Cloning, Expression, and Characterization of Recombinant Fab Antibodies against Dioxin

Nanju Lee,* Carol K. Holtzapple, and Larry H. Stanker

Food Animal Protection Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 2881 F&B Road, College Station, Texas 77845-9594

Using two hybridoma cell lines (DD1 and DD3) secreting anti-dioxin monoclonal antibodies as a source for messenger RNA and cDNA, light and heavy chain gene fragments of Fab domains were amplified by the polymerase chain reaction (PCR). The amplified gene fragments were cloned into the pFabUSDAI vector for expression of recombinant Fab antibodies in *Escherichia coli*. Expression of the soluble and functional recombinant Fab antibodies (designated rFab1-1 and rFab3-3) was confirmed by an indirect immunoassay using dioxin conjugated to rabbit serum albumin. On the basis of these rFabs, two competitive inhibition immunoassays using 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) as a competitor were developed. The concentration of 2,3,7,8-TCDD required to inhibit color development by 50% (IC_{50}) determined from the dose response curves for rFAB1-1 and rFAB3-3 were 10.4 ± 2.4 and 12.2 ± 6.0 ng/mL, respectively. The binding properties of both rFab antibodies for other chemically related compounds were relatively similar to those of their respective monoclonal antibodies and enzymatically derived Fab fragments.

Keywords: *Dioxin; recombinant antibody; cloning antibody genes; Fab; expression vector; Escherichia coli; immunoassay*

INTRODUCTION

The development of hybridoma technology in 1975 (Kohler and Milstein, 1975) allows the production of an unlimited supply of homogeneous antibody. Since then, monoclonal antibodies have been used successfully and extensively in many areas of science. In environmental diagnostics, sensitive immunoassays using monoclonal antibodies have been developed for numerous compounds. These include dioxin (Stanker et al., 1987), atrazine (Giersch and Hock, 1990), metolachlor (Schlaeppi et al., 1991), paraquat (Niewola et al., 1986), and picloram (Deschamps et al., 1990). Monoclonal antibody production requires fusion of differentiated B-lymphocytes and myeloma cells, and an extensive and laborious screening process must be performed to obtain the desired hybridomas. The drawback of the technology, however, is that the selection is limited to the more prevalent populations of clones that may exhibit lower affinity for the analyte. Clones with high affinity may not have the opportunity to be selected due to either the screening strategy itself or perhaps chromosomal instability of specific clones. More importantly, the properties of the monoclonal antibody cannot be readily altered, and improvements can be achieved only through conventional approaches, for example, by synthesis of a new immunogen.

Rapid developments in molecular biology and protein engineering now make it possible to engineer "tailor-made" polypeptides or proteins such as immunoglobulins with either specific or improved functionality. Since the early reports describing recombinant antibody technology in the mid 1980s, significant progress has been made, especially in the design of expression vectors, phage display systems, isolation of heavy and light chain encoding genes by polymerase chain reaction (PCR), and various expression systems for bulk production. Different antibody fragments such as Fv (Skerra and Plückthun, 1988), single-chain Fv (scFv; Bird et al., 1988), Fab (Better et al., 1988), $(Fab')_2$ (Carter et al., 1992), miniantibodies (Sollazzo et al., 1995), and diabodies (Holliger et al., 1993) have now been expressed. Also, antibody molecules fused to reporter enzymes or affinity tags can be easily engineered. Furthermore, modification of an existing antibody or design of a new one can be achieved at the molecular level via site-directed mutagenesis, chain shuffling, or complementarity-determining region (CDR) grafting.

Antibody engineering technology was developed initially for medical and biochemical diagnostics and for therapeutic applications. More recently, several research groups have examined the potential of this technology for developing more useful antibodies for environmental diagnostics. Although many recombinant antibodies have been engineered for biomedical and clinical applications, only a few recombinant antibodies have been reported for environmental diagnostics. These include recombinant Fab antibodies for atrazine (Ward et al., 1993) and diuron (Karu et al., 1994; Scholtholf et al., 1997) and scFv fragments for *s*-triazine (Kramer and Hock, 1996), parathion (Garrett et al., 1997), and cyclohexanedione (Webb et al., 1997). Although the technology has proven to be useful for generating more appropriate antibody molecules, many researchers have found technical difficulties in synthesizing recombinant antibodies routinely. The inability to generate high-affinity recombinant antibodies from synthetic combinatorial libraries caused a number of researchers to use hybridomas secreting relevant mono-

* Author to whom correspondence should be addressed [telephone (409) 260-9306; fax (409) 260-9332; e-mail lee@usda.tamu.edu].

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clonal antibodies as the starting point for developing recombinant antibodies. Even with the use of hybridomas, problems arose due to unequal production of the light and heavy chains during PCR amplification, contamination of high-affinity clones capable of recognizing hapten in solution with nonspecific clones during the panning step, and poor recombinant protein production and recovery during expression. A number of alterations in the methodology have improved the technology. These include development of more effective primer mixes for amplification of both the heavy and light chains (Zhou et al., 1994), use of an immunomagnetic separation method to reduce contamination of high-affinity clones (Kramer and Hock, 1996), and development of better purification protocols (Byrne et al., 1996; Kipriyanov et al., 1997) for improved recombinant antibody production. Despite the difficulties, antibody engineering has the potential to produce more effective antibodies for environmental analyses.

