Properties of Polyclonal, Monoclonal, and Recombinant Antibodies Recognizing the Organophosphorus Pesticide Chlorpyrifos-ethyl

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A rabbit polyclonal antiserum and two murine monoclonal antibodies recognizing the organophosphorus pesticide chlorpyrifos-ethyl were produced. The two hybridoma cell lines were then used as sources of immunoglobulin genes for the generation of recombinant scFv antibodies in *Escherichia coli*. The two scFvs showed either similar or improved limits of detection in an ELISA when compared with the monoclonal antibodies. Cross-reactivity studies showed that all of the antibodies were specific toward the chlorinated aromatic ring. Furthermore, scFv gene sequences were linked directly to sequences coding for either a c-Myc tag, a His-tag, or alkaline phosphatase. The fusion products generated were functional, and their properties were determined. The problems associated with producing scFvs and scFv derivatives for detection of pesticide residues from hybridoma are addressed and discussed.

Keywords: *Variable region; antibody engineering; immunoassay; recombinant antibody; pesticide analysis*

INTRODUCTION

The organophosphorus pesticide chlorpyrifos, *O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphothioate, has a broad range of insecticidal activity and is widely used in agricultural and domestic applications (Worthing and Hance, 1991; Racke, 1993). Due to its widespread use, chlorpyrifos contaminant residues have been reported in food (U.S. Food and Drug Administration, 1994) and environmental samples (Hallberg, 1989). Despite significant advances in the study of acute detoxification of chlorpyrifos by serum paraoxonase in mice (Li et al., 1995; Shih et al., 1998) and humans (Davies et al., 1996), there is still a requirement for monitoring and surveillance programs for chlorpyrifos residues (Cochran et al., 1995).

As an alternative to laborious chromatographic procedures for chlorpyrifos analysis (AOAC, 1995), several immunoassay methods have been developed to determine the chlorpyrifos methyl ester (Skerrit et al., 1992; Hill et al., 1994), the ethyl derivative (Manclus et al., 1994, 1996; Manclus and Montoya, 1996a), and the main environmental breakdown product of chlorpyrifos, 3,5,6-trichloro-2-pyridinol (Manclus and Montoya, 1996b). In general, immunoassay methods are simple to perform, cost-effective, robust, and amenable to on-site monitoring. However, the increasing demand for even simpler and faster methods in new formats (including biosensors) requires more rapid generation of novel antibodies and antibody fragments to meet these increasing demands (Hock, 1997). In this role, recombinant antibody technology is becoming a powerful tool in the field of medicine (Hudson, 1998).

Recombinant antibodies may offer several advantages over polyclonal and monoclonal antibodies, including speed of antibody generation, the ability to generate novel and rare functionalities, and the possibility of altering affinity and specificity (Huston et al., 1993; Pluckthun and Pack, 1997). Spleen cells, hybridomas, and synthetic gene libraries have been used as sources of recombinant antibody fragments (Hudson, 1998), although the generation of functional fragments from all three sources has been problematic. For hybridomas, the presence of aberrant mRNAs has been extensively reported (Carroll et al., 1988; Duan and Pomerantz, 1994; Nicholls et al., 1993; Ostermeir and Michel, 1996). The levels of aberrant mRNAs can greatly exceed the levels of normal antibody transcripts (Ostermeir and Michel, 1996) and hinder the successful generation of recombinant antibody fragments. Since the problem was recognized, several procedures have been proposed to eliminate the nonfunctional pseudogenes, including RNase H digestion (Ostermeir and Michel, 1996), ribozyme cleavage (Duan and Pomerantz, 1994), in vitro translation (Nicholls et al., 1993), and phage display selection (Krebber et al., 1997). A critical discussion on the advantages of the different methods is given by Krebber et al. (1997). For synthetic libraries, the recovery of low-affinity antibody fragments characteristic of the in vivo primary response is the norm, and the problem has been thoroughly reported (Garrard and Henner, 1993; Hoogenboom and Winter, 1992; Karu et al., 1994).

Recombinant antibodies with a restricted range of affinity are required in immunoassays for small analytes, and the identification of such antibodies by gene technology is not as straightforward as it has sometimes been made to appear in the scientific literature or with commercially available kits. Nevertheless, functional scFvs against the herbicide paraquat (Graham et al., 1995), the herbicide atrazine (Byrne et al., 1996), the herbicide *s*-triazine (Kramer and Hock, 1996), the mycotoxin zearalenone (Yuan et al., 1997), and the pesticide parathion (Garrett et al., 1996) have been

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isolated and characterized, and Fab fragments against the herbicide diuron (Bell et al., 1995) have been described.

In the present paper we describe the production of polyclonal and two monoclonal antibodies from different fusion experiments against the pesticide chlorpyrifos. The two hybridoma cell lines were used as sources of immunoglobulin genes for the generation of functional recombinant scFv antibodies in *Escherichia coli* by a recently revised methodology. ELISA characteristics for the polyclonal, monoclonal, and recombinant antibodies in a microtitration format were determined. Furthermore, functional derivatives of the anti-chlorpyrifos scFv antibodies were produced, and the results are discussed.

