

Derivation and Properties of Recombinant Fab Antibodies to Coplanar Polychlorinated Biphenyls

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Recombinant Fab antibodies (rFabs) specific for coplanar polychlorinated biphenyls (PCBs) were derived from a hybridoma cell line (Chiu et al. *Anal. Chem.* **1995**, *67*, 3829–3839). Immunoglobulin V_H-C_{H1} and V_L-C_L sequences from S2B1 messenger RNA were amplified by PCR and cloned into the M13 phagemid vector pComb3H. Phage displaying rFab were enriched by panning on a PCB hapten conjugate and expressed as soluble rFabs in *Escherichia coli* XL-1 Blue. Two rFab clones competitively bound PCBs 77 and 126 with half-maximal inhibition (*I*₅₀) of 10–13 ppb in indirect and direct enzyme immunoassays (EIAs), with selectivity nearly identical to that of whole S2B1 IgG and its Fab fragments prepared by papain digestion. These results, and comparison of N-terminal amino acid sequences of MAb S2B1 and the rFab, indicated that rFab S2B1 is a functional copy of the MAb. The rFab S2B1 sequences have 75–89% sequence identity with antibodies that bind nitrophenyl haptens and are being used to construct a three-dimensional computational model of the PCB binding site.

Keywords: *Recombinant Fab antibody; PCB; polychlorinated biphenyl; congener-specific; ELISA*

INTRODUCTION

Polychlorinated biphenyls (PCBs) are man-made compounds of worldwide concern because of their broad range of toxic effects, wide distribution, persistence, and ability to bioaccumulate (Dobson and van Esch, 1993; Research Triangle Institute, 1997; WHO, 1975). Industrial PCB formulations were a type of combinatorial chemistry. The structures of all 209 congeners are similar (C₁₂Cl_{*n*}H_{10–*n*}) (Ballschmitter and Zell, 1980), and the Aroclors and other commercial products that polluted the environment were mixtures consisting of different mole fractions of numerous congeners (U.S. Department of Health and Human Services, 1993).

Over the past 30 years, PCBs have been measured with increasingly sophisticated and sensitive instrumental and molecular biological methods (Diaz-Ferrero et al., 1997; Erickson, 1997). Analysis of individual congeners or groups of congeners has been a long-sought goal that would greatly advance toxicological and epidemiological studies, as well as risk assessment and regulatory monitoring. Congeners have been categorized by abundance, bioavailability, and toxicological modes of action, on the basis of existing data (Creaser et al., 1992; Hansen, 1998; Jones, 1988; McFarland and Clarke, 1989; Mes et al., 1993; Risby et al., 1990; Wolff et al., 1997). However, congener-specific instrumental analysis remains difficult and expensive. Some *ortho*-chlorinated and non-*ortho*-chlorinated species coelute in gas chromatography (GC) (Draper, 1990). Carbon columns and similar methods to resolve the non-*ortho*-chlorinated PCBs are time- and labor-intensive (Erickson, 1997). No presently available single GC column can

separate all 209 congeners. Procedures involving at least two types of columns and advanced methods such as high-resolution or multidimensional gas chromatography (HRGC or MDGC) are required (Font, 1996).

The complexity and costs of instrumental PCB analysis prompted development of faster, less expensive, field-portable techniques, such as immunoassays (Diaz-Ferrero et al., 1997) and immunosensors (Zhao et al., 1995). Several commercially available PCB immunoassay kits have been validated by the EPA for field use on extracts from soil or nonaqueous liquids, in accordance with EPA method 4020 (U.S. EPA OSWER, 1996). All of these kits detect the most abundant, noncoplanar PCBs present in the commercial Aroclors (Karu et al., 1998). The kits are useful and cost-effective for environmental problem-solving and decision-making, but they cannot be used for congener-specific toxicological studies. Prototypes of more advanced multianalyte detection systems have been reported. These will require panels of different antibodies and new ways to interpret the cross-reactivity patterns (Ekins et al., 1990a,b; Healey et al., 1997; Jones et al., 1994; Walt et al., 1995; Wortberg et al., 1995).

The hydrophobicity and molecular symmetry of PCBs pose serious difficulties for antibody-based analysis. We previously derived a monoclonal antibody (MAb), S2B1, that was very specific for the coplanar congeners (Chiu et al., 1995). However, the mono- and di-*ortho*-chlorinated congeners are so similar and the practical options for hapten synthesis are so limited (Carlson, 1995) that it may not be possible to derive additional antibodies with the desired selectivities by conventional immunization and hybridoma technology. We undertook the present study to define the architecture and bonding interactions of the S2B1 binding site and to determine whether that information may be used to create new PCB recognition properties by genetic engineering.

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MATERIALS AND METHODS

Reagent and Culture Media. All chemicals and solvents were of molecular biology grade or Spectrograde and were purchased from Aldrich Chemical Co. (Milwaukee, WI), Fisher Scientific (Pittsburgh, PA), or Sigma Chemical Co. (St. Louis, MO). Water was purified in a Milli-Q apparatus (Millipore Corp., Bedford, MA). Alkaline phosphatase-conjugated, affinity-purified goat anti-mouse IgG was from Boehringer-Mannheim Corp., Indianapolis, IN. The casein-based blocking agent I-Block was from Tropix, Inc. (Bedford, MA). PCB congeners of >99% purity and other reference standards were purchased from AccuStandard, Inc. (New Haven, CT), and prepared and stored as described previously (Chiu et al., 1995). Ingredients for bacterial culture media and agar plates were from Difco Laboratories (Detroit, MI). Luria-Bertani (LB) and SOC media and super broth (SB) were prepared as described (Barbas and Burton, 1994; Maniatis et al., 1989). All methods for bacterial selection by tetracycline, carbenicillin, and kanamycin resistance were as specified by Barbas and Burton (1994).

Safety Precautions. Hapten syntheses were done in a chemical fume hood with charcoal filters. All synthetic byproducts and wastes and solutions of PCB analytes and haptens from assays were disposed of as hazardous materials. All experiments using PCBs and related analytes were conducted in a chemical fume hood lined with disposable Kimpack paper. Goggles, spill-resistant gowns, and double-PCB-impermeable nitrile gloves were worn. EIA washing steps that contained PCBs were done in a stainless steel pan. Solutions in microplates were aspirated into a glass waste container using a vacuum manifold (Nunc Immunowash 12). All liquid and solid wastes were collected and disposed of by the Office of Environmental Health and Safety at the University of California, Berkeley.

Molecular Cloning Reagents. DNA restriction enzymes and buffers were purchased from New England Biolabs, Inc. (Beverly, MA). T4 DNA ligase and isopropyl β -D-thiogalactoside (IPTG) were from GIBCO BRL (Grand Island, NY). The pComb3H cloning vector was provided by Dr. Carlos Barbas, The Scripps Research Institute, La Jolla, CA. Phagemid DNA was prepared with a Qiagen Plasmid Purification Kit (Qiagen, Inc., Santa Clarita, CA). DNA fragments for library construction were resolved by electrophoresis in a low melting point agarose gel (BRL Life Technologies, Inc., Gaithersburg, MD) and recovered by digesting the agarose with β -agarase I (New England Biolabs) according to the manufacturer's instructions. DNA and RNA concentrations were estimated by UV spectrophotometry or by ethidium bromide fluorescence in agarose gel as described by Maniatis et al. (1989).

