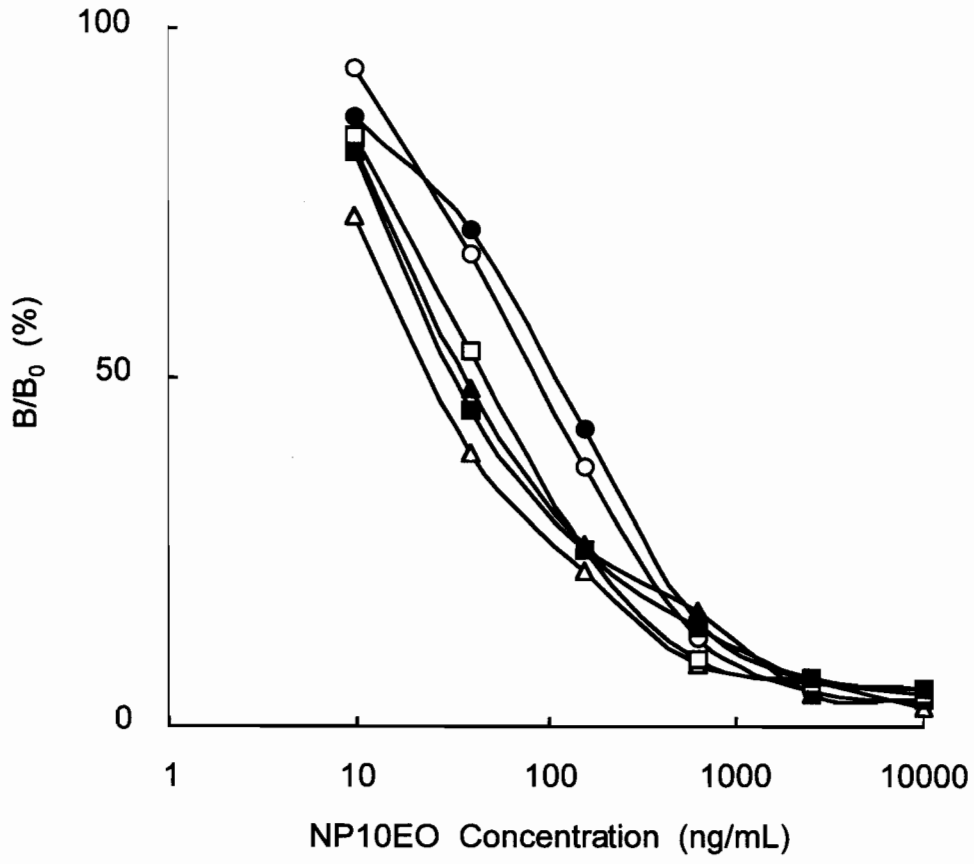
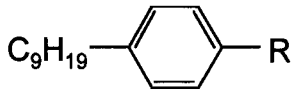


Fig. 4-9. Standard curves obtained with the wild-type and hybrid scFv antibodies for NP10EO in ELISA
AP-14HL/scFv, (□); MOF3-139HL/scFv, (■); AH-ML/scFv, (○); ML-AH/scFv, (●); MH-AL/scFv, (△); AL-MH/scFv, (▲).

with both. As expected, the wild-type scFv antibodies showed the cross-reactivity similar to the corresponding mabs. On the other hand, the hybrid scFv antibodies were obviously different in the cross-reactivity. The AH-ML/scFv cross-reacted with NP1EC and NP at 101% and 14% in ELISA, respectively, while the cross-reactivity to NP1EC and NP of the MH-AL/scFv was at 1.3% and 2.4%, respectively. Thus, it was found that the scFv antibodies containing the VH domain of AP-14 tended to cross-react with both compounds, and the other scFv antibodies containing the VH domain of MOF3-139 were not reactive to these compounds and specific to APEOs. A similar cross-reactivity was also found with the LH type of the wild-type and hybrid scFv antibodies. As mentioned above, there were many

Table 4-2. Cross-reactivity of the two mabs, two wild-type and two hybrid scFv antibodies with nonylphenolic compounds in ELISA



R	Compound	mab		scFv			
		AP-14	MOF3-139	AP-14scFv	MOF3-139scFv	AH-ML/scFv	MH-AL/scFv
OC_2H_4OH	NP1EO	15	4.4	7.5	8.5	17	9.6
$OC_2H_4OC_2H_4OH$	NP2EO	12	9.4	14	17	21	17
$(OC_2H_4)_5OC_2H_4OH$	NP6EO	20	17	29	44	41	31
$(OC_2H_4)_9OC_2H_4OH$	NP10EO	100	100	100	100	100	100
$(OC_2H_4)_{14}OC_2H_4OH$	NP15EO	51	54	56	75	67	36
OCH_2COOH	NP1EC	85	1.7	87	2.8	101	1.3
$OC_2H_4OCH_2COOH$	NP2EC	114	73	95	94	186	79
$O(C_2H_4O)_3CH_2COOH$	NP4EC	63	65	64	81	111	52
OH	*n* -NP	< 1.0	< 1.0	< 1.0	< 1.0	1.6	< 1.0
OH	NP	32	< 1.0	42	2.3	14	2.4

[a]Cross-reactivity (%) = [IC_{50} (NP10EO)/IC_{50} (a tested compound)] x 100.

differences of amino acid sequences in the VH domains of both antibodies. These amino acid residues in the VH domains seemed to be important for determination of the cross-reactivity of both antibodies. By the way, all mabs and scFv antibodies did not bind to *n*-NP. Since it is known that technical NP does not contain *n*-NP,[142] the cross-reaction of the antibodies with *n*-NP was not so important.