Dioxins are environmental contaminants that are considered carcinogenic and that can pose a potential threat to human health if adulterated food is consumed. Milk was found to be contaminated with polychlorinated dibenzo-*p*-dioxin and polychlorinated dibenzofurans (Ryan et al., 1991; Schuler et al., 1997). More recently, chicken carcasses were contaminated with dioxin; the source of contamination was the clay used in the feed (*Food Chemical News*, 1997). These incidents demonstrate that dioxins can find their ways into the food chain through contaminated raw materials. Conventional instrumental analyses require laborious multistep cleanup procedures and are very expensive. In an effort to provide simplified and routine analyses, a number of immunoassays for dioxin have been developed previously (Albro et al., 1979; Kennel et al., 1986; Stanker et al., 1987). A panel of monoclonal antibodies developed by Stanker et al. (1987) provides sufficient sensitivity, specificity, and accuracy for analysis of dioxin in industrial samples and in soil (Vanderlann et al., 1988; Watkins et al., 1989). Two of the monoclonal antibodies, DD1 and DD3, have been fully characterized (Stanker et al., 1995).

We have cloned the heavy and light chain genes, obtained from the DD1 and DD3 hybridoma cell lines that secrete anti-dioxin monoclonal antibodies, into the pFabUSDAI vector. Two recombinant Fab fragments (rFab) were expressed in *Escherichia coli*. Both of the rFabs displayed competitive inhibition with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) in an indirect ELISA. We chose to express Fab fragments rather than scFv for several reasons. A number of examples, including previous experience in our laboratory, indicated that scFv fragments are likely to be less stable than Fab fragments, partly due to the lack of intramolecular disulfide bonds (Reiter et al., 1996). The constant domains contribute greatly to the interaction between the light and heavy chain, thus increasing the architectural stability of the antibody molecule. For environmental diagnostics, recombinant antibodies are required that are sufficiently stable in various matrixes for analysis. Thus, Fab fragments would be more useful as analytical reagents. Here we report the construction of an expression vector, cloning and expression of recombinant Fab fragments, characterization of these fragments for sensitivity and specificity, and comparison of their binding properties with those of parent antibodies and enzymatically derived Fab fragments.

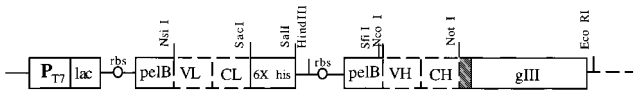

Figure 1. Map of pFabUSDAI vector containing a dicistronic operon for expression of anti-dioxin rFab. This construct consists of a T7*lac* promoter (P_{T7} lac), *pelB* regions for secretion of light and heavy chain fragments in the periplasmic space, ribosomal binding sites (rbs), a polyhistidine tag (6x his) for detection and purification, and various restriction enzyme sites for insertion of light and heavy chain genes (cloning sites are indicated by the dashed lines).

MATERIALS AND METHODS

Reagents. Dibenzodioxin (DD), 2,3,7,8-tetrachloro-DD (TCDD), and 3,3′,4,4′-tetrachlorobiphenyl (TCBP) were obtained from Chem Service Chemicals (West Chester, PA). 2,4,5-Trichlorophenol and 2,5-dichloronitrobenzene were purchased from Aldrich Chemical Co. (Milwaukee, WI). Both γ- and δ-hexachlorocyclohexane were obtained from Sigma Chemical Co. (St. Louis, MO).

Construction of a Recombinant Fab Antibody Expression Vector. The pFabUSDAI expression vector containing appropriate restriction endonuclease sites for cloning purposes was constructed from the existing vectors pET22b+ (Novagen) and pHEN (a generous gift of H. R. Hoogenboom) as follows. The *Eco*RI, *Nco*I, and *Not*I sites in pET22b+ were removed by sequential Klenow fill-in and religation reactions. An *Eco*RI site was then introduced between the *Hin*dIII and *Xho*I sites in the vector using an adapter. A 6x histidine site followed by a stop sequence (UGA) was added after the *Sac*I site so that the recombinant product could be easily purified by metal chelate affinity chromatography. Finally, the *Hin*dIII−*Eco*RI sequence of pHEN containing a ribosomal binding site, *pelB* leader sequence, and the gene encoding the phage gIII protein was inserted into the *Hin*dIII−*Eco*RI site of the altered pET22b+. A diagram of the pFabUSDAI expression vector showing the sites of insertion of the antibody genes is shown in Figure 1.

Cloning of Recombinant Fab Fragments into the Expression Vector. Messenger RNA was isolated from DD1 and DD3 hybridoma cell lines (Micro-Fast Track kit; Invitrogen, CA), which secreted monoclonal antibodies against the dioxins (Stanker et al., 1987). First-strand cDNA was synthesized from the mRNA using an oligo-d(T) primer (First-Strand cDNA Synthesis kit; Pharmacia Biotech, Uppsala, Sweden), and the immunoglobulin heavy and light chain genes were amplified from cDNA by PCR using *Taq* DNA polymerase and specific oligonucleotide primers with the desired restriction sites. To produce Fab fragments, the primers were designed to anneal to the 5′-sequences of framework region 1 of the variable regions and the 3′-sequences of the constant regions. Heavy chain genes were reamplified using a set of second primers designed to attach an additional endonuclease cleavage site (*Sfi*I). The sequences of 5′-primers are as follows (the underlined sequences indicate restriction sites): for DD1 heavy chain, 5′-AGC CGG CCA TGG CCC AGG TCC AAC TGC AGC AGC-3′ (designated 5′DD1H-*Nco*I) and 5′-ACT CGC GGC CCA GCC GGC CAT GGC CCA GGT CCA A-3′ (5′DD1H-*Sfi*I); for DD1 light chain, 5′-ATG GCC ATG CAT GGT GAT GTT GTG ATG ACC CAA GC-3′ (5′DD1-*Nsi*I); for DD3 heavy chain, 5′-GGC CAT GGC CGA CGT AAA CCT GGT GGA GTC-3′ (5′DD3H-*Nco*I) and 5′-ACT CGC GGC CCA GCC GGC CAT GGC CGA CGT AAA-3′ (5′-DD3H-*Sfi*I); for DD3 light chain gene, 5′-ATG GCC ATG CAT GGT GAC ATC CTG ATG ACC CAA TC-3′ (5′DD3L-*Nsi*I). The sequences for 3′-primers are 5′-GTC GAT GCG GCC GCA ATT TTC TTG TCC ACC TTG-3′ (3′CH1-*Not*I) for the heavy chains and 5′-TCG ACG GAG CTC ACA CTC ATT CCT GTT GAA-3′ (3′LC-*Sac*I) for the light chains. PCR was performed for 30 cycles as follows: denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 2 min. PCR products exhibiting a size of approximately 700 bp were excised from agarose gels and purified using microspin columns (Pharmacia, Piscataway, NJ).