Antibody Purification. Monoclonal IgG was affinity purified on protein A-Sepharose as described by Harlow and Lane (1988). Papain-digested Fab fragments were prepared from purified S2B1 IgG using an Immunopure Fab preparation kit (Pierce Chemical Co., Rockford, IL).

Synthesis of PCB Haptens. The haptens used for this study that mimic full-sized PCBs are shown in Figure 1. 3,3',4'-Trichloro-2-hydroxybiphenyl (**2a**), 3,3',4'-trichloro-4-hydroxybiphenyl (**2b**), 3',4,4'-trichloro-3-hydroxybiphenyl (**2c**), and 2,3',4'-trichloro-3-hydroxybiphenyl (**2d**) were synthesized as reported previously (Chiu et al., 1995), with the following modifications. 3,4-Dichloroaniline (9 g) was dissolved in 60 mL of 2-chloroanisole at 40 °C. Isoamyl nitrite was added dropwise to the stirred solution under argon with vigorous bubbling. The solution was heated at 150 °C for 2.5 h. Excess anisole was distilled off under reduced pressure, and the residue was purified by silica gel flash chromatography (hexane/methylene chloride 19:1). Two fractions were collected: the first contained mostly isomer **1a** (~600 mg), and the second fraction (3.1 g) consisted of **1b**, **1c**, and **1d**. Solvent was evaporated, and the residue of fraction 2 was dissolved in dry methylene chloride under argon and cooled to -78 °C; 5 mL of 1 M BBr₃ in methylene chloride was added slowly. The mixture was warmed and stirred overnight at room temperature. The reaction was then quenched with 15 mL of saturated KH₂PO₄.

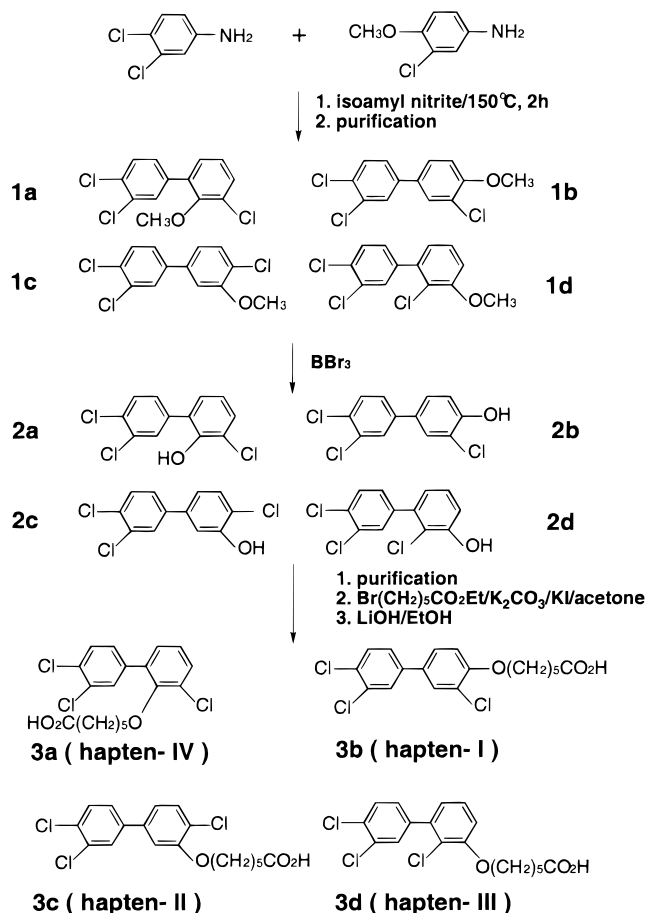


Figure 1. Hapten structures and synthesis.

The methylene chloride layer was separated from the aqueous layer and dried over anhydrous sodium sulfate. Solvent was evaporated, and the crude product was purified by silica gel flash chromatography (methylene chloride/hexane 1:9). Two fractions were obtained: the first contained roughly equal amounts of **2c** and **2d** (1.3 g), and the second was pure biphenylol **2b** (166 mg). Products **2c** and **2d** (0.6 g each) were purified by silica gel flash chromatography (triethylamine/methylene chloride/hexane, 1:1:8).

Ethyl 6-bromohexanoate (149 mg) and **2a**, **2b**, **2c**, or **2d** (166 mg) were dissolved in 40 mL of acetone, and anhydrous potassium carbonate (110 mg) and potassium iodide (10 mg) were added. The solution was refluxed for 18 h and then filtered and evaporated to dryness to yield a crude residue. To this residue were added 12 mL of absolute ethanol and 2.5 mL of 1 N LiOH, and the mixture was stirred at room temperature overnight. The solution was acidified with 3 mL of 1 N HCl to yield 6-[(3,3',4'-trichlorobiphenyl-4-yl)oxy]hexanoic acid (**3b**, hapten I), a white solid (99 mg), and the other corresponding products (haptens II-IV). ¹H NMR spectral data of this preparation of hapten I, obtained using a Nicolet NT-300 MHz instrument, were identical to the previously reported values (Chiu et al., 1995). **3a** (hapten IV): ¹H NMR (CDCl₃) δ 7.11 (t, $J = 7.8$ Hz), 7.20 (dd, $J = 2.0, 7.8$ Hz), 7.39 (dd, $J = 2.2, 8.1$ Hz), 7.40 (dd, $J = 1.8, 7.8$ Hz), 7.49 (d, $J = 8.3$ Hz), and 7.69 (d, $J = 2.0$ Hz). **3b** (hapten I): ¹H NMR (CDCl₃) δ 6.96 (d, $J = 8.5$ Hz), 7.34 (dd, $J = 2.2, 8.3$ Hz), 7.36 (dd, $J = 2.2, 8.5$ Hz), 7.48 (d, $J = 8.3$ Hz), 7.55 (d, $J = 2.4$ Hz), 7.60 (d, $J = 2.2$ Hz). **3c** (hapten II): ¹H NMR (CDCl₃) δ 7.02 (s, br), 7.05 (dd, $J = \text{br}, 7.8$ Hz), 7.36 (dd, $J = 2.2, 8.5$ Hz), 7.41 (dd, $J = 7.8$ Hz), 7.49 (d, $J = 8.3$ Hz), 7.61 (d, $J = 2.2$ Hz). **3d** (hapten III): ¹H NMR (CDCl₃) δ 6.89 (t, $J = 7.8$ Hz), 6.96 (d, $J = 8.3$ Hz), 7.23 (d, $J = 7.8$ Hz), 7.28 (dd, $J = 2.0, 8.0$ Hz), 7.49 (d, $J = 8.3$ Hz), and 7.51 (d, $J = 2.2$ Hz). The competitor hapten 4-(3,4-dichlorobenzoyl)butyric acid (3,4-keto) (Chiu et al., 1995) was prepared by the Friedel-Crafts

acylation of *o*-dichlorobenzene with glutaric anhydride (Rosowsky et al., 1971). The structure was verified by NMR: ^1H NMR (CDCl_3) δ 2.07 (2H, tt, $J = 7.1$ Hz), 2.51 (2H, t, $J = 7.1$ Hz), 3.04 (2H, t, $J = 7.1$ Hz), 7.55 (1H, d, $J = 8.4$ Hz), 7.79 (1H, dd, $J = 2.0, 8.4$ Hz), and 8.04 (1H, d, $J = 2.0$ Hz).

