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Development and characterization of new monoclonal antibodies specific for coplanar polychlorinated biphenyls

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Abstract

Three polychlorinated biphenyls (PCBs) widely recognized as being among the most toxic, 3,3′,4,4′-tetrachlorobiphenyl (PCB77), 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) and 3,3′,4,4′,5,5′ -hexachlorobiphenyl (PCB169) were each chemically substituted at the para position and attached to BSA via carboxylic acid spacers by the *N*-hydroxysuccinimide active ester method. The three resultant immunogens were used to stimulate immune responses in mice. Three of the resultant monoclonal antibodies were selected on the basis of target recognition and dimethyl sulfoxide tolerance and taken forward for further study and affinity measurement using a flow based immunoassay platform. The high sensitivity of the immunoassay platform used allows use of antibody concentrations below the K_d (=1/affinity) of the antibody offering ideal conditions to achieve K_d limited detection, the theoretical limit for the antibody used. One of the three antibodies exhibits high affinity binding to all three of the target PCBs, showing dynamic ranges in 2% (v/v) dimethyl sulfoxide of approximately 200 ng l^{-1} to 14 μg l^{-1} for PCB77, 160 ng l^{-1} to 6 μg l^{-1} for PCB126 and 22 ng l^{-1} to 1.4 μg l^{-1} for PCB169. In addition, this antibody showed low cross reactivity to a panel of structurally similar PCBs, and to four common commercial mixtures of PCBs, Kanechlor 300, 400, 500 and 600. Data are included showing that the detection limit for PCB169 is not affected by the presence of a 50-fold excess of commercial PCB mixture Kanechlor 300.

Keywords: Coplanar PCB; PCB; Immunoassay; Antibody

1. Introduction

Polychlorinated biphenyls (PCBs) were widely used in industrial applications, including dielectric fluid in capacitors and transformers, because of their desirable physical and chemical properties, including their low water solubility and very high thermal and chemical stability. Their industrial use was sharply curtailed and then discontinued in the 1970s as their carcinogenic and other toxicological properties became known. The exact properties that made PCBs desirable originally have made them particularly pernicious as environmental toxins. Because of their bioaccumulation in fatty tissues [1], high toxicity [1,2], and long term stability in the environment, assay techniques for PCBs continue to generate a great deal of effort [3–6]. Of the 209 PCB congeners, four non-*ortho* (no chlorines in the 2,2′,6 or 6′ positions) and eight mono-*ortho* (chlorine in only one of the same four positions) congeners are currently recognized by the World Health Organization (WHO) as "dioxin like" in their toxic effects. These 12 congeners have been assigned toxic equivalent factors (TEFs) by the WHO relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the most toxic of the dioxins [7]. The highest TEF for a PCB congener is 0.1 and is assigned to the non-*ortho* coplanar congener, 3,3′,4,4′5-pentachlorobiphenyl (PCB126), the second highest TEF for a PCB, 0.01, is assigned to 3,3′,4,4′,5,5′-hexachlorobiphenyl (PCB169). Congener 3,3′,4,4′ tetrachlorobiphenyl (PCB77) is included in the 12

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and has been reported in the past [8] as one of the most toxic but the most recent WHO-TEF numbers [7] give it a TEF of 0.0001, which is in the middle of the remaining 10 congeners (TEFs ranging from 0.00001 to 0.0005). Structures of these and several other PCBs can be seen in Table 3. Because of their high toxicity relative to the other congeners, it has been recognized that an assay capable of quantifying one or all of these congeners would be highly desirable [8]. It is also widely recognized that this goal is difficult because the measurement will usually take place in the presence of an unknown mixture of some or all of the other 206 congeners, often at much higher concentrations [9].

There are a variety of commercially available immunoassay test kits available for PCBs [3,9]. The commercially available kits are intended for screening, usually of soil samples, and are targeted toward current regulatory practice which specifies limits for total PCB, without regard to congener content. Chiu et al. [4,8,10] and Franek et al. [11] have both reported enzyme-linked immunosorbent assay (ELISA) based immunoassays capable of recognizing PCB77 and PCB126. Although, Chiu used a monoclonal antibody and Franek a polyclonal mixture, both authors report high specificity to PCB77 and/or PCB126 and poor recognition of PCB169. Franek reports a cross reactivity to PCB169 of 3.3% while Chiu reports no reaction up to one part per million PCB169 in 5% dimethyl sulfoxide (DMSO) or methanol. Interestingly, Chiu found about 5% to 20% cross reactivity in higher solvent concentrations.