Reactivity of Region-Grafted ScFv Antibodies

There were many differences in the primary sequences of CDR1, CDR2, CDR3, FR1 and FR3 in the VH domains as described above. We attempted to clarify the importance of these regions on the cross-reactivity of AP-14. Five regions CDR1, CDR2, CDR3, FR1 and FR3 in the VH domain of AP-14HL/scFv were each grafted into the corresponding regions of MOF3-139HL/scFv, which were named as CDR1scFv, CDR2scFv, CDR3scFv, FR1scFv and FR3scFv, respectively, as shown in Fig. 4-10. The expression plasmids for five scFv antibody genes were constructed and each transformed into *E. coli* cells. These CDR- and FR-grafted scFv antibodies were each produced in the recombinant *E. coli* cells.

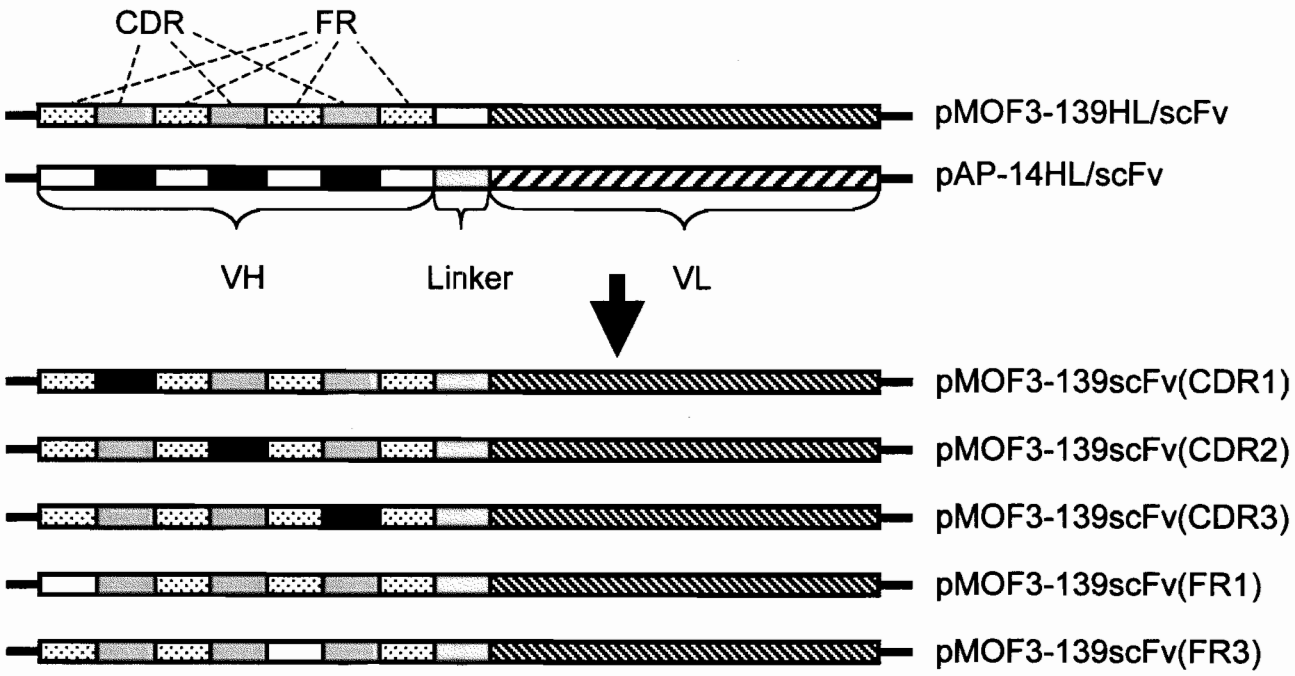


Fig. 4-10. The schematic diagrams of the expression plasmids for CDR- and FR-grafted MOF3-139scFv mutaints
Each of CDR1, CDR2, CDR3, FR1 and FR3 regions in the VH domain of AP-14 was grafted into the corresponding regions of MOF3-139HL/scFv.