Hapten Conjugates. Conjugates of haptens I–IV and 3,4-keto were activated and coupled to bovine serum albumin (BSA) and cytochrome C in two steps as previously described (Chiu et al., 1995; Karu et al., 1994), with the following modifications. Hapten (0.01 mmol), in 20 μL of dimethylformamide (DMF), was activated by adding 0.01 mmol (2.17 mg) of *N*-hydroxysulfosuccinimide in 13 μL of 50% DMF and 0.01 mmol (1.9 mg) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide in 13 μL of 50% DMF. The mixture was shaken gently at room temperature for 3.5 h and then centrifuged to remove urea precipitate. The resulting active esters were stored at -80°C .

Five micromoles of active ester (a 20-fold molar excess over carrier protein) was added to 1.7 mL of borate buffer ($\text{H}_3\text{BO}_3/\text{Na}_2\text{B}_4\text{O}_7$, 0.1 M, pH 9.0) containing BSA (17 mg, 0.25 μmol) or cytochrome C (31 mg, 0.25 μmol), stirred on a roller overnight at 4°C , and then dialyzed overnight against PBS (three changes of 700 mL). The final concentrations of all conjugates were ~ 10 mg/mL, estimated by using bicinchoninic acid (BCA) protein assay (Smith et al., 1985) (Pierce Chemical Co.). A horseradish peroxidase (HRP) conjugate of the 3,4-keto hapten was made essentially the same way. The HRP (1.4 mg, EIA grade, Boehringer-Mannheim Corp.) was reacted with ethylenediamine to introduce additional free amino groups, as described by Hsiao and Royer (1979). The 3,4-keto active ester (1 μmol , 5 μL) was added to the modified HRP in 1 mL of 0.1 M Bicine–NaOH (pH 8.5), reacted overnight at 4°C , and dialyzed against PBS as described above.

Preparation of Antigen-Coated Magnetic Beads for Phage Panning. Underivatized and hapten-conjugated BSA and cytochrome C were covalently attached to long-chain alkylamine-derivatized paramagnetic porous glass beads (5 μm bead diameter, 50 nm pore diameter; product MLCA0502, CPG Inc., Lincoln Park, NJ) in two steps as recommended by the manufacturer, with the following modifications. The coupling buffer was 0.01 M boric acid/sodium tetraborate (pH 7.6), instead of 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.5). Wash buffer was coupling buffer containing 1 M NaCl, and storage buffer consisted of coupling buffer containing 150 mM NaCl, 0.1% BSA, and 0.02% NaN_3 . Hapten-specific binding of S2B1 MAb and proteolytic Fab was verified by EIA (Scholthof et al., 1997).

Library Construction. Messenger RNA (mRNA) was extracted and purified from 5×10^6 cells of hybridoma line S2B1 using a QuickPrep mRNA purification kit, and complementary DNA (cDNA) was synthesized from the mRNA with a First-Strand cDNA synthesis kit (both from Pharmacia Biotech Inc., Piscataway, NJ). The H sequences were amplified from the cDNA by PCR in separate reactions with 16 5' primers (H 1.5', H 2.5' to H 9.5' and MVh1Xho.1, MVh2Xho.1 to MVh7Xho.1), and 1 3' primer (MIG2b3'Spe) was used to amplify the H sequences. The L sequences were amplified using 7 5' primers (L 1.5', L 2.5' to L 7.5'), and 1 3' primer (Kappa L3') was used for cloning the L sequences (Table 1). The L PCR products were digested with *SacI* and *XbaI*, and the H products were cut with *XhoI* and *SpeI* and then resolved by agarose gel electrophoresis. Only six of the H-sequence 5' primers [H 1.5', 5.5', 6.5', 7.5' (Kang et al., 1991) and MVh3XhoI and MVh4&8XhoI (Bell et al., 2000)] and five of the L-sequence 5' primers (L 3.5', 4.5', 5.5', 6.5' and 7.5') gave products of the expected size (~ 680 bp). These were recovered by digesting the gel with β -agarase I (Lamb et al., 1993).

The pComb3H vector was cut with *SacI* and *XbaI*, electrophoretically purified to remove the stuffer fragment, and a mixture containing equal amounts of the five L PCR products was ligated in as described by Bell et al. (2000), except that the ligation reaction was run at 14 – 16°C .

The construct was introduced into *XL-1* blue cells by electroporation (Hanahan, 1985). Transformed cells were selected by carbenicillin resistance. The pComb3H DNA containing L sequences was electrophoretically purified, cut

with *XhoI* and *SpeI* to remove the H stuffer, and electrophoretically purified again. The six preparations of H segments were ligated into this DNA in separate reactions, using the amounts, concentrations, and conditions described by Barbas and Burton (1994). *XL-1* Blue transformants were selected, and restriction digests of phagemid DNA from 24 colonies were analyzed to verify that the vector contained H as well as L inserts. A 250 mL culture of the transformed cells was infected with VCSM13 helper phage (Stratagene, La Jolla, CA). Phage displaying rFab were harvested, concentrated by precipitation with PEG and NaCl (Barbas and Lerner, 1991), and resuspended in 2 mL of PBS containing 0.2% I-block.

Panning of Hapten-Binding Phage. Phage selection on magnetic beads was done essentially as described previously (Scholthof et al., 1997), with the following modifications. To remove phage that might bind to the carrier proteins, aliquots (1 mL) of phage suspension ($\sim 3.45 \times 10^{14}$ cfu/mL) were first incubated for 30 min with 0.3 mL of a 1:1 mixture of beads coated with underivatized BSA or underivatized cytochrome C. The supernate, containing phage that did not bind to these beads, was then transferred onto beads coated with hapten I–BSA for 2 h at room temperature. Beads were collected with a magnet and washed five times with 0.2% I-block in PBST and then three times with PBST (PBS with 0.01% Tween 20). Bound phage were eluted with two 30-min washes of 0.5 mL of 0.1 M HCl–glycine (pH 2.2), and the pH was neutralized with 60 μL of 2 M Tris base and then amplified by infecting new host cells and adding helper phage. Three rounds of amplification and panning were done, using beads coated with hapten I–BSA for the first and third rounds and beads coated with hapten I–cytochrome C for the second panning. The phage titer before and after each panning was determined by assay of carbenicillin-resistant colonies.

Preparation of Soluble rFab. After the third panning, phagemid DNA was recovered by miniprep, the gene III was excised by digestion with *SpeI* and *NheI*, and the vector DNA was electrophoretically purified, re-ligated, and transformed into *XL-1* Blue cells (Barbas and Burton, 1994; Scholthof et al., 1997). Eighty-eight $\text{tet}^r\text{carb}^r$ colonies were transferred into wells of a flat-bottom 96-well cell culture plate (Costar) containing 100 μL of SB medium with 50 $\mu\text{g}/\text{mL}$ carb, 20 $\mu\text{g}/\text{mL}$ tet, and 20 mM MgCl_2 . The plate was shaken for 6 h at 37°C , and then aliquots (25 μL) were transferred into 1 mL tubes (Bio-Rad, Hercules, CA) containing 0.6 mL of the same medium. After an additional 4 h at 37°C , IPTG was added to 0.1 mM final concentration to induce rFab expression (Bell et al., 2000). The tubes were shaken overnight at 30°C and then centrifuged (1000g, 10 min, in an IEC PR-6000 with microplate carrier) to collect the cells. The medium was removed, the cells were resuspended in 0.2 mL of 20% sucrose/0.05 M Tris–HCl (pH 8.0), and periplasmic lysates containing soluble rFab were prepared (Karu and Belk, 1982).