We report here on the development and characterization of three new monoclonal antibodies showing excellent affinity and specificity for three non-*ortho* coplanar PCBs, PCB126, PCB169 and PCB77. The high affinity and specificity of one of these antibodies to these three dioxin like PCBs has never been shown previously, and provides the basis for an immunoassay with ng l^{-1} sensitivity for the three coplanar PCBs.

2. Materials and methods

2.1. Preparation of PCB-bovine serum albumin (BSA) conjugates for use as immunogens

Three hapten derivatives, 6-[(3,3′,4′-trichlorobiphenyl-4-yl)oxy]hexanoic acid (PCB77-COOH), 6-[(3,3′,4′,5′-tetrachlorobiphenyl-4-yl)oxy]hexanoic acid (PCB126-COOH), 6-[(3,3′,4′,5,5′-pentachlorobiphenyl-4-yl)oxy]hexanoic acid (PCB169-COOH), were newly synthesized as described in Japanese Patent JP2000-191699A and their structures were verified by NMR. These derivatives were reacted with *N*-hydroxysuccinimide to obtain active esters and isolated esters were then reacted with BSA. The procedure for the coupling of these haptens to BSA was as follows. To a vigorously stirred solution of 15 mg of BSA in phosphate buffer (1 ml, pH 8.0) was added DMSO (545 μl) at 0 °C. A solution of the ester (4.55 μmol) of hapten in DMSO (455 μl) was added drop wise to the stirred BSA solution over a 1 min period, and stirring was continued overnight at room temperature. Conjugates were purified by PD-10 column (Amersham Bioscience, RPN 1231 San Francisco, CA, USA), using PBS as eluent. The conjugates, PCB77-BSA, PCB126-BSA, PCB169-BSA, were subsequently used for immunization of mice and for solid phase coatings in immunoassays.

2.2. Procedure for production of monoclonal anti PCB antibodies

Seven week old mice were injected with 25–50 μg per body of one of the PCB–BSA conjugates emulsified in RIBI Adjuvant Systems (Corixa, Seattle, WA, USA). Each mouse was injected 6–10 times at two weeks intervals. Three days following a final booster shot (50 μg/body) the spleen was harvested. Myeloma cells (P3x63Ag8.653; ATCC No. CRL-1580) were mixed with spleen cells at a ratio of 1:5 with polyethylene glycol (PEG) 1500 added to promote cell fusion. Cells were cultured in RPMI 1640 (gibco BRL, Gaithersburg, MD, USA) (10% FBS) + HAT + P/S for 7–10 days. Since, water miscible organic solvents are commonly employed to enhance the solubility of PCBs in aqueous solutions [8,11], preliminary screening for antigen binding capability was done (see procedure below) in 50% (v/v) DMSO (Nacalai Tesque, Kyoto, Japan) to assure solvent tolerance of the selected antibodies. Three hybridomas were chosen for further work. A seven week old Balb/c mouse (Charles River Labs., Charles River, Canada) was injected with 0.5 ml of 2,6,10,14-tetramethylpentadecane (Nacalai Tesque). Three weeks later, 10^7 hybridoma cells were also injected. After 10–14 days, mouse ascites fluid was collected, centrifuged and introduced into a protein G column for IgG separation.

2.3. ELISA screening of hybridomas for anti PCB activity

Hybridoma supernatants were screened on the basis of three competition assays for each supernatant. Separate assays were performed for each of the three target PCBs. The assay strategy employed used immobilized antibody and looked for competition between biotinylated PCB and pure PCB. Pursuant to this, 96-well plates (Corning, Corning, NY, USA) were first coated with Fc specific anti-mouse antibody. Fifty microliters of 10 μg ml^{-1} Goat anti-mouse IgG(Fc) antibody (ICN Biomedicals, Irvine, CA, USA) in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.4) was incubated in each well at 37 °C for 1 h. The role of this antibody was to act as a specific capture reagent for the mouse anti-PCB antibodies. Supernatant was discarded and plates washed three times with 0.005% (v/v) Tween 20 (Bio-Rad Labs., Hercules, CA, USA) PBS. To reduce non-specific binding, 300 μl of 1% (w/v) Gelatin (Bio-Rad Labs., CA, USA) in PBS was added and incubated 2 h at 37 °C. Supernatant was discarded and