The reactivity of the grafted scFv antibodies was examined towards NP10EO, NP1EC and NP in ELISA. The standard curves were prepared for these compounds and IC_{50} values were calculated as to each grafted scFv antibody. The results are shown in Fig. 4-11 as a ratio ((IC_{50} of the wild-type MOF3-139HL/scFv)/(IC_{50} of a tested scFv antibody)). The grafted scFv antibodies showed the reactivity similar to the wild-type MOF3-139HL/scFv towards NP10EO, except for the decrease in the reactivity of the CDR1-grafted scFv antibody, although the FR3-grafted scFv antibody increased the reactivity by 50%. Then, the cross-reactivity of the grafted MOF3-139HL/scFv antibodies towards NP1EC and NP was determined in ELISA. The CDR1-grafted scFv antibody was found to increase the reactivity towards NP1EC and showed over 15-fold higher reactivity than the wild-type MOF3-139HL/scFv, whereas the other grafted scFv antibodies did not change the reactivity towards NP1EC. In addition, the CDR2-grafted scFv

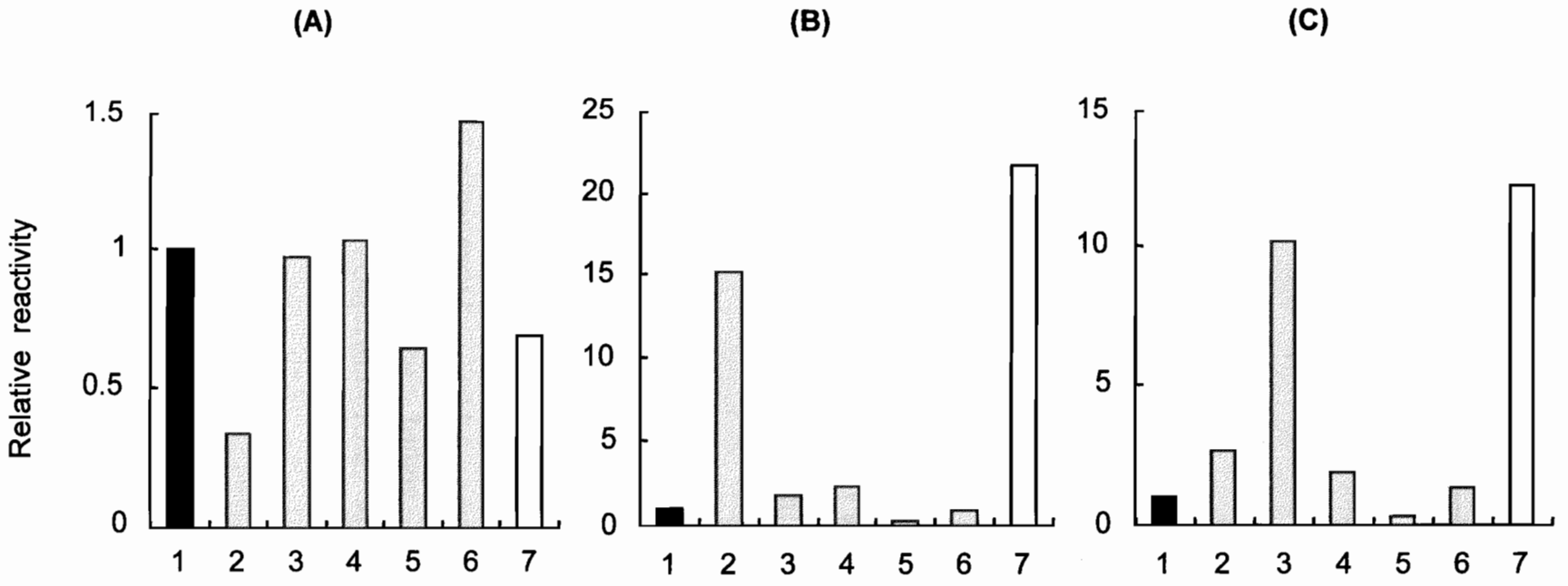


Fig. 4-11. Relative reactivity of the CDR- and FR-grafted MOF3-139HL/scFv mutants with nonylphenolic compounds in ELISA

Relative reactivity = (IC_{50} of the wild-type MOF3-139HL/scFv)/(IC_{50} of a MOF3-139scFv mutant). 1, wild-type MOF3-139HL/scFv; 2, CDR1-grafted MOF3-139scFv; 3, CDR2-grafted MOF3-139scFv; 4, CDR3-grafted MOF3-139scFv; 5, FR1-grafted MOF3-139scFv; 6, FR3-grafted MOF3-139scFv; 7, wild-type AP-14HL/scFv.

(A), NP10EO; (B), NP1EC; (C), NP.

antibody showed 10-fold higher reactivity than the wild-type MOF3-139HL/scFv towards NP, although the other grafted scFv antibodies did not significantly change in the reactivity. These results suggested that the CDR1 and CDR2 regions in the VH domain of AP-14 would be involved in the cross-reactivity with NP1EC and NP, respectively.

Reactivity of Single Point Mutants of MOF3-139HL/scFv

The single amino acid changes in the CDR1 and CDR2 regions of the VH domain of MOF3-139HL/scFv were introduced by replacing with the corresponding amino acid residues of AP-14. Totally, 11 single point mutants of MOF3-139HL/scFv were produced as described in Table 4-3. Six of them had a point mutation in the CDR1 region and the other 5 had in the CDR2 region. The reactivity of these mutants of scFv antibodies was examined towards NP10EO in