Direct and Indirect EIA Methods. Procedures and reagents for EIAs were those reported by Chiu et al. (1995), unless otherwise indicated. An indirect EIA was first used to identify positive lysates from the 88 rFab clones. Indirect competition EIAs were performed as before except that the diluent for all analytes was aqueous 10% methanol with 0.005% Tween 20, and dilutions of MAb, Fab, and rFab lysates (previously determined to be optimal by checkerboard EIA) were mixed with the analytes for 2 h instead of overnight before they were added to the EIA wells. Direct competition EIAs were done in wells coated with 200 ng of affinity-purified goat anti-mouse Fab-specific IgG as capture antibody, and 3,4-keto–HRP (1:24000, 100 μL per well) was used as competitor. Both indirect and direct competition EIAs were used to identify rFabs that competitively bound various PCBs, but only the indirect competition EIA was used to test recognition of other organochlorine compounds.

BstNI Restriction Profiling. Phagemid DNA minipreps from EIA-positive clones were purified with a Qiagen plasmid purification kit, digested with *BstNI*, a frequent-cutting restriction enzyme (Marks et al., 1991). Fragments were resolved by electrophoresis on 2.5% agarose gels in TBE buffer (45 mM Tris–borate/1 mM EDTA), and the patterns were compared.

Table 1. PCR Primers Used for Murine Immunoglobulin (IgG_{2b}κ) Fab Region Gene Amplification^a

Name	Sequence	Priming region
Set A		
Hc 1.5'	5'-AG GTC CAG CTG <u>CTC GAG</u> TCT GG-3'	γ H-chain 5' primers ^b
Hc 2.5'	5'-AG GTC CAG CTG <u>CTC GAG</u> TCA GG-3'	variable domain aa 1-8
Hc 3.5'	5'-AG GTC CAG CTT <u>CTC GAG</u> TCT GG-3'	
Hc 4.5'	5'-AG GTC CAG CTT <u>CTC GAG</u> TCA GG-3'	
Hc 5.5'	5'-AG GTC CAA CTG <u>CTC GAG</u> TCT GG-3'	
Hc 6.5'	5'-AG GTC CAA CTG <u>CTC GAG</u> TCA GG-3'	
Hc 7.5'	5'-AG GTC CAA CTT <u>CTC GAG</u> TCT GG-3'	
Hc 8.5'	5'-AG GTC CAA CTT <u>CTC GAG</u> TCA GG-3'	
Set B		
MVh1Xho.1	5'-SAK GTG CAG <u>CTC GAG</u> SAG TCA GGA CCT-3'	γ H-chain 5' primers ^c
MVh2Xho.1	5'-GAG GTY CAG <u>CTC GAG</u> CAR TCT GGA CCT-3'	variable domain aa 1-9
MVh3Xho.1	5'-CAG GTC CAA <u>CTC GAG</u> CAG YCT GGG KCT-3'	
MVh4&8Xho.1	5'-GAG GTT CAG <u>CTC GAG</u> CAG TCT GGR GCW G-3'	
MVh5Xho.1	5'-GAR GTG AAG <u>CTC GAG</u> GAG WCT GGA SGA-3'	
MVh6Xho.1	5'-GAG GTG AAG CTT <u>CTC GAG</u> TCT GGA GGT-3'	
MVh7Xho.1	5'-GAA GTG MAG <u>CTC GAG</u> GAG TCT GGG GGA-3'	
MIg2b3'Spe	5'-GG <u>ACT AGT</u> GGG CCC GCT GGG CTC AAG TTT T-3'	γ H-chain Fd 3' primers ^d constant domain aa 232-222 (IgG2b)
κ L-chain 5' primers ^b		
Lc 1.5'	5'-CC AGT TCC <u>GAG CTC</u> GTT GTG ACT CAG GAA TCT-3'	κ L-chain 5' primers ^b
Lc 2.5'	5'-CC AGT TCC <u>GAG CTC</u> GTG TTG ACG CAG CCG CCC-3'	variable domain aa 1-7
Lc 3.5'	5'-CC AGT TCC <u>GAG CTC</u> GTG CTC ACC CAG TCT CCA-3'	
Lc 4.5'	5'-CC AGT TCC <u>GAG CTC</u> CAG ATG ACC CAG TCT CCA-3'	
Lc 5.5'	5'-CC AGA TGT <u>GAG CTC</u> GTG ATG ACC CAG ACT CCA-3'	
Lc 6.5'	5'-CC AGA TGT <u>GAG CTC</u> GTC ATG ACC CAG TCT CCA-3'	
Lc 7.5'	5'-CC AGT TCC <u>GAG CTC</u> GTG ATG ACA CAG TCT CCA-3'	
Lc 3'	5'-GCG CCG <u>TCT AGA</u> ATT AAC ACT CAT TCC TGT TGA A-3'	κ L-chain 3' primers ^b constant domain aa 209-214

^a Underscored sequences are restriction sites. ^b From Kang et al. (1991) and modified to include degeneracies from the antibody sequence database. ^c Sequences from Dr. D. Burton and modified to include major degeneracies from the antibody database by Kabat et al. (1991). Primers were designed to bind the following subclasses: MVh1Xho.1, Vh sub Ia and Ib; MVh2Xho.1, Vh sub IIa; MVh3Xho.1, Vh sub IIb; MVh4&8Xho.1, Vh sub IIc and Va; MVh5Xho.1, Vh sub IIIa and c; MVh6Xho.1, Vh sub IIIb; MVh7Xho.1, Vh sub IIIc. The IUPAC uncertainty codes are M = (A/C); R = (A/G); W = (A/T); S = (C/G); Y = (C/T); K = (G/T). ^d From Bell et al. (2000).

Protein Electrophoresis, Western Blotting, and Sequencing. Samples of EIA-positive lysates, MAb, and proteolytic Fab were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 4–12% Bis-Tris gradient gels under conditions that did not reduce disulfide bonds, using the manufacturer's reagents and instructions

(Novex, San Diego, CA). The resolved bands were electrophoretically transferred to an Immobilon membrane (Millipore, Inc.), and western blot analysis was done by standard methods (Harlow and Lane, 1988).

For amino-terminal sequence analysis, the MAb S2B1 H and L polypeptides were separated by SDS-PAGE, electrophoreti-

cally transferred from the gel to an Immobilon membrane, and visualized by Coomassie Blue staining. The H and L chain bands were cut from the membrane and sent to Dr. Carol Beach (University of Kentucky, Microchemistry Facility, Lexington, KY) for N-terminal sequence analysis.

DNA Sequencing and Analysis. The H and L sequences from both DNA strands were determined at the University of Georgia, Molecular Genetics Facility, using the dideoxy termination method (Maniatis et al., 1989). The primers were 5'-GCCGCTGGATTGTTATTAC (S2B1.Hc.XhoI.for) and 5'-ACGCTTACAATTTCCC (S2B1.Hc.NheI.rev) for H and 5'-GATTGCAGTGGCACTG (S2B1.Lc.SacI.for) and 5'-CCGTAGGC-AATAGG (S2B1.Lc.XbaI.rev) for L. The sequences were aligned and analyzed using AssemblyLIGN (Eastman Kodak, New Haven, CT) and MacVector v5.0 software (Oxford Molecular Group, Campbell, CA). The amino acids were numbered according to the Kabat numbering system using AbCheck (Martin, 1996), and the most homologous sequences were identified from the Kabat database (Kabat et al., 1991) using the program Seqhunt II [http://immuno.bme.nwu.edu/seqhunt.html (Johnson et al., 1995)]. AbCheck was also used to identify CDR loops that had canonical structures (Chothia and Lesk, 1987). The Brookhaven Protein Data Bank (PDB) was searched to identify crystallographically solved structures with the greatest homology to the H and L sequences from S2B1 (Bernstein et al., 1977).