plates were again washed in 0.005% Tween20-PBS three times. Next, 50 μl of mouse anti-PCB antibody, excreted from myeloma-spleen cell fusion, at 5 $\mu g\,ml^{-1}$ in 0.1% Gelatin-PBS was added and incubated at room temperature for 1 h. Supernatant was discarded and the plates washed three times in 0.005% Tween 20-PBS. Next, 25 μl of Biotin-PCB (Biotin-PCB77: 1.6×10^{-7} M, Biotin-PCB126: 8.0×10^{-7} M, Biotin-PCB169: 4.0×10^{-8} M, respectively) in 50% DMSO-PBS was added to each well and incubated 10 min at room temperature. Twenty five milliliters of PCB at various concentrations in 50% DMSO-PBS was added to each well and incubated 1 h at room temperature. This two-step screening procedure was used to select antibodies specific for the PCBs themselves rather than the biotin-PCB analogs. In the first step, the biotin analogues were incubated alone, leading to a signal from antibodies able to bind the analog. In the second step, unaltered PCBs were added and only antibodies whose binding to the biotin analog was displaced by the PCB were taken as positives. Supernatant was discarded and plates washed three times in Tween20–PBS. Fifty microliters of HRP-Streptavidin (Amersham Bioscience, RPN 1231, San Francisco, CA, USA) at 1:1000 dilution with 0.1% Gelatin-PBS was added and incubated 1 h at room temperature. Supernatant was again discarded and plates washed three times with Tween20–PBS. Fifty microliters of TMB Microwell Peroxidase Substrate System ($0.2\,g\,l^{-1}$ of 3,3′,5,5′-tetramethylbenzidine, 0.01% H_2O_2) was added and incubated 5–10 min at room temperature. Fifty microliters of 1 M phosphoric acid (Nacalai Tesque) was added to stop the reaction. Plates were read for absorbance at 450 nm.

These biotin-PCBs were prepared as follows. An active ester of hapten (0.02 mmol) and biotin-hydrazide (0.024 mmol, Dojindo Labs., Kumamoto Japan) were dissolved in DMSO (0.3 ml). After standing overnight at room temperature, biotin-PCB was purified by reversed-phase HPLC on ODS-80Tm column (Tosoh, Tokyo, Japan).

2.4. Methodology used to measure antibody affinity and cross reactivity

Except for preliminary screening as described above, all binding measurements were carried out on the KinExA 3000 binding analysis platform (Sapidyne Instruments, Boise, ID, USA). Fig. 1 shows a schematic diagram of the system. Briefly, equilibrated antibody antigen solutions are flowed over micro particles coated with a binding partner of the antibody. The particles are used as a probe to capture and quantify a fraction of the free (unoccupied) antibody in the mixture [12]. Continuous flow of the sample through the flow cell containing the particles permits significant accumulation of antibody on the particles even from very low antibody concentrations. The quantity of antibody bound to the particles is measured by staining with fluorescent dye labeled-secondary antibody and correlated to the free antibody concentration. More detail of the principles and operation of this instrument can be found elsewhere [13,14]. The K_d determination in this study followed the method of the previous report [13].

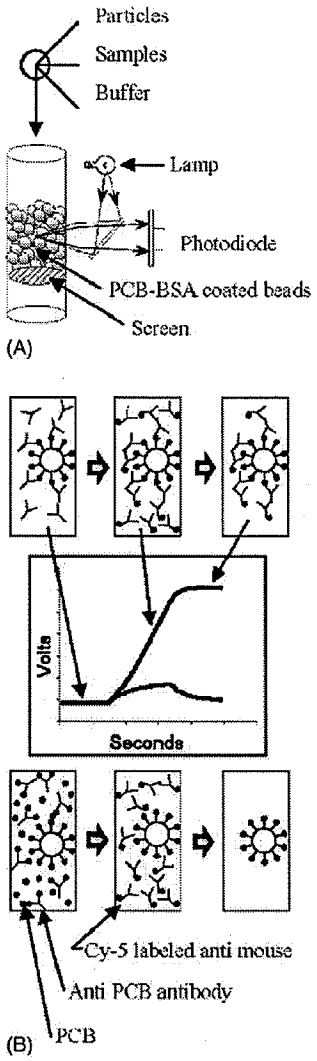


Fig. 1. Schematic diagram of the flow based immunoassay system employed. (A) PCB–BSA coated particles are flowed into a capillary flow cell and trapped by a screen at the focus of an epi-illumination fluorometer. Sample containing anti-PCB antibody and PCB (bottom of panel B) or no PCB (top of panel B) is flowed over the particles. These materials are not fluorescent and do not cause a signal, shown in (B) as volts of photodiode response, above baseline. In a second step, fluorescently labeled anti species antibody is flowed through to stain any primary antibody retained on the particles. Fluorescent signal accumulates or plateaus depending on the quantity of primary antibody retained. In the final step, unbound materials are washed out.

2.5. Preparation of antigen-coated beads for use in the flow based immunoassay

Poly(methyl methacrylate) (PMMA) beads with an average diameter of 100 μm were obtained from Sapidyne Instruments. Two hundred mg (dry wt) of beads were coated

with 1 ml of PBS (pH 7.4) containing 100 μg of PCB–BSA conjugate in accordance with the supplier's suggestions for coating BSA conjugates. After agitating 2 h at room temperature, the supernatant was removed. The beads were then blocked against non-specific binding by immersing them in 1 ml of PBS containing 10 mg of BSA and incubated for 2 h at room temperature.