Table 4-3. Nucleotide sequences of single point MOF3-139HL/scFv mutants

	HCDR1						HCDR2				
	28	30	31	33	34	35	52	53	54	57	59
MOF3-139HL/scFv	T	R	S	W	I	E	L	V	G	S	K
MOF3-139scFv(T28R)	R	-	-	-	-	-	-	-	-	-	-
MOF3-139scFv(R30T)	-	T	-	-	-	-	-	-	-	-	-
MOF3-139scFv(S31R)	-	-	R	-	-	-	-	-	-	-	-
MOF3-139scFv(W33T)	-	-	-	T	-	-	-	-	-	-	-
MOF3-139scFv(I34M)	-	-	-	-	M	-	-	-	-	-	-
MOF3-139scFv(E35H)	-	-	-	-	-	H	-	-	-	-	-
MOF3-139scFv(L52N)	-	-	-	-	-	-	N	-	-	-	-
MOF3-139scFv(V53P)	-	-	-	-	-	-	-	P	-	-	-
MOF3-139scFv(G54S)	-	-	-	-	-	-	-	-	S	-	-
MOF3-139scFv(S57D)	-	-	-	-	-	-	-	-	-	D	-
MOF3-139scFv(K59E)	-	-	-	-	-	-	-	-	-	-	E
AP-14HL/scFv	R	T	R	T	M	H	N	P	S	D	E

The same amino acid residue as that in MOF3-139HL/scFv was described with a bar.

ELISA. The results are shown in Fig. 4-12A and Fig. 4-12B. All of the mutants containing a point mutation in the CDR1 region showed lower reactivity than the wild-type MOF3-139HL/scFv except for the replacement of 30th Arg with Thr which was higher in the reactivity. Two scFv antibodies mutated at 28th and 34th residues showed significant decrease in the reactivity with NP10EO in ELISA. The results seemed to be consistent with the results of the CDR1-grafted scFv antibody showing lower reactivity than the wild-type MOF3-139HL/scFv. On the other hand, all of the mutants containing a point mutation in the CDR2 region showed the reactivity similar to the wild-type MOF3-139HL/scFv.

The cross-reactivity of these mutants towards NP1EC was examined in ELISA. The results are shown in Fig. 4-12C and Fig. 4-12D. Two antibodies mutated at 33rd and 35th residues in the CDR1 region increased 8-fold higher reactivity than the wild-type MOF3-139HL/scFv towards NP1EC. The results corresponded to the fact that the CDR1-grafted scFv antibody showed strong cross-reactivity towards NP1EC in ELISA. On the other hand, two scFv antibodies mutated at 28th and 31st residues decreased the reactivity as compared with the wild-type MOF3-139HL/scFv towards NP1EC in ELISA. All other scFv mutants showed the reactivity similar to the wild-type MOF3-139HL/scFv with NP1EC. The results of cross-reactivity tested with NP are shown in Fig. 4-12E and Fig. 4-12F. Six among 11 scFv mutants showed more than 3-fold higher reactivity than the wild-type MOF3-139HL/scFv in ELISA. Especially, 3 scFv mutants replaced at 33rd, 57th and 59th residues with Thr, Asp and Glu, respectively, showed the cross-reactivity compared to the wild-type AP-14HL/scFv. Since the CDR1-grafted scFv antibody did not change the reactivity with NP, it was not expected that the replacement of 33rd Trp with Thr would increase the binding ability to NP. No significant changes in the binding activity to NP10EO, NP1EC and NP were found in both scFv mutants altered at 53rd and 54th amino acid residues with Pro and Arg, respectively.