RESULTS AND DISCUSSION

Gene Amplification and rFab Construction. Because MAb S2B1 was an IgG_{2b} κ , 3' primers for the C_{H1} and C_L consensus sequences of this subtype were used. In general, sequences at the 5' ends of different subgroups or families of V_H and κ V_L mRNA are sufficiently similar that they may be amplified by a limited number of "family-based" primers, although cross-family priming may occur (Barbas and Burton, 1994). Although several PCR primers gave products of the correct size, some did not produce hapten-binding display phage or functional soluble rFab. The two functional rFab clones we eventually isolated were designated H2 and D4. The DNA sequence at the 5' end of rFab D4 V_L was that of primer Kang's primer L 6.5', and V_L of rFab H2 matched primer L 7.5' after the SacI site (GAGCTC). The H sequences of both clones belonged to Kabat subgroup II(b). However, the 5' end of D4 V_H matched Kang's primers H 1.5', 5.5', and 7.5' after the XhoI site (CTCGAG), whereas V_H of rFab H2 matched both MVh primers after the XhoI site. This functional H chain resulted from cross-family priming by MVh4&8XhoI, which was originally designed to amplify V_H subgroups II(c) and V(a).

Figure 2 is the map of pFabS2B1. The pComb3H vector used for this work had some advantages over pComb3, from which it was derived (Barbas and Burton, 1994; Barbas and Wagner, 1995), pComb3H has an *ompA* leader for the L chain and a *pelB* leader on the H chain. This prevents recombinational loss of the L chain that occurs occasionally in pComb3, in which both leaders are *pelB* (Barbas and Wagner, 1995; Janda et al., 1997). The H and L stuffer sequences in pComb3H, 300 and 1200 bp, respectively, facilitated DNA library construction by providing accurate size markers in gel electrophoresis. The L inserts of ~680 bp were ligated into the vector DNA first (Bell et al., 2000). Nine of 10 transformants had L segments that could be excised by digestion with *SacI* and *XbaI* (data not shown). Electrophoresis on a long agarose gel separated the vector DNA that contained L chain (4.4 kb) from uncut vector that contained the L stuffer sequence (4.9 kb) and from

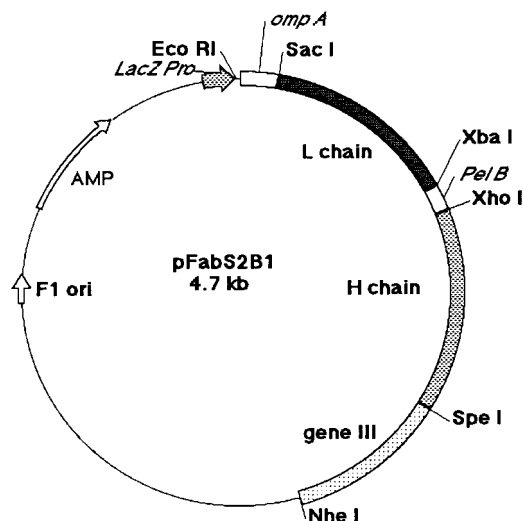


Figure 2. PFab S2B1 constructed from pComb 3H-ss. Shown are the cloning and restriction sites for H and L chains. The V_H-C_{H1} gene is fused with gene III in antibody-displaying phage for panning purpose. Excision of gene III with a simple *NheI*/*SpeI* digest allows for the expression of soluble rFabs. Lac promoter (*LacZ Pro*) is induced by IPTG for the transcription of both chains. L and H chains are targeted to the periplasm by attaching with leader peptide, *ompA* and *pelB*, respectively. The vector contains the gene (*AMP*) for the enzyme β -lactamase that confers ampicillin resistance to bacteria that harbor pComb 3H-ss DNA.

vector that had neither L stuffer nor L chain (3.7 kb). The same procedure was used to separate vector with H and L chain (4.7 kb) inserts from vector that had L chain with H stuffer (4.4 kb) or without H stuffer (4.1 kb).

The ligation conditions used to insert the L sequences did not work for inserting H sequences. The higher amounts and concentrations of DNA and the higher temperature proved to be important for ligation of the H sequences. When ligation was done as described under Materials and Methods, 15 of 24 transformants had an H sequence and 14 of these also had L inserts (data not shown).

Hapten Preferences for Phage-Displayed and Soluble rFab. To set up a phage panning strategy, indirect EIAs were run to compare the relative binding of MAb S2B1 and its proteolytic Fab fragment to the PCB haptens. Whole S2B1 IgG bound to BSA and cytochrome C conjugates of haptens I, II, and 3,4-keto (Figure 3A). The 3,4-keto hapten, which represents half of a PCB, was a weaker competitor than hapten I in competition EIAs (Chiu et al., 1995). S2B1 proteolytic Fab recognized the hapten I conjugates, but conjugates of hapten II and 3,4-keto were bound too weakly for practical purposes. Neither MAb nor proteolytic Fab bound to conjugates of haptens III and IV, which are mimics of *ortho*-chlorinated biphenyls (data not shown). Together, these results indicated that only hapten I conjugates would be useful for panning display phage, with the possibility that some of the selected rFabs may have weaker, but usable, binding to 3,4-keto hapten. As described under Materials and Methods, phage were first panned on underivatized BSA and cytochrome C and then alternately on the hapten I conjugates. The enrichment of hapten binders over three rounds of panning is shown in Table 2.

Binding Characteristics of MAb, Proteolytic Fab, and rFab. Past experience indicated that phage-