Alternately, 1 to 5 ml of NHS activated Sepharose for Fast Flow (Amersham Biosciences, no. 17-0906-01, Uppsala, Sweden) were placed in a 50 ml plastic centrifuge tube (Sumitomo Bakelite). After spinning for 3 min at 4000 rpm the supernatant was withdrawn and discarded. The particles were then suspended in 10 to 50 ml (in each case 10-fold above the starting volume of Sepharose) of cold (4 °C) 0.1 mM HCl. Particles were again spun down and the supernatant discarded. This process was repeated a minimum of three times with 0.1 mM HCl and then repeated a minimum of three times with cold PBS. After the final PBS wash, the particles were distributed into 1.5 ml Eppendorf micro centrifuge tubes (approximately 0.5 ml of sepharose gel per tube) and 15 μg of the PCB–BSA conjugate was added. The particles were rotated at room temperature for 2 h, after which 50 μl of concentrated ethanolamine was added to each tube to cap any remaining NHS esters and reduce non-specific binding. Particles were rotated for a minimum of 1 h before use and were kept refrigerated for up to several weeks. Prior to use, both types of particles were suspended into 30 mL of PBS in the KinExA 3000 bead reservoir.

2.6. Preparation of PCB samples for measurement

Samples were prepared in PBS (PBS, 137 mM NaCl, 3 mM KCl, 20 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) with 1 mg ml^{-1} BSA (Sigma, St. Louis, MO, USA, catalog no. A9647), and a final concentration of 2% DMSO (Wako, Osaka, Japan, catalog no. 346 03615) to keep the PCBs in solution. The fluorescently labeled second antibody used here was F(ab')2 fragment Cy-5 labeled goat anti-mouse IgG (H&L) from Jackson Immunochemical (West Grove, PA, USA). Kanechlor technical mixtures of PCBs were obtained from GL Sciences (Tokyo, Japan).

3. Results and discussion

3.1. Screening of monoclonal antibody with ELISA

The antibodies produced from all successfully cloned cell lines were screened against the three target antigens using ELISA, as described above. The ELISA screening was performed in the presence of 50% DMSO to assure the selected antibodies would be tolerant of this solvent, chosen for use in stabilizing PCB solutions. The three clones exhibiting the strongest signal inhibition in response to the target PCBs were selected for affinity determination and cross reactivity analysis. Antibody clones PCB77-A and PCB77-B were obtained by the immunization with PCB77 conjugated to BSA, and the clone PCB169-E was obtained with the PCB169–BSA conjugate. None of the mice injected with the PCB126–BSA conjugate produced antibodies that passed the initial screening. Fig. 2 shows ELISA data for the selected clones. For this figure, the three selected clones were inhibited by their target antigen, thus, the PCB concentration shown in the figure indicates the concentration of PCB77 for clones PCB77-A and -B, and the concentration of PCB169 for the clone PCB169-E.

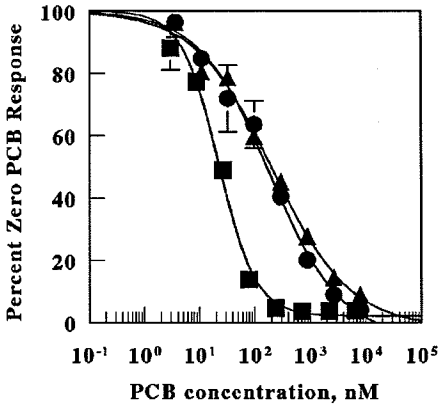


Fig. 2. ELISA results from preliminary screening. Percentage of zero PCB response for monoclonal antibody clone PCB77-A (●), and clone PCB77-B (▲) reacting with PCB77, and for clone PCB169-E (■) reacting with PCB169. Error bars represent plus and minus one standard deviation based on triplicate measurement. In some cases, error bars are smaller than the symbols used and are concealed.