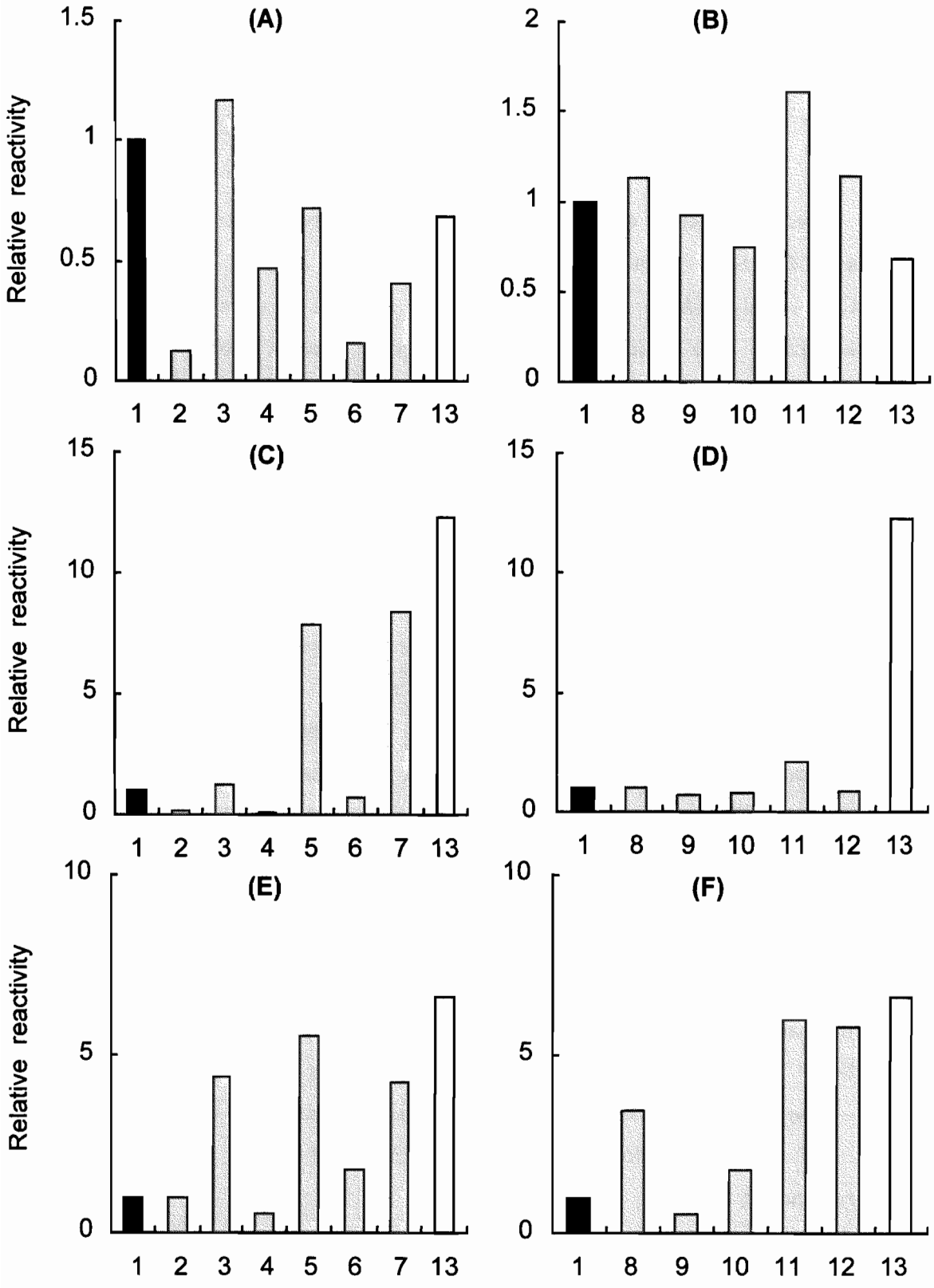


Fig. 4-12. Relative reactivity of the single point MOF3-139HL/scFv mutants with nonylphenolic compounds in ELISA

1, wild-type MOF3-139HL/scFv; 2, MOF3-139scFv(T28R); 3, MOF3-139scFv(R30T); 4, MOF3-139scFv(S31R); 5, MOF3-139scFv(W33T); 6, MOF3-139scFv(I34M); 7, MOF3-139scFv(E35H); 8, MOF3-139scFv(L52N); 9, MOF3-139scFv(V53P); 10, MOF3-139scFv(G54R); 11, MOF3-139scFv(S57D); 12, MOF3-139scFv(K59E); 13, wild-type AP-14HL/scFv.

(A, B), NP10EO; (C, D), NP1EC; (E, F), NP.

DISCUSSION

Based on the comparison of the primary structures between two mabs MOF3-139 specific to APEOs and AP-14 cross-reacting with APEOs and APs, various scFv mutants were produced and characterized for understanding molecular basis on reactivity and specificity of both mabs in this paper. In the first attempt, HL types of two scFv antibodies MOF3-139HL/scFv and AP-14HL/scFv were produced in recombinant *E. coli* cells and subjected to ELISA. The reactivity of both scFv antibodies was comparable to that of the corresponding parent mabs with NP10EO in ELISA. The results indicated that both scFv antibodies were functionally folded in the recombinant *E. coli* cells without forming aggregation, and that the Fv fragments covalently linked with a flexible peptide linker $(Gly_4Ser)_3$ retained the innate conformation of binding pocket of the corresponding parent mabs. In addition to the HL type of the scFv antibodies, LH type of two scFv antibodies MOF3-139LH/scFv and AP-14LH/scFv were generated. The reactivity of them was compared with that of the HL type of scFv antibodies to examine the impact of the order of both VH and VL domains via a linker, because it would affect the reactivity of scFv antibodies or production in recombinant *E. coli* cells in some cases.[92,93] As the results the reactivity of the LH type of scFv antibodies was similar to that of HL type of scFv antibodies. The results indicated that the order of the VH and VL domains of MOF3-139 and AP-14 would not affect their binding activity.

Four hybrid scFv antibodies where the VH and VL domains of MOF3-139 and AP-14 were heterologously assembled were prepared and the reactivity of them was examined. Both AL-MH/scFv and MH-AL/scFv showed the reactivity similar to the wild-type scFv antibodies, although AH-ML/scFv and ML-AH/scFv had lower reactivity towards NP10EO than the wild-type scFv antibodies in ELISA. The results indicated that both of the VH domain of MOF3-139 and the VL

domain of AP-14 stably interacted with each other to bind to NP10EO, however, the association of the VH domain of AP-14 and the VL domain of MOF3-139 seemed to be less stable. Therefore, the VL domain of AP-14 was able to bind to NP10EO with both VH domains of AP-14 and MOF3-139. However, the VL domain of MOF3-139 decreased the binding ability to NP10EO with both VH domains of MOF3-139 although retained the reactivity with the VH domains of AP-14. These observations suggested that the difference between both VL domains of MOF3-139 and AP-14 would be involved in the interaction with the VH domain of AP-14. The alignment of amino acid sequences between both mabs showed that there were only 5 amino acid residues different from each other in the VL domains. Then, these residues of MOF3-139 would be involved in the interaction with the VH domain of MOF3-139.