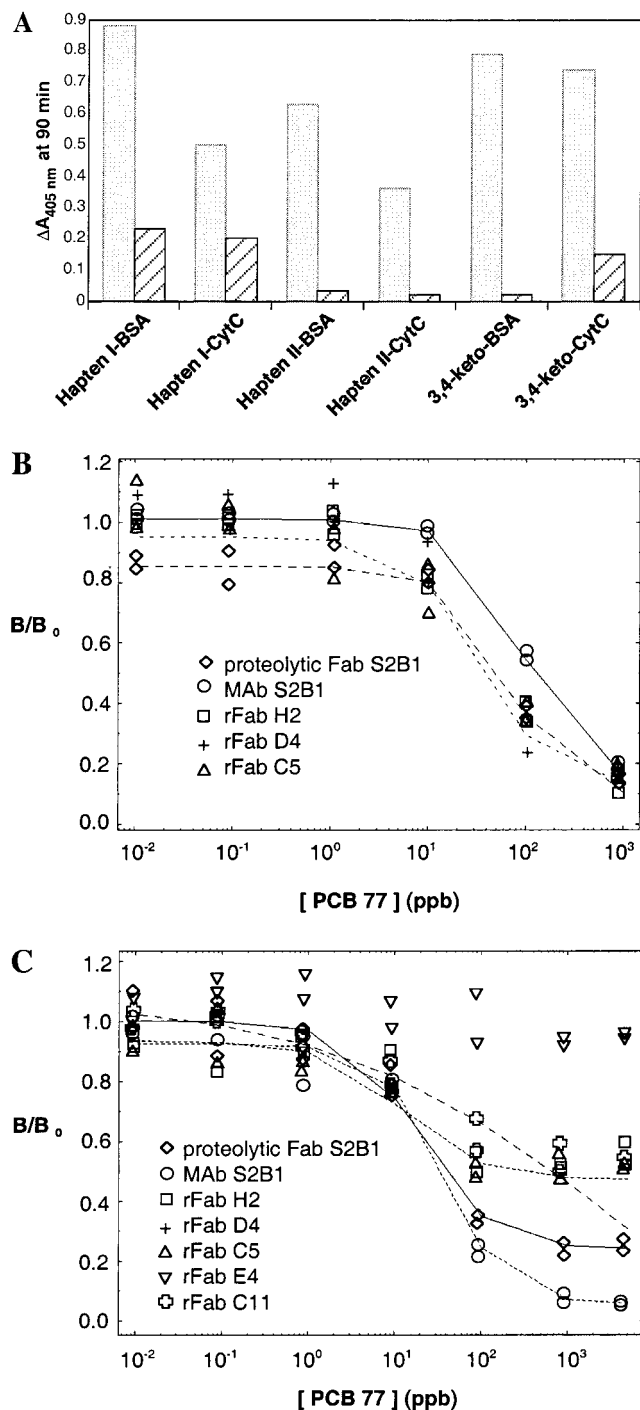


Figure 3. Hapten binding preferences. Binding of S2B1 MAb (lightly shaded bars) and proteolytic Fab (slashed bars) to PCB-hapten conjugates in an indirect EIA (A). Bars are the average of duplicates in each experiment. Relative binding of rFabs H2, D4, and C5, S2B1 MAb, and proteolytic Fab, compared in the indirect EIA (B) and direct EIA (C).

Table 2. Enrichment of Display Phage in the Three Rounds of Panning

round	panning conjugate	input (cfu/mL) ^a	output (cfu/mL)	ratio (out-put/input)
1	hapten I-BSA	3.5×10^{14}	1.4×10^6	4.1×10^{-9}
2	hapten I-cytochrome C	5.7×10^{13}	2.1×10^6	3.6×10^{-8}
3	hapten I-BSA	8.9×10^{13}	1.2×10^8	1.2×10^{-6}

^a Colony forming units per milliliter of phage.

displayed and soluble rFabs may differ in binding to some haptens (Bell et al., 2000; Scholthof et al., 1997).

In fact, of 88 phage clones recovered from the third panning, only five (designated H2, D4, C5, E4, and C11) produced soluble rFab that bound hapten I-BSA, and none detectably bound 3,4-keto-BSA coating conjugate in indirect EIAs. These five also bound 3,4-keto-HRP conjugate in the direct EIA (hapten I-HRP was not tested). However, only rFabs H2, D4, and C5 competitively bound the coplanar congeners (PCBs 35, 77, and 126) in both the indirect and direct EIA formats (Figure 3B,C, respectively). The I_{50} values were similar to those obtained with S2B1 IgG and proteolytic Fab (Table 3). Binding of rFab C5 was weaker than that of H2 and D4. Assays could be run using higher concentrations of C5 lysate, but this clone was not characterized further. In the direct competition EIA, rFab E4 did not competitively bind PCB 77 at all, and binding of rFab C11 was inhibited only ~20% by 5 ppm of PCB 77. Consequently, these two clones were not tested in the indirect competition format. Like S2B1 MAb and proteolytic Fab, rFabs H2 and D4 showed no competitive binding of mono- and di-*ortho*-chlorinated PCB congeners, 3,4,5-trichloro-4-biphenylol, 3,4,3',4'-tetrabromobiphenyl, or *p,p'*-DDT, in either EIA format. Thus, in terms of sensitivity and specificity, rFab clones H2 and D4 were functional copies of MAb S2B1.

The physical form of the rFabs was tested by western blot analysis of *E. coli* lysates resolved by SDS-PAGE under nonreducing conditions. The H2, D4, and C5 lysates and proteolytic Fab S2B1 each had a single band of ~50 kDa that bound goat anti-mouse Fab-alkaline phosphatase conjugate (Figure 4).

Comparison of MAb and rFab Sequences. As a preliminary check for DNA sequence similarity, agarose gel electrophoresis patterns of *Bst*NI restriction digests were compared. The patterns for clones H2 and D4 were identical, but the pattern for clone C5 differed. This clone was not studied further.

Figure 5 shows the DNA sequences and deduced amino acid sequences of rFab clones H2 and D4. The L chain sequences of both rFab clones differed by only two nucleotides. These differences were silent; that is, they encoded the same amino acid in both clones. The N-terminal amino acid sequence of the MAb S2B1 L polypeptide was DIQMTQSPSSLSASL. The N terminus of the rFab L chain was identical except for the first three residues, which were ELV in the rFabs. This was because the PCR primers L 6.5' and L 7.5' introduced the codons for EL that provided the SacI site essential for cloning and the adjoining codon for V. The V_L domains of both clones belonged to κ L chain subgroup V (five) (Kabat et al., 1991).

The N-terminal amino acid sequence of MAb S2B1 H chain could not be determined. This is often caused by cyclization of an N-terminal glutamine and can sometimes be remedied by treatment with pyroglutamyl amino peptidase (PGAP), which has been used to remove N-terminal cyclized glutamine in other proteins (Hirano, 1997) (C. M. Beach, personal communication). However, the H polypeptide still could not be sequenced after this was done.

The H sequences of rFab clones H2 and D4 belonged to H chain subgroup II(B) (Kabat et al., 1991). Both started with EVQLLE and differed by only one nucleotide and one amino acid. The first four amino acids were encoded by the pComb3H vector, and the next two residues (LE) comprised the *Xho*I restriction site introduced by the primers. The rFab clone H2 had an extra

Table 3. Sensitivity of Competition EIAs for Selected PCB Congeners and Other Organochlorines

PCB (IUPAC no.)	chlorination pattern	I_{50}^c (parts per billion)			
		MAb S2B1	Fab S2B1	rFab H2	rFab D4
direct cEIA ^a					
77	3,4,3',4'-tetrachloro-	10–30	49	25	93
126	3,4,5,3',4'-pentachloro-	10–20	46	10	68
35	3,4,3'-trichloro-	310	142	158	266
79	3,4,3',5'-tetrachloro-	1500	— ^d	500	240
52	2,5,2',5'-tetrachloro-	NC ^e	NC	NC	NC
76	3,4,5,2'-tetrachloro-	NC	NC	—	—
indirect cEIA ^b					
35	3,4,3'-trichloro-	40	372	11	282
77	3,4,3',4'-tetrachloro-	32	27	13	25
126	3,4,5,3',4'-pentachloro-	18	32	13	45
52	2,5,2',5'-tetrachloro-	NC	NC	NC	NC
76	3,4,5,2'-tetrachloro-	NC	NC	NC	NC
	3,4,3',4'-tetrabromobiphenyl	100	NC	NC	NC
	3,4'5-trichloro-4-biphenylol	NC	NC	NC	NC
	<i>p,p'</i> -DDT	NC	NC	NC	NC

^a Direct EIA with 3,4-keto-HRP as competitor. ^b Indirect EIA with hapten I-BSA coating as competitor. ^c I_{50} values were obtained by fitting the dose-response curve of duplicate samples of each analyte at seven different concentrations, using the four-parameter logistic equation. ^d —, not tested. ^e NC, no competition by the analyte at 5 ppm.