3.2. Selection of solid phase for use in antibody capture

Recently, it has been described that a flow-based immunoassay (KinExA system) allows accumulative signal buildup from relatively large (several milliliters) volumes of dilute (pM range) solutions of antibody [13]. The same reference also describes how using an antibody at a concentration below its K_d offers ideal conditions to achieve theoretical (antibody affinity based) limits of detection for the target analyte. In order to maximize PCB sensitivity, the selected antibodies in this study were applied to this platform. Because the solid phase in this system is used only as a probe to capture and quantify a fraction of the free antibody, the binding partner used to coat the solid phase for capturing the primary antibody need not be the same as the antigen in solution, so long as they both bind to the active site of the primary antibody. Therefore, each of PCB–BSA conjugates was covalently coupled to the sepharose particles or adsorbed onto the PMMA particles and evaluated as a solid phase for each of the antibody clones. The evaluation was done in two steps, in the first step, the signal levels were compared for all six solid phases at an arbitrary but constant 1 nM concentration of primary antibody. Table 1 summarizes the results and shows that the highest overall

Table 1
Signal level at 1 nM primary antibody concentration for each of three antibodies on each of six solid phase variations

Solid phase	PCB–BSA conjugate	Antibody PCB77-A	PCB77-B	PCB169-E
Aepharose	77	3.15	1.49	5.00
	126	2.57	0.64	2.28
	169	1.16	0.22	0.87
PMMA	77	4.91	0.85	2.28
	126	0.32	0.07	0.29
	169	0.14	0.01	0.14

The values are given as an average of three individual deteminations. The units are voltage of the fluorometer output.

signals are achieved with PCB77–BSA covalently immobilized on sepharose. In a second series of experiments, the signal response for the PCB77–BSA sepharose solid phase checked as a function of the primary antibody concentration. In calculating K_d, the signal is interpreted in terms of the concentration of free antibody concentration, and a linear relationship between signal and free antibody concentration simplifies the calculation. To check this, 0.5 mL aliquots of primary antibody at various concentrations were drawn past PCB77–BSA sepharose beads followed by 0.5 ml of secondary antibody at 1 nM. Fig. 3 shows the signal measured for all three antibodies. The fluorescence intensity of the instrument response was a nearly linear function of the free antibody concentration in each sample solution. Subsequent experiments were conducted using PCB77–BSA and the sepharose solid phase, regardless of which antibody was used in a particular experiment.

3.3. Dynamic range

The dynamic range for the three target compounds with each of the three antibodies was measured. PCBs were prepared at double the final concentration by serial dilution in PBS with 4% DMSO and 1 mg ml^{-1} BSA. These solutions were mixed with equal volumes of primary antibody (also double the final concentration) prepared in PBS with 1 mg ml^{-1} BSA, and allowed to incubate to equilibrium. Concentrations of primary antibody were chosen to be as low as easily practical (lower antibody concentrations result in lower binding signals, we chose to work with signals of 0.5 V or greater) in expectation that the resulting measurement curves would give accurate K_d information (see discussion of error analysis below). The final concentrations of antibody used were 200 pM (clone PCB77-A), 500 pM (clone PCB77-B), and 150 pM (clone PCB169-E). After packing the flow cell with PCB77–BSA coated solid phase material, flow was initiated with 30 s of PBS/BSA at a rate of 0.25 ml min^{-1}. Next, equilibrated sample was flowed, also at 0.25 ml min^{-1}, for 4 min, followed by 4 min of secondary antibody (F(ab') 2 fragment Cy-5 labeled anti-mouse). A two stage wash was used, first 30 s of PBS/BSA at 0.25 ml min^{-1} followed by 60 s at 1.5 ml min^{-1}. Fig. 4 shows percentage of free antibody for each of the three antibodies against each of the three target analytes. Referring to Fig. 4C for clone PCB169-E, the dynamic range (calculated as the range from10% to 90% inhibition of response) of the assay for each of the three target PCBs is seen to be approximately 200 ng l^{-1} to 14 μg l^{-1} (0.7–48 nM) for PCB77, 160 ng l^{-1} to 6 μg l^{-1} (0.5–18 nM) for PCB126 and 22 ng l^{-1} to 1.4 μg l^{-1} (0.06–4 nM) for PCB169.

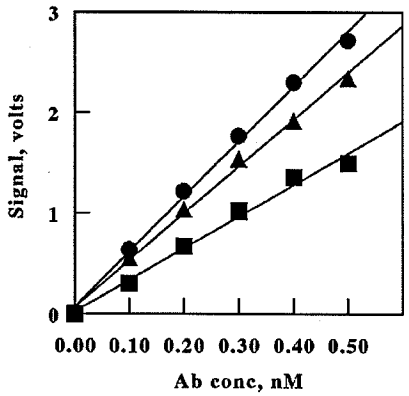


Fig. 3. Signal vs. primary antibody concentration using PCB77-BSA, PMMA solid phase. Monoclonal antibody clone PCB77-A(●), clone PCB77-B (▲) and clone PCB169-E (■). Each point was measured in triplicate. Error bars are included but are smaller than the symbols employed.