Both the PCR products and the pFabUSDAI vector were digested with the appropriate pairs of restriction enzymes. The digested heavy and light chain DNA fragments were directionally cloned into the vector, and the ligated products were used to transform *E. coli* NovaBlue cells (Novagen, Madison, WI). Plasmid DNA was isolated from individual colonies and digested with appropriate restriction enzymes, and positive clones were identified by the presence of an approximately 700 bp insert.

Expression of Recombinant Fab Fragments to Dioxin. For expression of recombinant Fab fragments, *E. coli* BL21-(DE3) and BL21(DE3)pLysS strains (Novagen) were transformed with plasmid DNA from the positive clones. Transformed cells were plated onto LB agar plates containing carbenicillin (50 μg/mL) for the BL21(DE3) strain or carbenicillin (50 μg/mL) and chloramphenicol (20 μg/mL) for the BL21-(DE3)pLysS strain. Plates were incubated at 37 °C overnight, and then each colony was transferred to 5 mL of LB medium containing appropriate antibiotics. Cultures were incubated overnight at 37 °C with continuous shaking. Five hundred microliters of culture was then transferred to 50 mL of LB medium containing appropriate antibiotics and the incubation continued at 30 °C until $OD_{600} = 0.8-1.0$. Cells were induced by adding IPTG to final concentrations of 0.4 and 1 mM, for BL21(DE3) and BL21(DE3)pLysS strains, respectively, and incubating for 3 h or overnight at 30 °C.

Preparation of Periplasmic Extract. Periplasmic extract was prepared using a method described by Polymenis and Stollar (1995). Cells were harvested by centrifugation at 5000*g* for 10 min. Supernatant was collected in a separate tube, and the bacterial pellet was resuspended in cold lysis buffer (200 mM Tris-HCl, 0.5 mM EDTA, 0.5 M sucrose; 10 μL/10 mL cell culture). The resuspended bacterial pellet was added to a tube containing 12 mL of cold purified water and 4 mL of cold lysis buffer. The sample was mixed well and incubated on ice for 30 min. Periplasmic extract was obtained by centrifuging the suspension at 15000*g* for 10 min at 4 °C and collecting the supernatant. Culture supernatant and periplasmic extract were filtered through a 0.22 μm cellulose acetate membrane and stored at 4 °C until further purification.

Purification of Recombinant Fab Antibodies. Recombinant Fab fragments were purified either by cobalt immobilized metal affinity chromatography (IMAC, Clontech, CA) or protein G−Sepharose affinity chromatography. For IMAC purification, 20 mL of culture medium or periplasmic extract was added to pre-equilibrated resin in a gravity flow column and gently agitated for 20 min. Periplasmic extracts were initially dialyzed against Tris-buffered saline (TBS; 20 mM Tris-HCl and 100 mM NaCl, pH 8.0) to remove EDTA. The suspension was centrifuged at 700*g* for 5 min, and the supernatant was discarded. The resin was washed three times with 10 mL of IMAC wash buffer (20 mM Tris-HCl, 100 mM NaCl, and 10 mM imidazole, pH 8.0). Polyhistidine-tagged antibody was eluted after agitating with 2 mL of IMAC elution buffer (20 mM Tris-HCl, 100 mM NaCl, and 50−100 mM imidazole, pH 8.0) for 10 min.

Preparation of Fab Fragments from the Parent Antibodies. Fab fragments were produced by papain digestion of purified IgG (Andrew and Titus, 1991). The papain (2.5 mL of 0.1 mg/mL solution in PBS containing 0.02 M EDTA and 0.02 M cysteine) was added to an equal volume of antibody solution (1.6 mg/mL for DD1 and 2.2 mg/mL for DD3). The mixtures were incubated at 37 °C for 24 h, and enzyme digestion was stopped by adding iodoacetamide to a final concentration of 0.03 M. The solution was dialyzed against PBS for 3 days and purified on a protein G−Sepharose affinity column. The papain digestion was assessed using a polyacrylamide gel electrophoresis (SDS−PAGE) by the disappearance of a band at ~50 kDa that corresponded to the reduced intact heavy chain.