The cross-reactivity of two mabs, HL type of two wild-type scFv antibodies and two hybrid scFv antibodies was examined with various nonylphenolic compounds in ELISA. Both wild-type scFv antibodies had the cross-reactivity similar to the corresponding parent mabs. Similar observation was reported with other scFv antibodies against the insecticide parathion[52] and the endocrine disruptor bisphenol A.[140] The cross-reactivity of hybrid scFv antibodies AH-ML/scFv and MH-AL/scFv with NP1EC and NP was similar to that of AP-14HL/scFv and MOF3-139HL/scFv, respectively. The results suggested that the VH domains of both mabs would be involved in the cross-reactivity with NP1EC and NP. The alignment of amino acid sequences between both mabs showed that the identity in the VL domains was extremely high (95%) and that in the VH domains was relatively low (65%). This fact also supported the idea that the VH domains of both mabs would be key components of cross-reactivity.

For extensive analysis on the cross-reactivity, all three CDRs, FR1 and FR3 in the VH domain of AP-14 were each grafted into the corresponding regions of MOF3-139HL/scFv, where a number of amino acid residues different from MOF3-139 were found. The amino acid

sequences in the FR2 and FR4 regions of VH domains between both mabs were very similar. Therefore, the FR regions of AP-14 would not be involved in the cross-reactivity with these compounds. The reactivity of these CDR- and FR-grafted scFv antibodies was examined in ELISA. The CDR1-grafted scFv antibody and the CDR2-grafted scFv antibody showed 15-fold and 10-fold higher binding activity than the wild-type MOF3-139HL/scFv with NP1EC and NP, respectively. The results suggested that there would be amino acid residue(s) critical to the interaction with these compounds in both regions of AP-14. The CDR1-grafted scFv antibody also showed lower reactivity by 33% than the wild-type MOF3-139HL/scFv with NP10EO. Since both wild-type MOF3-139HL/scFv and AP-14HL/scFv showed high reactivity with the compound, this observation seemed to be inconsistent. Therefore, the association of the CDR1-grafted VH domain and the innate VL domain of MOF3-139 in this scFv antibody would mismatch to some extent. While, the hybrid scFv antibodies AH-ML/scFv and ML-AH/scFv showed lower reactivity by 33% with NP10EO than the wild-type scFv antibodies and 5 amino acid residues in the VL domain of MOF3-139 different from the corresponding ones of AP-14 would interact with the VH domain of MOF3-139 as mentioned above. Then, it was considerable that these 5 amino acid residues might interact with the CDR1 region in the VH domain of MOF3-139 and the CDR1-grafted scFv antibody would lose the interaction with the VL domain of MOF3-139. As a result, the CDR1-grafted scFv antibody decreased the reactivity to NP10EO as much as AH-ML/scFv and ML-AH/scFv antibodies.

Both FR1- and FR3-grafted scFv mutants behaved in a similar manner as the wild-type MOF3-139HL/scFv in ELISA. Grafting the CDR3 region of AP-14 into MOF3-139HL/scFv had little effect on the specificity, although the amino acid residues in this region were significantly different from each other. Many aromatic amino acid residues were fond in the CDR3 region of the VH domains. Therefore, these residues were assumed to interact with an apolar

nonylphenolic group of these compounds. Because a spacer deposited on the opposite site of a nonylphenolic group in the APEO hapten used for immunization, it was expected that the CDR3 region of the VH domain of both mabs would be mostly buried in the binding pocket and both CDR1 and CDR2 regions would exist in upper place of the binding pocket.

For further analysis on the cross-reactivity, 11 single point mutations were each introduced into the CDR1 and CDR2 regions in the VH domain of MOF3-139HL/scFv and the reactivity of these scFv mutants was examined in ELISA. Five among 6 scFv antibodies mutated in the CDR1 region decreased the binding activity to NP10EO, although all scFv antibodies mutated in the CDR2 region did not. The results were consistent with the fact that the CDR1-grafted scFv antibody decreased the reactivity to NP10EO. Especially, the replacements of 28th Thr with Arg and 34th Ile with Met significantly decreased the binding activity of the scFv mutants to NP10EO. As mentioned above, the CDR1 region in the VH domain of MOF3-139 might interact with some of the 5 amino acid residues in the VL domain of MOF3-139, which were different from the corresponding ones of AP-14. Therefore, both amino acid residues 28th Thr and 34th Ile would play an important role in the interaction with such residues in the VL domain of MOF3-139. Thus, these two single point scFv mutants would lose the interaction between the VH and VL domains, resulting in the significant decrease of the reactivity with NP10EO.