3.4. Measurement of antibody affinity to each of the target analytes

One of benefits in the assay used here is that it allows easy and accurate determination of antibody affinity in the solution phase. Because of a limited contact time (less than 500 ms) the binding of the antibody to the solid phase takes place without significantly perturbing the solution equilibrium, and therefore, provides a direct measure of the concentration of free (i.e. not complexed to analyte) antibody in solution [12–14]. The information obtained as a fractional occupancy of antibody binding sites versus the analyte concentrations in Fig. 4, was used to determine the equilibrium binding constant (K_d) of all three antibodies towards the individual coplanar PCBs. Since the signal is proportional to the free antibody in solution, the well understood and relatively simple solution phase binding equations provide an appropriate model. Eq. (1), an exact solution for a standard 1:1 bimolecular binding interaction [13] was fitted to each data set.

$$\text{Signal} = \frac{1}{2}\frac{\text{Sig}_0 - \text{NSB}}{Ab_0}[Ab_0 - K_d - Ag_0 + (Ab_0^2 + 2Ab_0K_d - 2Ab_0Ag_0 + K_d^2 + 2Ag_0K_d + Ag_0^2)^{(1/2)}] + \text{NSB} \quad (1)$$

In Eq. (1), Sig_0 is the signal with zero antigen. NSB is the non-specific binding signal. Ab_0 is the antibody concentration. K_d is the equilibrium dissociation constant (1/affinity). Ag_0 is the total antigen concentration. The K_ds of the

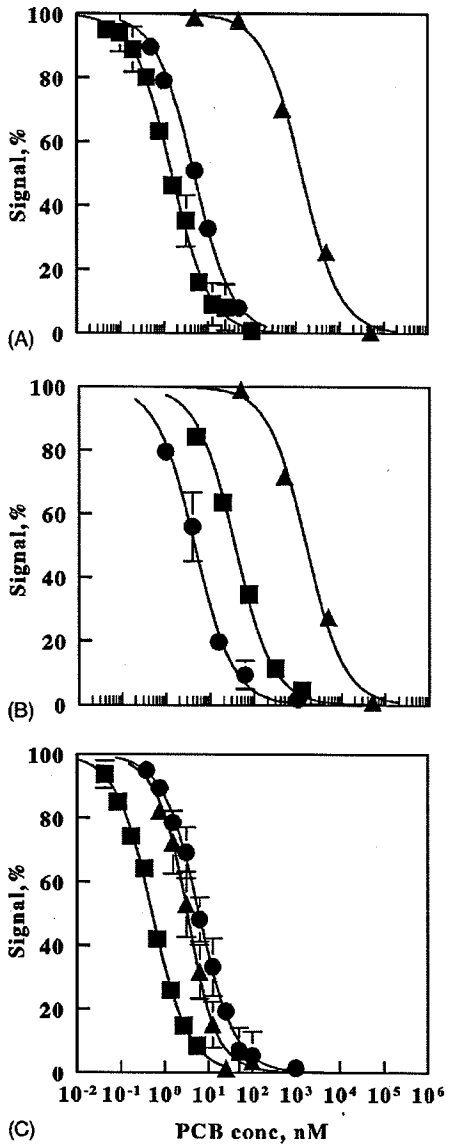


Fig. 4. Percentage free antibody at equilibrium vs. antigen concentration. In all three panels, the analytes represented are PCB77 (●), PCB126 (▲), and PCB169 (■), (A), (B), and (C) show results for monoclonal antibody clones PCB77-A, PCB77-B, and PCB169-E, respectively. Solid lines are best fit theoretical lines for a standard 1:1 binding. Error bars of plus and minus one standard deviation (based on triplicate measurement) are included but in some cases are obscured by the symbols used.

antibodies towards each of the coplanar PCBs are summarized in Table 2. Antibody clone 169-E exhibited the best overall reactivity towards the group of three PCBs examined, and is the only one to bind PCB126 with a nM K_d.

3.5. Analysis of residual errors (after fitting) between theory and data

K_d values determined by fitting Eq. (1) were checked by an error analysis in which the residual error between the data and the fitted theory (Eq. (1)) was examined as a function of K_d. For this analysis, the K_d was shifted away from the global optimum and the other fit parameters were optimized for the new K_d. In conjunction with a measurement of system reproducibility, this error analysis allows estimation of a 95% confidence interval for the K_d. The 95% confidence interval gives the range, over which the measured K_d would be expected to vary as a result of system reproducibility.

Table 2
Equilibrium binding constants of antibodies towards coplanar PCBs

IUPAC#	Antibody		
	PCB77-A	PCB77-B	PCB169-E
PCB77	4.8	4.5	5.3
PCB126	1380.0	1560.0	1.7
PCB169	1.4	37.0	0.50

All values were calculated from fitting the data in Fig. 3 to the Eq. (1) and expressed as nM.