DNA Sequencing. The cloned antibody genes were sequenced using a commercial kit (Sequenase 2.0, Amersham, Arlington Heights, IL) and reverse primers designed to bind either to the framework 2 regions or to the 5′-end of the constant regions. The sequences of the primers are as follows: for DD1 heavy chain framework 2, 5′-CCA ATC CAC TCA AGG CCT TG-3′; for DD1 light chain framework 2, 5′-ATA GAT TAG GCG TTT TGG AG-3′; for DD3 heavy chain framework 2, 5′-TGC GAC CCA CTC CAG CCT CT-3′; for DD3 light chain framework 2, 5′-ATA GAT CAG GCC CTT AAA TG-3′; for the constant region of the heavy chain, 5′-CAG GGG CCA GTG GAT AGA C-3′; and for the constant region of the light chain, 5′-CTG CTC ACT GGA TGG TGG GA-3′.

Electrophoresis. *Gel Electrophoresis.* SDS−PAGE was performed using either 4−20% gradient gels or 12% acrylamide gels in a Bio-Rad Mini Protein II gel electrophoresis apparatus at 200 V for 40−60 min (Bio-Rad, Hercules, CA). The protein bands were stained with either Coomassie Bright Blue R or silver nitrate for visualization.

Immunoblotting. Total proteins from SDS−PAGE were transferred to a poly(vinylidene difluoride) (PVDF) membrane using a semidry immunoblotting apparatus (Bio-Rad). The membrane was blocked with 1% BSA−PBS/T (1% bovine serum albumin in PBS containing 0.01% Tween 20) for 1 h. Following three washes with PBS/T, the membrane was incubated with goat anti-mouse antibody conjugated to horseradish peroxidase (1/1000 in 1% BSA−PBS/T) for 1 h. Unbound conjugate was removed by incubating the membrane in PBS/T three times for 10 min each time. 3,3′,5,5′-Tetramethylbenzidine (1-Step TMB blotting substrate, Pierce, Rockford, IL) was added for color development. All incubations were performed with constant agitation. For detection of the polyhistidine tag, the membrane was incubated for 1 h in mouse anti-polyhistidine monoclonal antibody (diluted 1/3000 in 1% BSA−PBS/T, Sigma) prior to incubation with HRP-labeled goat anti-mouse Fc.

Indirect Enzyme Immunoassays. *Detection of 6x His-Tagged Recombinant Fab.* Wells were coated with 0.5 μg/well of 1-*N*-(adipamino)-3,7,8-trichlorodibenzo-*p*-dioxin conjugated to rabbit serum albumin (tri-CDD-A-RSA; Stanker et al., 1987). After unbound sites were blocked with blocking buffer (3% nonfat dried milk in PBS, pH 9), 100 μL of supernatant or periplasmic extract was added to each well and incubated for 2 h at room temperature. Wells were washed 10 times with washing buffer (0.1% Tween 20) and 100 μL of mouse anti-polyhistidine monoclonal antibody (diluted 1/3000 in 1% BSA−PBS/T) was added to each well. After 1 h of incubation at room temperature, unbound antibodies were removed by washing as previously described, and 100 μL of goat anti-mouse conjugated to horseradish peroxidase (diluted 1/1500 in 1% BSA−PBS/T) was added and incubated for 1 h. Washing was performed to remove unbound conjugate, and then 150 μL of K-blue substrate (ELISA Technologies, Lexington, KY) was added and incubated for 30 min. Color development was stopped by adding 50 μL of 2 M sulfuric acid. Absorbance was read at both wavelengths, 450 and 655 nm.

Competitive Indirect Enzyme Immunoassay To Detect 2,3,7,8-TCDD and Related Compounds. An indirect immunoassay using a solid-phase antigen format was used for the competitive inhibition studies. Wells were coated with coating antigen at 0.25 μg/well. After incubation with blocking buffer, 100 μL of dioxin standard and 100 μL of purified antibody solution were added to each well and incubated for 1.5 h. Wells were washed three times with washing buffer using a microwell plate washer (Bio-Rad). Dioxin waste generated from this step was retained in a waste bottle for appropriate disposal. Goat anti-mouse conjugated to HRP (100 μL per well) was incubated for 1 h, and the unbound conjugate was removed by washing. Color development was performed as described earlier, and absorbance was read at both 450 and 655 nm.

Preparation of Dioxin Standards. Dioxin standards were prepared as described by Vanderlaan et al. (1988) with some modifications. 2,3,7,8-TCDD in toluene (10 μg/mL) was dried under nitrogen and redissolved in an equal volume of methanol containing 1% Tween 20. The solution was diluted 1/10 with 1% BSA−PBS/T to obtain 1 μg/mL and then serially diluted 1/3 in the same buffer to give 333, 111, 37, 12, 4, 1.37, 0.46, and 0.15 ng/mL. The final concentrations of methanol and Tween 20 in microwells did not exceed 10 and 0.1%, respectively. Other chlorinated chemicals were prepared in a similar