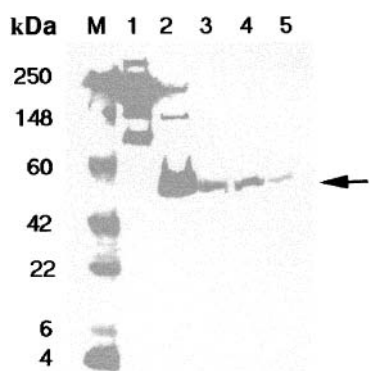


Figure 4. Western blot of MAb S2B1, proteolytic Fab, and rFab after SDS-PAGE under nonreducing conditions. The samples were MULTIMARK [multicolored standard (M)], purified IgG of MAb S2B1 (lane 1), proteolytic Fab S2B1 (lane 2), periplasmic lysates from clone rFab H2 (lane 3), rFab D4 (lane 4), and rFab C5 (lane 5). The unit for protein standard is kilodaltons.

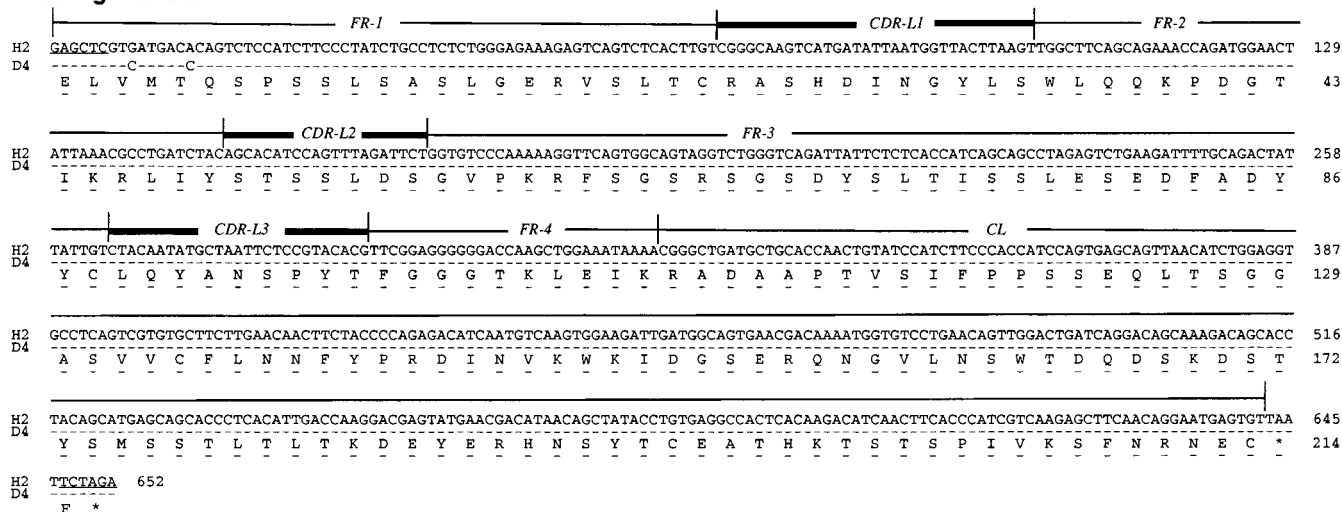
glutamine (Q) inserted between the sixth and seventh residues, immediately after the *Xho*I restriction site. rFab D4 did not have this insertion. The extra residue was apparently introduced by the MVhXho.1 5' PCR primers, which encoded either glutamine (Q) or glutamic acid (E) after the *Xho*I site. A primer from the same set introduced an additional glutamic acid (E) between the sixth and seventh H chain residues of an IgG_{2b} rFab that bound polynuclear aromatic hydrocarbons (Bell et al., 2000). The ninth V_H residue was threonine (T) in rFab clone D4 and alanine (A) in rFab clone H2. This was due to a G → A nucleotide substitution. The Kang 5' primers that produced clone D4 did not extend to the ninth residue, but the MVhXho.1 5' primers that produced clone H2 were long enough to introduce the codon for A at that position. Thus, although we did not have the N-terminal amino acid sequence of the H polypeptide for comparison, we inferred that the ninth residue of MAb S2B1 H chain was more likely to be T. Together, the sequence and the EIA data supported the premise that rFab clones H2 and D4 were structural as well as functional copies of MAb S2B1. The C_L and C_{H1} sequences of both clones (Figure 5) verified the presence of cysteine residues at positions L214 and

H128, which form the interchain disulfide bridge essential for Fab assembly in IgG_{2b} antibodies (Padlan, 1994).

Evidence and Inferences Regarding PCB-Binding Site Structure. Antibody binding site structures are determined primarily by sequences of the six complementarity-determining region loops (CDRs) in the V_H and V_L domains (Chothia et al., 1989; Padlan, 1994). Irrespective of the antigen, most mutations that affect ligand binding occur in these loops. However, each of the respective CDRs in rFab clones H2 and D4 had identical sequences (Figure 5). In most antibodies, five of the six CDRs—L1, L2, L3, H1, and H2—may assume one of a few conserved shapes, known as canonical structures (Chothia et al., 1989; Lesk and Tramontano, 1992; Tramontano and Lesk, 1992). Analysis with the program AbCheck showed that CDRs L2, L3, and H1 each have canonical class 1 structures of their respective loops. The 11 amino acid CDR L1 was similar, but not identical, to canonical class 2 of L1 loops, and CDR H2 was similar to class 2 or 3 of H2 loops.

The Seqhunt II program revealed high sequence similarity between rFab clones H2 and D4 and antibodies specific for substituted phenyl and nitrophenyl haptens. The V_H sequence was 75–76% homologous to those of mouse antibody T099'CL that binds nitrophenyl hapten [Kabat ID 019387 (Lane et al., 1994)], hybridoma-derived mouse antibody B1–8 that binds 4-hydroxy-3-nitrophenylacetate [Kabat ID 001816 (Bothwell et al., 1981)], and mouse antibody B1–8.DELTA1V3 that binds 4-hydroxy-3-nitro-5-iodophenylacetate [Kabat ID 001821 (Radbruch et al., 1985)]. The V_L sequence of rFab clones H2 and D4 had 89% sequence identity with V_L of mouse antibody 48G7, which binds nitrophenyl phosphonate transition state analogue 3 hapten and catalyzes hydrolysis of several nitrophenyl esters and carbonates [Kabat ID 006672 (Lesley et al., 1993)]. The binding site structures of some of these antibodies have been determined by X-ray crystallography at a resolution of 1.9–3.2 Å. The murine N1G9 Fab fragment [PDB 1NGQ (Mizutani et al., 1995)] and the B1–8 recombinant Fv fragment [PDB 1A6V (Simon et al., 1996; Simon and Rajewsky, 1992)], both of which bind 4-hydroxy-3-nitrophenyl acetate, have high sequence identity with the V_H portion of the S2B1 rFabs. The Fab fragments of mouse antibody CNJ206