Probably, the most useful feature of the error analysis is that the resultant graph provides an immediate visual indication of whether the primary antibody concentration in the experiment was low enough to allow an accurate determination of K_d. If the primary antibody concentration is significantly higher than the K_d then the resultant curve can be fit equally well by a wide range of K_d values, tending all the way down to zero (i.e. irreversible binding). If this occurs in a given experiment, the investigator is immediately alerted to the need to repeat the experiment at a lower primary antibody concentration. Fig. 5 shows an error graph generated for clone PCB77-A against PCB169, with the 95% confidence interval indicated. This clearly defined minima in the residual error indicates that the 200 pM antibody concentration used in this experiment was low enough to allow reasonably accurate determination of the K_d, with the 95% confidence interval between 0.6 and 2.2 nM (see Fig. 5). Error graphs

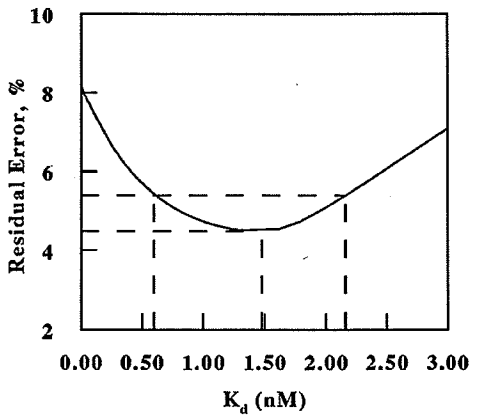


Fig. 5. Goodness of fit vs. K_d. The residual fit error after optimized fitting of the theoretical equation to the data from clone PCB77-A binding PCB169 is shown as a solid line. The minimum residual error occurs at 1.49 nM K_d and the 95% confidence interval for K_d, based on measurement reproducibility is from 0.6 to 2.2 nM, as shown by the dashed lines.

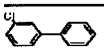
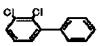
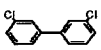
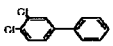
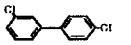
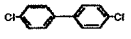
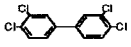
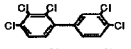
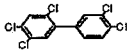
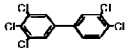
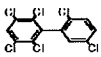
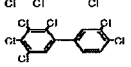
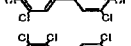
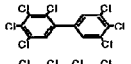
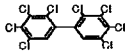
for the other eight K_d determinations show similar minima, validating the initial choices of antibody concentration.

3.6. Antibody cross reactivity to other PCB congeners, and detection of PCB169 in the presence of Kanechlor 300

The cross reactivity of the three antibodies was checked against a panel of 13 other PCBs chosen for structural similarities. Inhibition was checked at three antigen concentrations (0.03 nM, 30 nM, and 3 μM PCB) with a 1 nM antibody concentration. The results are summarized in Table 3. Antibody clone PCB169-E exhibits the best specificity, with cross reactivity of approximately 1.0% or less to the congeners checked. Also, this clone is the only one of the three to bind PCB126 with a nM binding constant.

Clone PCB169-E was also checked for reactivity with commercial PCB mixtures, Kanechlor 300, Kanechlor 400, Kanechlor 500, and Kanechlor 600. For these experiments clone PCB169-E (200 pM) was mixed with titrations of each of the Kanechlor mixtures in PBS BSA supplemented with 2% DMSO. The mixtures were allowed to equilibrate for 30 min and were then measured on the KinExA instrument.

Table 3
Cross reactivity of antibodies towards related PCBs

Structure	IUPAC #	Cross reactivity (%)		
		PCB77-A	PCB77-B	PCB169-E
	PCB2	ND	≈0.2	ND
	PCB5	ND	ND	ND
	PCB11	ND	≈0.2	≈0.01
	PCB12	≈0.1	≈15	<1.3
	PCB13	≈0.1	<15	<1.3
	PCB15	≈0.1	<15	≈0.01
	PCB77	29	100	7.6
	PCB105	<4.7	≈15	<1.3
	PCB118	<4.7	<15	<1.3
	PCB126	1	2.8	23.5
	PCB151	ND	ND	ND
	PCB156	≈20	≈0.2	≈1.3
	PCB169	100	12.2	100
	PCB189	≈0.1	≈0.2	<1.33
	PCB194	≈0.1	ND	≈0.01
	PCB209	≈0.1	ND	ND

ND indicates no cross reactivity was detected.