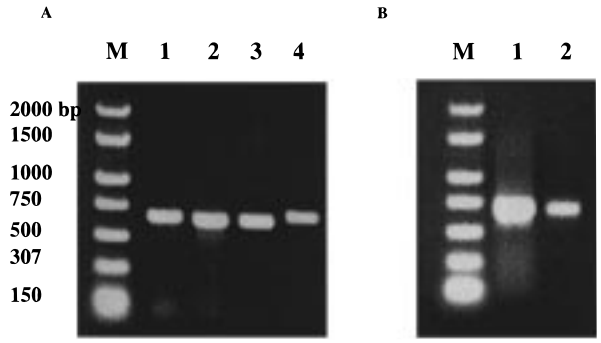

Figure 2. Agarose gel electrophoresis of heavy and light chain genes: (A) Lane 1 is DD1 light chain gene amplified from cDNA using 5′DD1L-*Nsi*I and 3′LC-*Sac*I primers; lane 2 is DD1 heavy chain gene amplified using 5′DD1H-*Nco*I and 3′CH1-*Not*I primers; lane 3 is DD3 light chain gene amplified using 5′DD3L-*Nsi*I and 3′LC-*Sac*I primers; and lane 4 is DD3 heavy chain gene amplified using 5′DD3H-*Nco*I and 3′CH1-*Not*I primers. (B) Lane 1 is DD1 heavy chain reamplified using 5′DD1H-*Sfi*I and 3′CH1-*Not*I primers; and lane 2 is DD3 heavy chain re-amplified using 5′DD3H-*Sfi*I and 3′CH1-*Not*I primers. DNA molecular weight markers in bp are indicated to the left of each panel.

manner. Suitable protective clothing, gloves, and eye protection were worn when dioxin was handled, and the immunoassays were performed in an approved fume hood. The generation of waste material containing dioxin and its congeners was kept to a minimum. The disposal of the solid and liquid waste that was generated was handled by a hazardous waste broker adhering to all federal, state, and local regulations.

RESULTS AND DISCUSSION

Cloning and Expression of Recombinant Fab Fragments to Dioxin. The use of hybridoma cell lines that secrete dioxin monoclonal antibodies as a source for mRNA provided several advantages for our studies. First, the DNA sequences from the cDNA of DD1 and DD3 have been published (Recinos et al., 1994; Accession Nos. in EMBL database: DD1-γ Z21788, DD3-κ Z19575, DD3-γ X58884, and DD3-κ X59052), which enabled specific primers to be designed. Second, immunoassays using these monoclonal antibodies were fully developed and characterized. This provided a helpful reference regarding the affinity of these antibodies, thus permitting a quick assessment of and comparison with the parent antibodies to determine whether correct folding of the light and heavy chains had been achieved. Finally, the published molecular modeling data describing the antigen binding sites of these antibodies (Stanker et al., 1995) will be beneficial when mutagenesis is considered for future study.

To prepare heavy and light chain genes, mRNA isolated from DD1 and DD3 hybridoma cell lines was used to synthesize first-strand cDNA using a random primer. The heavy and light chain genes were amplified by PCR using primers complimentary to the 5′-end of framework 1 and the 3′-end of constant regions. The agarose gel electrophoretic analyses of the heavy and light chain gene PCR products are shown in Figure 2. Each primer contained a specific restriction enzyme site for directional cloning. For the light chain gene cloning, the primers were designed to contain sequences complimentary to *Sac*I and *Nsi*I. Our initial attempts to use *Nco*I for directional cloning of heavy chain genes were unsuccessful. We found two *Nco*I sites present in the variable region of the DD3 heavy chain and also in the constant regions of both heavy chains. The frequency of occurrence of *Nco*I sites in mouse heavy and light chain variable regions is reported to be 6.2 and 5.6%, respectively, and occurrence of *Sfi*I sites has not been reported for mouse heavy and light chain variable regions (Ge et al., 1995). Thus, both DD1 and DD3 heavy chain genes were initially amplified with primers containing *Nco*I and *Not*I and then reamplified using primers containing *Sfi*I and *Not*I. Light chain genes were cloned into pFabUSDAI, and the vectors were used to transform *E. coli* Nova Blue cells. Positive clones were determined by isolating plasmid DNA followed by restriction enzyme digestion and electrophoresis of the digested products. One clone containing the DD1 light chain gene and one containing the DD3 light chain gene were selected for cloning the heavy chain genes. The final clone carrying the DD1 light and heavy chains is designated pRFAB1-1, and the clone carrying the DD3 light and heavy chains is designated pRFAB3-3. The PCR-amplified antibody gene sequences in these clones were identical to the published DNA sequences, indicating that no errors were introduced during PCR amplification.

Recombinant Fab antibodies were initially expressed by inducing the transformed BL21(DE3)pLysS strain with 1 mM IPTG. The induction was performed in 50 mL batches for 3 h or overnight at 30 °C with continuous shaking. The lower temperature during induction is an essential factor for the expression of soluble recombinant antibodies, because it allows expression and transport of expressed proteins into the periplasmic space to occur at a slower rate (Schein and Noteborn, 1988; Takagi et al., 1988). Overexpression of recombinant antibodies can result in the formation of insoluble aggregates. Also, it has been noted that an antibody is more likely to fold correctly at a lower temperature. Cosecretion of both light and heavy chains into the periplasmic space was facilitated by the use of the *pelB* signal sequence placed immediately upstream of each cloning site in the expression vector. Secretion of the rFabs into the bacterial periplasm is preferred, because the periplasm contains disulfide isomerase. This enzyme enhances the formation and rearrangement of disulfide bonds (Bardwell et al., 1991) and assists in the correct folding of proteins. Antibody domains expressed in a nonoxidative environment such as the cytoplasm require in vitro refolding to form functional recombinant antibodies. Secretion of both light and heavy chains into the periplasmic space has an added advantage of eliminating in vitro refolding of the expressed recombinant domains.