The cross-reactivity tests of these single point scFv mutants were examined with NP1EC in ELISA. The results showed that the replacements of either 33rd Trp with Thr or 35th Glu with His was found to be important for cross-reaction with NP1EC. The former may strengthen a hydrogen bond network via a carbonyl group of NP1EC. The latter may form a salt bridge with a carboxyl group of NP1EC to hold the antigen. Since no mutants increased their cross-reactivity with NP1EC except for these two scFv mutants, these results

corresponded to the fact that the CDR1-grafted scFv antibody strongly cross-reacted with NP1EC. On the other hand, the replacement of either 28th Thr or 31st Ser with Arg significantly decreased the reactivity with NP1EC. It was assumed that a guanidium group of Arg would affect the nearby amino acid residues by electrostatic interactions or steric hindrance in the binding pocket.

The cross-reactivity of the scFv mutants was determined with NP in ELISA. Because the CDR2-grafted scFv antibody increased the reactivity with NP, NP was assumed to form specific hydrogen bonds to some polar residues in the CDR2 region of the VH domain of AP-14. As a result, several scFv mutants introduced with single point mutations into the CDR1 and CDR2 regions were found to increase the reactivity with NP. The results did not corresponded to the observation that the CDR2-grafted scFv antibody increased the binding activity to NP in spite of unchanged reactivity of the CDR1-grafted scFv antibody. Three scFv antibodies mutated at 33rd Trp with Thr, 57th Ser with Asp or 59th Lys with Glu showed stronger reactivity with NP. The scFv mutant altered at 33rd Trp with Thr also increased the cross-reactivity with NP1EC, but did not with NP10EO. Then, this amino acid residue would affect the common interactions between the scFv mutant and both NP1EC and NP. The replacement of polar amino acid residues at 57th Ser and 59th Lys with acidic ones also highly increased the cross-reactivity with NP in ELISA. Then, electrostatic interactions or hydrogen bonds between a negatively charged carboxyl group of the amino acid residues and NP or the nearby residues may account for the observed cross-reactivity with NP equivalent to the wild-type AP-14HL/scFv. Some mutants also showed certain reactivity with NP other than the mutants showing a strong cross-reactivity. Then, small changes of single amino acid residue may make hydrogen bonds between side chains of the nearby residues and a phenol group of NP.

We could identify the amino acid residues of two anti-APEO mabs critical to the cross-reactivity based on the comparison of the

primary structures and production of various scFv mutants without a modeling simulation. Therefore, comparing with amino acid sequences of antibodies with relatively high homology to the desired antibody seemed to be effective to analyze the molecular interaction between antigen and antibody. Especially, building up the information about the reactivity of anti-hapten antibodies will provide great value for cross-reactivity analysis. On the other hand, it is difficult to obtain antibodies specific to NP only by the traditional hapten design. Then, generation of a scFv antibody specific to NP may be available by introduction of certain point mutations into AP-14HL/scFv. At this stage, it is still unclear how the scFv mutants interacted with NP10EO, NP1EC and NP in detail. To make more obvious, determination of the three-dimensional structure of the scFv antibodies bound to antigen by X-ray crystallographic analysis will be effective.

CHAPTER V

CONCLUDING REMARKS

Expansion of contamination of the environment and agricultural products with certain chemicals is a serious global problem. Public concerns on the adverse effects by such compounds have been increasing in the world. To assess the risks, determining the concentration of chemicals in the environment and agricultural products is of great significance, and immunoassays are suitable for the routine monitoring. Then, in this study, it was described to produce mabs and recombinant antibodies against the organophosphorus pesticide malathion, the industrial compounds bisphenol A and alkylphenol ethoxylate, and to develop sensitive ELISA systems based on them. The scFv antibodies obtained in this study showed the reactivity and specificity similar to the corresponding mabs in ELISA. The ELISA based on the mabs MLT2-23 and MLT40-4 could detect low ppb levels of malathion. Because the maximum residue limits of malathion in agricultural foodstuff are equal to or more than 0.1 ppm, this ELISA would be useful for screening of agricultural products, especially wheat grain, for detection of the malathion residues. The ELISA based on anti-bisphenol A antibodies developed in this study could determine the concentration of bisphenol A at ppb to higher ppt levels. Since bisphenol A was detected at ppb to ppt levels in surface water, the ELISA would be possible to determine the concentration of bisphenol A in environmental water samples without complicated and time-consuming clean-up steps. The ELISA based on MOF3-139 was able to determine the amount of alkylphenol ethoxytes, and the ELISA based on AP-14 could determine the total amounts of alkylphenol ethoxytes, alkylphenol ethoxylate carboxylic acids and alkylphenols. Because simultaneous detection of alkylphenol ethoxylates, alkylphenol ethoxylate carboxylic acids and alkylphenols is very difficult by instrumental analysis due to the difference of physical property, the ELISA assays

would be useful for environmental monitoring of these compounds.

The scFv antibodies have some advantages over traditional mabs. By cultivation of recombinant *Escherichia coli* cells, scFv antibodies were obtained even more rapidly and readily than the mabs. Gene manipulation will make mutated antibodies altering their property. Thus, scFv antibodies are powerful tools as immunoreagents, and it is expected that scFv antibodies can be applied to the immunological technology such as immunoaffinity chromatography and immunosensor as well as immunoassays.