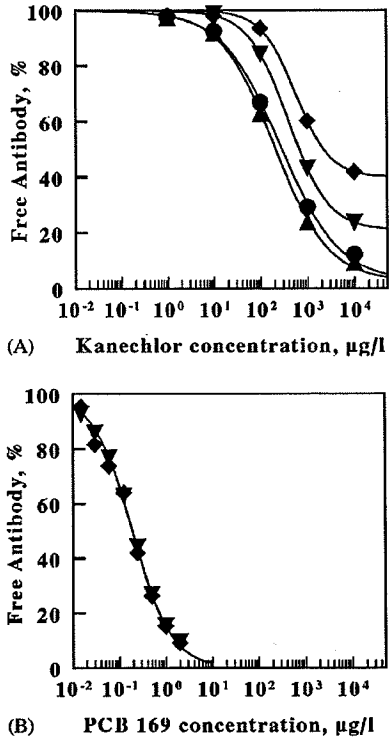


Fig. 6. Effect of Kanechlor on clone 169-E. In(A), circles (●) represent Kanechlor 300, upright triangles (▲) represent Kanechlor 400, inverted triangles (▼) represent Kanechlor 500 and diamonds (◆) represent Kanechlor 600. In(B), circles (●) represent response from PCB169 in the presence of 1 $\mu g l^{-1}$ Kanechlor 300 and diamonds (◆) represent the response from PCB169 alone. All measurements were made in triplicate and error bars of plus and minus one standard deviation are included. In many cases, excellent measurement reproducibility resulted in error bars small enough to be concealed by the plotting symbols used.

The sample was flowed at a rate of 0.25 ml min^{-1} for 2 min and followed by a labeling solution of 2 nM Cy-5 labeled F(Ab)2 anti-mouse antibody flowed for the same time and rate. Results are summarized in Fig. 6A where it can be seen that antibody is most cross reactive to the Kanechlor 300 and 400, with an I_{50} for these compounds of approximately 200 $\mu g l^{-1}$. In a follow up experiment, a standard curve for PCB169 was measured in PBS BSA 2% DMSO spiked with 1 $\mu g l^{-1}$ Kanechlor 300. This curve is shown in Fig. 6B plotted along with the same curve measured without the Kanechlor. The sensitivity to PCB169 (approximately 20 $ng l^{-1}$ based on 10% inhibition) is not affected by the presence of approximately 50-fold excess of the Kanechlor mixture.

It is interesting to speculate about the structure of the binding site on clone PCB169-E. For PCBs, Coplanar refers to the orientation of the two phenyl rings. The lowest energy state of all PCBs is with planes of the phenyl rings perpendicular [1]. The absence of chlorine in any of the *ortho* positions (position 2, 2′, 6 or 6′) reduces the energy barrier that disfavors the coplanar configuration and PCBs with 0 or 1 *ortho* position chlorines are generally considered

coplanar. PCBs with two or more *ortho* chlorines are usually considered non-coplanar as the steric hindrance of the chlorines is thought to effectively prevent the coplanar configuration. In the Kanechlor experiments, the clone shows stronger cross reactivity to the lower numbered Kanechlor mixtures. The lower numbers correspond to a lower average number of chlorines per PCB molecule in these mixtures (approximately half of the Kanechlor 300 mixture is composed of trichlorobiphenyls), resulting in a larger percentage of PCBs with 0 or 1 *ortho* position chlorines. For example, Kanechlor 300 has approximately 49% (w/w) PCBs with one or fewer *ortho* position chlorines, compared to Kanechlor 600 which has about 9%. The binding site of the antibody may be a narrow pocket that coplanar PCBs can slide into. The hypothesized pocket may be deep enough to accommodate nearly the entire PCB molecule, as evidenced by the approximately four-fold reduction in binding brought about by elimination of a single chlorine in the 5 (*para*) position (PCB126) or the nearly 100-fold decrease brought about by the addition of a single chlorine in the two position (PCB189).

4. Conclusion

Clone PCB169-E provides the potential for highly sensitive and selective detection of three of the most toxic PCBs. Compared to the only previously published account of a monoclonal coplanar specific anti-PCB antibody [4], the present clone shows about three-fold lower affinity for PCB77, and about two-fold higher affinity for PCB126. Direct comparison to the polyclonal antibody described by Franek et al. [11] is more difficult, the IC_{50} value they describe for PCB126 is 19.2 $\mu g l^{-1}$ (59 nM) which is over 30-fold higher than the midpoint of the curve shown here (1.7 nM) but many factors could influence this. One advantage that the current antibody offers over both of those published previously is that it is also able to recognize PCB169 (second most toxic, after PCB126) with nM affinity.

While this antibody alone cannot definitively quantify sample toxicity (for example, its affinity for PCB77, whose TEF is 0.0001, is only about three-fold lower than its affinity for PCB126, whose TEF is 1000 times greater) it is hoped it can provide an additional tool in identifying which samples are dangerous to living organisms.

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