Detection of rFab1-1 and rFAB3-3 was performed using both indirect ELISA and immunoblotting experiments. The ELISA results indicated that both the culture supernatant and periplasmic extract contained recombinant Fab fragments. rFab1-1 samples showed strong antigen binding as determined from the color development, whereas rFab3-3 samples showed weak binding. To obtain sufficient quantities for the competitive inhibition study, periplasmic extract was initially purified by Talon metal affinity chromatography. A larger quantity of both culture supernatant and periplasmic extract was purified by a protein G−Sepharose affinity chromatography. Protein concentrations of 5.9 and 0.04 μg/mL of induced cell culture were recovered from the protein G purification for rFab1-1 and rFab3-3, respectively. Since unpaired heavy chain could be present in these solutions, these concentration values

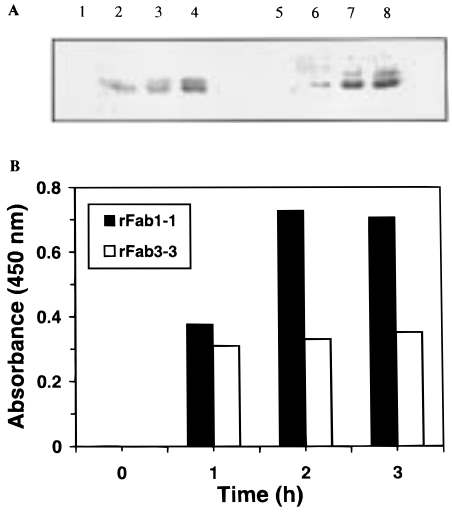

Figure 3. (A) Immunoblot of reducing gel electrophoresis of recombinant Fab fragments in a time course study. Lane 1 is the rFab1-1 expression at 0 h, lane 2 is the rFab1-1 expression at 1 h, lane 3 is the rFab1-1 expression at 2 h, and lane 4 is the rFab1-1 expression at 3 h. Similarly, the rFab3-3 expression at 0, 1, 2, and 3 h is shown in lanes 5, 6, 7, and 8, respectively. The bands shown are approximately at 25 kDa. (B) Indirect ELISA data showing an increase in color development (A_{280}) with induction time (in h).

should be regarded as relative concentrations for the rFab1-1 and rFab3-3 expressions. The amount of the rFab3-3 antibody (4 μg/well) required to produce a similar color development in an ELISA was 100 times higher than that of rFab1-1 (0.04 μg/well). The ELISA titration data indicated that the amount of recombinant Fab antibodies produced by BL21(DE3) strain was similar to that produced by the pLysS strain. No antigen binding was observed for cell supernatant samples, which indicates BL21(DE3) was less prone to cell lysis during induction.

To determine the optimum time to harvest, a time course study was undertaken and the results are summarized in Figure 3. An increase in the rFab concentration with time was observed in both indirect ELISA and the immunoblot. From the band intensity of the immunoblot, it was obvious that the light chain was expressed in a larger quantity than the heavy chain. This observation was more prominent in the first 2 h, and expression appeared to reach optimum after 3 h of induction (i.e., the intensities of the ~25 kDa bands were similar for 3 h and overnight incubations). Stoichiometric expression of heavy and light chains is important for the formation of functional recombinant Fab antibody. The pFabUSDAI vector has been constructed to arrange heavy and light chain genes as a dicistronic operon controlled under one T7*lac* promoter. One explanation for the greater quantity of the light chain than that of the heavy chain would be that transcription by the T7*lac* promoter may be more efficient for the light chain gene, because it was cloned proximal to the T7*lac* promoter.

Comparison of rFabs to Parent Antibodies and Fab Fragments Derived from the Parent Molecules for Assay Sensitivity. Initial detection of rFab was performed using an indirect immunoassay with triCDD-A-RSA as a coating antigen. To detect the expression of rFab fused to a polyhistidine tag, an anti-polyhistidine antibody was incubated prior to the labeled antibody for color development. The same format

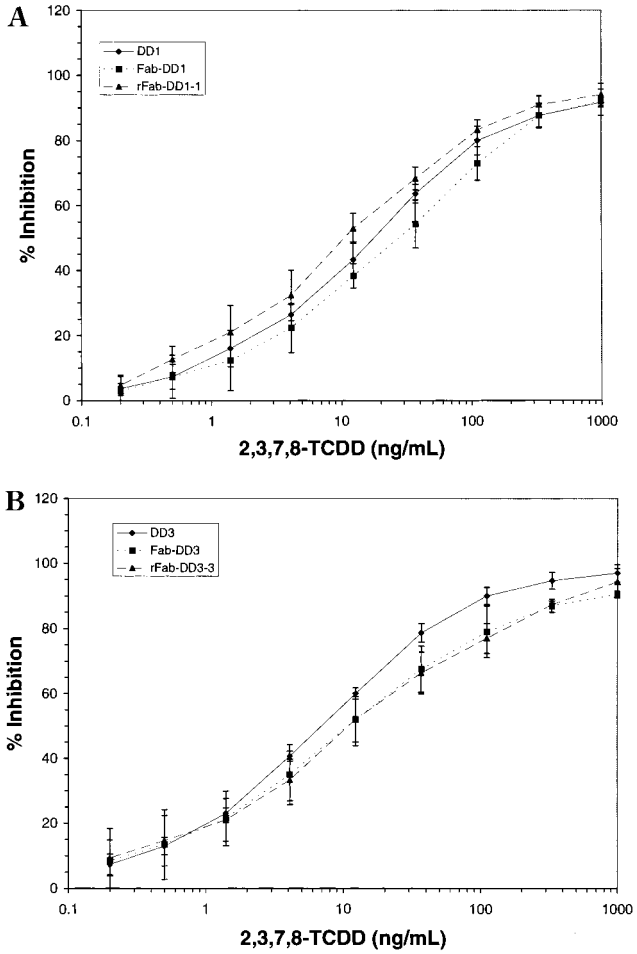

Figure 4. (A) Standard curves for 2,3,7,8-TCDD using DD1 monoclonal antibody, DD1 enzymatically derived Fab, and recombinant antibody rFab1-1. (B) Standard curves for 2,3,7,8-TCDD using DD3 monoclonal antibody, DD3 enzymatically derived Fab, and recombinant antibody rFab3-3.