rFab Light chain



rFab Heavy chain

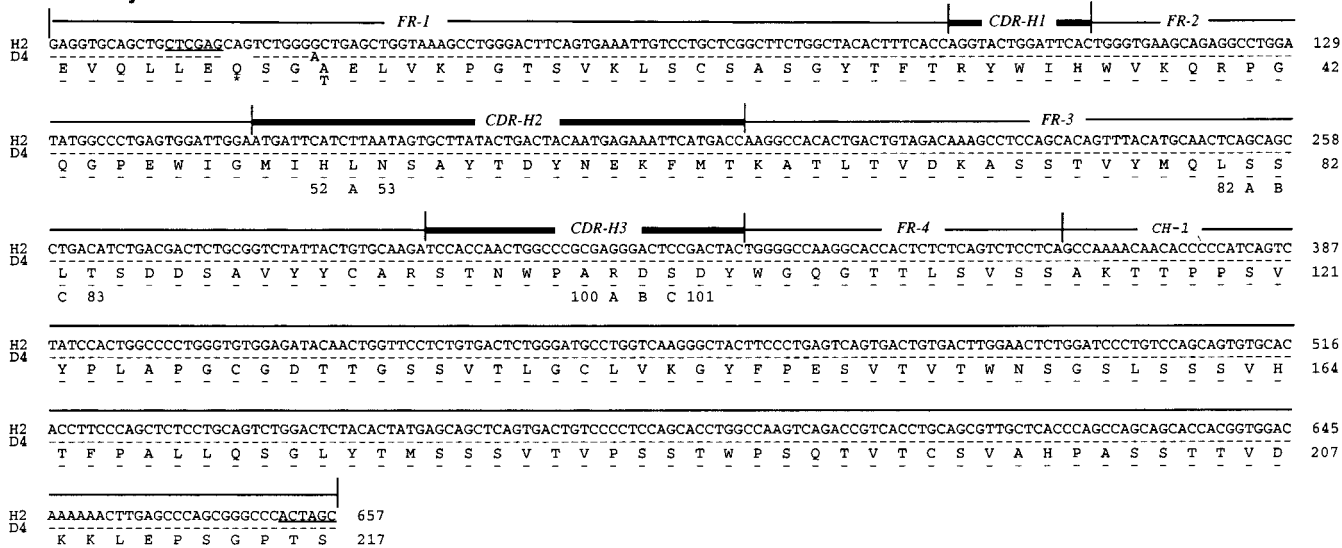


Figure 5. DNA and deduced amino acid sequences of rFab H2 and D4. Framework (FR) and complementarity-determining regions (CDRs) are marked by lines and bars. Sequences identical with the preceding line are represented by hyphens. Restriction sites are underscored in the DNA sequences. Amino acid numbering according to Kabat system is shown.

[PDB 1KNO (Charbonnier et al., 1995)], chimeric mouse-human rFab 48G7 [PDB 1GAF (Wedemayer et al., 1997)], and a third Fab [PDB 2GFB (Golinelli-Pimpaneau et al., 1994)], all of which catalyze hydrolysis of 5-(*p*-nitrophenyl phosphonate) esters, should be excellent templates for modeling the V_L domain of the PCB binding site.

A more puzzling observation was the high sequence homology (>75%) of V_H domains in the PCB-specific rFab clones and two DNA-binding antibodies characteristic of autoimmune disease (Kabat ID 001948 and 001949) and similar V_L domain homology between the PCB rFab and three other DNA-specific antibodies (Kabat ID 006663, 006668, and 006669).

MAbs that bound TCDD, and cross-reacted with a few other dioxin and dibenzofuran congeners, and PCB77 were originally derived by Stanker (1987). More recently, Stanker and co-workers prepared rFabs from two of these (DD-1 and DD-3), deduced the amino acid sequences, and modeled the binding sites, which proved to differ significantly from each other (Lee et al., 1998; Recinos et al., 1994). Thus, it was of interest to compare the sequences of S2B1 and these rFabs. The V_L domains

of DD-1 and DD-3 were 54 and 65% homologous to those of rFab S2B1. The respective V_H sequences were 67 and 41% homologous to V_H of S2B1. However, these homologies were confined to the framework (FR) regions. The length of CDR H3 was different in all three rFabs. Although the lengths of CDRs L2, L3, H1, and H2 were the same in all three, the sequences differed. Moreover, the S2B1 MAb did not detectably bind TCDD and DFs (Chiu et al., 1995). Thus, there are major structural and functional differences between rFab S2B1 and dioxin antibodies DD-1 and DD-3.

CONCLUSIONS

Immunoassay of specific PCB congeners is a challenging problem at the most fundamental level of molecular recognition. It raises the question of whether the desired specificities are beyond the limitations of hapten design, immunogenetics, and conventional polyclonal and monoclonal antibody methods. The advent of combinatorial phage display libraries, with repertoires orders of magnitude greater than the mammalian system, raised expectations that antibodies with useful

affinities and selectivities for almost any ligand could be found. However, the constraints of hapten design still determine what antibodies may be recovered. Even the largest libraries of germline variable-region sequences and combinatorial rearrangements may not be the best scaffolds to provide such subtle specificity differences for PCBs and similar man-made lipophilic compounds. Recent studies of human PCB-binding proteins indicate that there is more than one way to achieve PCB-protein interaction (Hard et al., 1995; Nord et al., 1995; Schantz et al., 1997). The work presented here was based on the premise that engineering of antibodies or other proteins may be the only practical way to achieve the differences required to distinguish among molecules as similar as the PCB congeners.

The most significant biological property of the non-ortho-chlorinated "coplanar" PCBs is that they mimic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other dioxin and dibenzofuran (DF) congeners in binding to aryl hydrocarbon receptor (Kafafi et al., 1993). Dioxins and dibenzofurans are rigidly planar. Although they are dimensionally similar to one conformational state of coplanar PCBs, the PCB phenolic rings can rotate about the bond that joins them. The specificity of the S2B1 antibody is novel and perhaps unique because it may involve induced fit based on flexibility of the PCB as well as parts of the binding pocket. No recognition of mono- and di-ortho-chlorinated PCBs by MAb S2B1 was observed in competition EIAs, which are based on equilibrium binding (Chiu et al., 1995). However, an assay that measures binding kinetics demonstrated that mono- and di-ortho-chlorinated PCBs do interact with S2B1, but the off-rates (k_{off}) are faster and the on-rates (k_{on}) are slower by 10–100-fold, respectively (Chiu et al., in preparation). We have begun computational homology modeling of rFab S2B1 toward visualizing the three-dimensional structure of the binding site and the putative bonding interactions.

ABBREVIATIONS USED

V_H, variable region of the immunoglobulin heavy chain; C_{H1}, first constant region of the heavy chain; V_L, variable region of the light chain, C_L, constant region of the light chain; rFab, recombinant fragment containing disulfide-linked V_H-C_{H1} and V_L-C_L polypeptides, referred to as H and L sequences, respectively; PCB, polychlorinated biphenyl, with congeners numbered according to the methodology of Ballschmitter and Zell (1980); PCB 77, 3,3',4,4'-tetrachlorobiphenyl; PCB 126, 3,3',4,4',5-pentachlorobiphenyl; PCR, Polymerase Chain Reaction; MAb, monoclonal antibody; mRNA, messenger RNA; bp, base pairs; kb, kilobases; CDR, complementarity-determining region; EIA, enzyme immunoassay; I₅₀, the concentration of analyte giving half-maximal inhibition in a competition EIA; amino acids are abbreviated either by the standard three-letter or one-letter code, e.g., tryptophan = Trp or W, and their position in the H and L chains is numbered following the Kabat system (Kabat et al., 1991); Carb, tet, kan, the antibiotics carbenicillin, tetracycline, and kanamycin, respectively.

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