On the other hand, molecular mechanisms of the interactions between environmental chemicals and specific antibodies and between the VH and the VL were analyzed by using various recombinant antibodies. Anti-malathion MLT40-4scFv was found to change the reactivity by replacing the order of the linkage between VH and VL domains. The unique framework region 2 of the VL domain of the anti-bisphenol A BBA-2187, which detected one amino acid residue was assumed to be important to interact with the VH domain of BBA-2187, and even considered to be involved in the higher reactivity with bisphenol A. From the mutation analysis of anti-alkylphenol ethoxylate scFv antibodies, single point mutation was found to be able to change the cross-reactivity dramatically. These information is considered to be important to analyze the molecular interaction between haptens and antibodies, and useful for preparation of recombinant antibodies specific to other environmental chemicals. Based on the knowledge obtained in this study would contribute to generate a novel recombinant antibody. For examples, anti-nonylphenol antibodies have been not available yet because of difficulty of hapten design. Then, the introduction of some point mutation into AP-14scFv or MOF3-139 may make a antibody specific to nonylphenol only.

These results were significant information for deducing the nature of antibodies against haptenic compounds such as environmental chemicals and for contributing to the improvement of recombinant antibodies. The knowledge would be useful to clarify the interaction

within immunoglobulin molecules and the reactivity and specificity of antigen-antibody binding. Therefore, this study would contribute to the improvements of the biological monitoring technology for environmental chemicals.

SUMMARY

In this study, production of anti-malathion mabs, anti-bisphenol A mabs, and anti-alkylphenol ethoxylate mabs and development of ELISAs were attempted.

In chapter II, two mabs MLT2-23 and MLT40-4 specific to the insecticide malathion were isolated and characterized in a dc-ELISA when mice were immunized with the conjugate EIT244-KLH containing the malathion-hapten with the P-NH-C bond instead of the P-S-C linkage. The other haptens with the P-S-C bond failed to raise antibodies against malathion. Malathion was determined in the ranges of 5.3 to 75 ng/ml and of 7.0 to 190 ng/ml in dc-ELISA based on the mabs MLT2-23 and MLT40-4, respectively. Then, the cDNA clones encoding heavy chain and light chain regions of both mabs were isolated from two individual cDNA libraries constructed from mRNA fractions extracted from the hybridoma cells producing the corresponding mabs. Two types of scFv antibody genes with the sequences of VH-linker-VL (HL) and VL-linker-VH (LH), respectively, were constructed on the basis of the cDNA clones of each mab, each inserted into the phagemid vector pCANTAB5E and expressed in *Escherichia coli* HB2151 cells. The IC_{50} values in ic-ELISA with MLT2-23/HL scFv and MLT2-23/LH scFv for malathion were 81 ng/ml and 72 ng/ml, respectively, in contrast to that of 60 ng/ml with the parent mab MLT2-23. On the other hand, MLT40-4/LH scFv showed the IC_{50} value of 150 ng/ml, in contrast to 75 ng/ml with the parent mab MLT40-4, while MLT40-4/HL scFv hardly reacted with malathion in ic-ELISA. It was found that the order of linkage of both VL and VH allowed the scFv antibodies to alter their antigen-binding affinity or antigen-antibody reactivity in the case of MLT40-4 scFvs.

In chapter III, four anti-BPA mabs were obtained and each characterized in ELISA. Among these mabs, BBA-2187 was the most reactive towards BPA. The quantitation limit of the ELISA assay for

BPA was 0.13 ng/mL, that was more sensitive than the other immunoassays reported. Then, the cDNA clones encoding variable heavy and variable light chains of these four mabs were isolated, and used for construction of four scFv antibody genes, which were expressed in *E. coli* cells. The reactivity of four scFv antibodies towards BPA in ELISA was comparable to those of the parent mabs. The most sensitive assay was achieved with BBA-2187scFv. Its cross-reactivity to the related compounds was similar to that of the parent mab. Based on the reactivity of heterologous combinations of VH and VL fragments, it was found that the unique structure of the framework region 2 in the VL of BBA-2187 appeared to be important for specific assembly together with the VH.

In chapter IV, the cDNA clones encoding the VH and the VL of each of two anti-alkylphenol ethoxylate mabs MOF3-139 and AP-14 were isolated from their hybridoma cell lines by 5'-RACE. The scFv genes were constructed from the cDNA clones and each expressed in recombinant *E. coli* cells. The produced scFv antibodies were characterized in ELISA. The reactivity of the scFv antibodies with nonylphenol ethoxylates and the cross-reactivity with nonylphenol ethoxycarbonates and NP were comparable to those of the corresponding parent mabs. Based on comparison of the primary structures between both mabs, various scFv mutants were produced in recombinant *E. coli* cells and characterized in ELISA. The results suggested that 33rd Thr and 35th His in the VH domain of AP-14 were involved in the cross-reaction with NP1EC and that 33rd Thr, 57th Asp and 59th Glu in the VH domain of AP-14 were involved in the cross-reaction with NP.

As mentioned above, establishment of ELISA assays for environmental chemicals and clarification of the interactions within the variable domains of antibody molecules and between antigen and antibody specific to environmental chemicals were achieved. These results would contribute to the improvement of the immunochemical technology for environmental monitoring.

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CHAPTER I

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CHAPTER II

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CHAPTER III

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CHAPTER IV

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