was employed in the initial development of competition immunoassays using rFab antibodies. Optimum conditions were determined initially by titration of both coating antigen and antibodies in a homologous system to give color development of ~1.0 absorbance unit. A comparison was made between detection of the polyhistidine tag (i.e., with anti-polyhistidine antibody) and detection of the rFab antibody (i.e., without anti-polyhistidine antibody) in the competitive ELISA. A ~3-fold improvement was achieved by use of direct detection of rFab fragments. Because our coating antigen was >10 years old, all antibodies were purified and assayed concurrently to ensure a direct comparison of the parent, enzymatically derived Fab, and rFab antibodies. Standard curves were performed at least three times to provide sufficient reproducibility, and the ELISA data shown in this paper were calculated using three sets of data obtained on three different days. The standard curves using monoclonal antibody, enzymatically derived Fab, and recombinant Fab are shown in partsA (for DD1) and B (for DD3) of Figure 4. The IC_{50} using the DD1 monoclonal antibody was 16.3 $\pm$ 4.2 ng/mL, which was comparable to the published data (Stanker et al., 1987). The standard curves for monoclonal antibody, enzymatically derived Fab, and rFab antibodies derived from DD1 were within the standard deviation of each other, indicating they exhibit a similar sensitivity for the competitor. The IC_{50} values for

Table 1. Cross-Reactivity of MAb, Enzymatically Derived Fab, and Recombinant Fab Fragments for Dioxin, Biphenyl, and Other Related Compounds

	cross-reaction (%)											
	DD1		Fab-DD1		rFab1-1		DD3		Fab-DD3		rFab3-3	
compound	IC_{50}[a]	CR[b]	IC_{50}[a]	CR[b]	IC_{50}[a]	CR[b]	IC_{50}[a]	CR[b]	IC_{50}[a]	CR[b]	IC_{50}[a]	CR[b]
dioxin												
2,3,7,8-TCDD	0.015	100	0.025	100	0.01	100	0.007	100	0.01	100	0.01	100
dibenzodioxin	ni[c]		ni[c]		ni[c]		ni[c]		ni[c]		ni[c]	
PCB												
3,3′,4,4′-TCBP	ni[d]		ni[d]		ni[d]		ni[d]		ni[d]		ni[d]	
other compounds												
2,4,5-trichlorophenol	510	0.003	250	0.01	300	0.003	ni[e]		ni[e]		>1000	<0.001
2,5-dichloronitrobenzene	90	0.02	75	0.03	42	0.02	ni[e]		ni[e]		>1000	<0.001
hexachlorocyclohexane (γ-isomer)	ni[e]		ni[e]		ni[e]		ni[e]		ni[e]		ni[e]	
hexachlorocyclohexane (δ-isomer)	41	0.04	28	0.09	10	0.1	ni[e]		ni[e]		>1000	<0.001

[a] IC_{50} values shown here are in microgram per milliliter. [b] CR (in percent) determined as IC_{50} (2,3,7,8-TCDD)/IC_{50} (test compound) × 100. [c] ni, no inhibition at 10 ppm. [d] ni, no inhibition at 50 ppm. [e] ni, no inhibition at 1000 ppm.

enzymatically derived Fab and rFab antibodies for DD1 were 28.7 ± 12.5 and 10.4 ± 2.4 ng/mL, respectively. For DD3 monoclonal antibody, the IC_{50} (6.8 ± 0.3 ng/mL of 2,3,7,8-TCDD) was ~3-fold less than the data reported previously (25 ng/mL; Stanker et al., 1987). It is probable that the sensitivity of DD3 is more likely to be affected by the amount of coating antigen because it is more specific to dioxin (see below), and the use of less coating antigen in this assay may have improved the sensitivity of this antibody. The standard curves of enzymatically derived Fab and rFab derived from DD3 were superimposable (IC_{50} = 14.2 ± 6.9 and 12.2 ± 6.0 ng/mL, respectively), showing both Fab fragments exhibit similar sensitivities.

Specificity of the Immunoassay and Comparison of Parent Antibodies, Enzymatically Derived Fab, and Recombinant Fab Antibodies. The cross-reactivity of rFab antibodies was examined with the corresponding monoclonal antibodies and enzymatically derived Fab fragments. A limited number of reactive compounds were selected for the cross-reactivity study. These include 3,3′,4,4′-tetrachlorobiphenyl, 2,4,5-trichlorophenol, 2,5-dichloronitrobenzene, and two isomers of hexachlorohexane (γ- and δ-isomers). The IC_{50} values and the percent cross-reactivity relative to 2,3,7,8-TCDD are listed in Table 1. Dibenzodioxin, a nonchlorinated dioxin, was also tested to determine whether these antibodies were specific to chlorinated compounds. As would be expected, no cross-reactivity was observed with dibenzodioxin, whereas cross-reactivity was observed with chlorinated compounds. Of two hexachlorohexane isomers tested, only the noninsecticidal δ-isomer showed displacement of antibody binding. Interestingly, although the cross-reactivity patterns of monoclonal antibodies for the related compounds were similar to the published data (Stanker et al, 1987), the IC_{50} values were 1−2 orders of magnitude lower, probably due to the aging antigen. The cross-reactivity patterns of the enzymatically derived Fab and rFab antibodies were comparable to their respective monoclonal antibodies. rFab3-3 showed slight cross-reactivity with 2,5-dichloronitrobenzene, 2,4,5-trichlorophenol, and the δ-isomer of hexachlorocyclohexane, but only at high parts per million levels (<0.001% cross-reactivity relative to 2,3,7,8-TCDD). From the consistency of the ELISA data obtained using 10% methanol solution, it would be reasonable to conclude that both enzymatically derived Fabs and rFabs are relatively solvent tolerant, a property required for analysis of hydrophobic compounds such as dioxin. Therefore, these rFabs can be used in the environmental analysis of dioxin given that the methanol concentration in the final sample preparation does not exceed 10%.

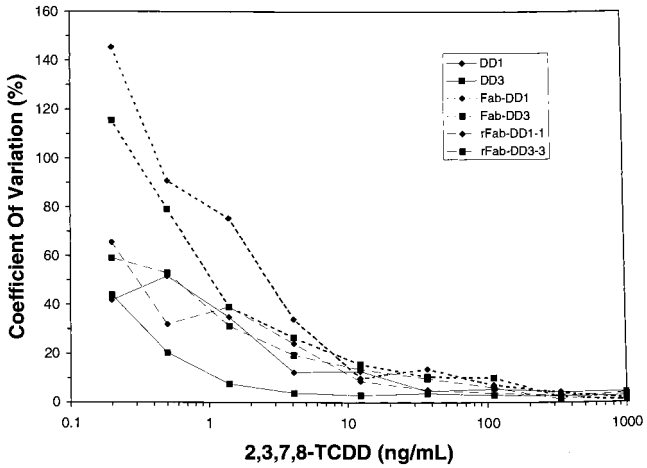

Figure 5. Coefficients of variation of MAb, enzymatically derived antibodies, and recombinant antibodies calculated from the standard curves using 2,3,7,8-TCDD as a competitor.

Precision of Immunoassays. The interassay precision was determined from three standard curves run on three different days. As would be expected from a typical immunoassay, the coefficient of variation (based on the percent inhibition) was lowest at high competitor concentrations and highest at low concentrations. This is shown in Figure 5. Of the three types of antibodies, the enzymatically derived Fab antibodies gave the greatest variation. The high imprecision of the enzymatically derived Fab antibody probably is due to either loss of antibody activity during the enzyme digestion or variation in the purity. The monoclonal antibodies and rFabs were comparable in their coefficient of variation and did not exceed 70%. These results suggest that rFab antibodies would be useful as analytical reagents.

CONCLUSION

We have expressed two functional recombinant antibodies for dioxin in *E. coli* strains, BL21(DE3) and BL21(DE3)pLysS, by cloning the heavy and light chain genes in the pFabUSDAI vector. Because the DNA sequences for both heavy and light chain genes were

known, PCR primers specific to dioxin antibody genes were easily designed, thereby decreasing the chance that undesirable transcripts from the splenocyte fusion partner would be amplified. Also, the sequences of the PCR-amplified genes were easily compared with DNA sequences obtained from the cDNA to determine if the PCR procedure has introduced errors in the sequences. The PCR-amplified DNA sequences of the heavy and light chains from both DD1 and DD3 were identical to those previously reported. Recombinant Fab antibodies were expressed in low micrograms per milliliter quantities, similar to that obtained in hybridoma cell culture. Like other researchers, we also found that even using the same vector, the quantity of functional recombinant antibodies expressed in the *E. coli* system can vary drastically. Thus, each recombinant antibody requires individual optimization for maximum expression, and other methods including in vitro refolding schemes may be required to obtain functional rFabs.

Unlike the rFab antibodies for diuron described by Karu et al. (1994) and Scholthof et al. (1997), the properties of our rFab antibodies were similar to those of Fab fragments obtained from papain digestion. Similar results were observed by Kramer and Hock (1996) with the scFv for *s*-triazine, by Webb et al. (1997) with the scFv for cyclohexanedione, and by Garrett et al. (1997) with the scFv for parathion. The antigen binding sites [calculated as buried surface area, $(Å)^2$] for small molecules such as agrochemicals are narrower than those for larger molecules such as lysozyme (Searle et al., 1995). Therefore, the small change in the folding pattern between heavy and light chains would significantly influence the sensitivity or the specificity of the recombinant antibody. For example, the antibody combining site of DD3 exhibits a cleftlike pocket, whereas the antibody combining site of DD1 antibody is more the shape of a bowl (Stanker et al., 1995). This explains the more stringent cross-reactivity pattern for dioxin congeners observed with DD3 monoclonal antibody. The slight cross-reactivity for a number of dioxin-related compounds observed with rFab3-3, but not with the parent or enzymatically derived Fab antibodies, was probably due to the heavy and light chains being less tightly bound to each other.

Many immunoassays for major agrochemicals have been developed, which have proven to be very useful as analytical tools and screening methods. Some exhibit high sensitivity and the desired specificity, whereas others require much improvement, mainly due to the difficulties in synthesizing the "correct" hapten. The recombinant antibody technology will serve as an alternative to hapten synthesis for improving the affinity of an existing antibody or for developing new ones without undergoing conventional procedures. We have described here the initial stage of this process, cloning, and expressing recombinant Fab fragment of the existing antibodies. Further studies to alter and improve the affinity of these rFabs by chain shuffling and mutagenesis will be undertaken to provide a clearer understanding of the nature of dioxin binding by these antibodies.

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