MATERIALS AND METHODS

Hapten Synthesis. Chlorpyrifos-ethyl was kindly donated by Dr. R. Maycock (Dow Chemical Co.). The hapten was prepared by the introduction of a mercaptoacid spacer arm into the aromatic ring of chlorpyrifos. The synthesis was accomplished by a one-step substitution of activated chlorine in the 6-position of the aromatic ring with mercaptopropionic acid as previously described by Manclús et al. (1994). The derivative [*O*,*O*-diethyl *O*-[3,5-dichloro-6-[(2-carboxyethyl)thio]-2-pyridyl] phosphorothioate] was then purified by HPLC (PU 4100-Philips, Cambridge, U.K.) on a silica C-18 chromatography column [Sphereclone, 5 μm, ODS(2), 250 × 4.6 mm, Phenomenex, Macclesfield, U.K.] using acetonitrile/water (isocratic, 60:40, 0.5 mL/min). The identity of the derivative was confirmed by mass spectroscopy according to standard procedures.

Preparation of Hapten—Carrier Conjugates. Chlorpyrifos-ethyl propionic acid derivative was coupled to carrier proteins [ovalbumin and keyhole limpet haemocyanin (KLH); Pierce Chemical Co., Rockford, IL] by the active ester method (Langone et al., 1975) and purified by gel filtration chromatography on Sephadex G-25 (Pharmacia, Uppsala, Sweden). The propionic acid derivative was also coupled to amino dextran 40 kDa (Molecular Probes Europe, Leiden, The Netherlands) essentially as previously described (Xiao et al., 1995).

Polyclonal Antibodies. Initially, New Zealand white rabbits were immunized subcutaneously with chlorpyrifos−KLH conjugate (100 μg) in phosphate-buffered saline (PBS) and Freund's complete adjuvant 1:1 (v/v, 800 μL) and subsequently at 4-week intervals, using Freund's incomplete adjuvant. Sera collected from the marginal ear vein 10 days after immunization were tested in an ELISA as described later. The tested and pooled rabbit antiserum was purified on a Sepharose−protein G column using PBS plus 0.05% Tween 20 (PBST) and glycine buffer, essentially as described in the manufacturer's handbook (Pharmacia).

Production of Monoclonal Antibodies (mAbs). Initially BALB/c mice were immunized subcutaneously with chlorpyrifos−KLH conjugate (50 μg) in PBS and Freund's complete adjuvant 1:1 (v/v, 200 μL) and subsequently at 4-week intervals using Freund's incomplete adjuvant. Animals given a positive test bleed, as assessed by ELISA, were killed 4 days after the next booster injection. Spleen cells were fused with myeloma cells [X63-Ag8−653, Imperial Laboratories (Europe) Ltd., Andover, U.K.] according to the established protocol of Galfré and Milstein (1981) and incubated in Opti-MEM following the manufacturer's instructions (GibcoBRL-Life Technologies Ltd., Paisley, U.K.). Hybridoma culture supernatants were screened for antibody activity by ELISA as described below, diluting the cell culture supernatants 1:1 in PBS containing 0.05% (v/v) Tween 20. Positive hybridomas were cloned three times by limiting dilution (Galfré and Milstein, 1981) on a microprocessor-controlled automatic workstation (Biomek 1000, Beckman Instruments Inc., High Wycombe, U.K.). Large quantities of mAbs were obtained by growth in tissue culture flasks. mAbs were purified by ammonium sulfate precipitation (50% saturation) followed by extensive dialysis in 10 L of PBS at 4 °C for 24 h. Dialyzed mAbs were stored at −20 °C.

Production of Recombinant scFv Antibodies. Messenger RNA was extracted from 10^6 hybridoma cells using the QuickPrep Micro mRNA purification kit (Pharmacia Biotech, St. Albans, U.K.). About 1 μg of mRNA was reverse transcribed using random hexamer primers and the first-strand cDNA synthesis kit (Pharmacia Biotech). scFvs were constructed by assembling the amplified V_H and V_L cDNA with an artificial 20 amino acid linker as described by Krebber et al. (1997) with minor modifications. Essentially, V_H and V_L were first amplified using Master Amp 2xPCR premix buffer B (Epicentre Technologies, Madison, WI) and AmpliTaq DNA polymerase (Applied Biosystems, Risley, U.K.). The V_H and V_L PCR products were agarose gel-purified (Quiaquick Kit, Qiagen Ltd., Dorking, U.K.) and the scFvs assembled by splicing using overlap extension PCR and MasterAmp 2xPCR premix J. The scFv fragments were gel-purified as previously mentioned and *Sfi*I digested following the manufacturer's instructions (Promega, Madison, WI). The scFv fragments were purified using MicroSpin S-400 HR columns (Pharmacia Biotech), ligated into pAK100 vector (kindly given by Dr. A. Plückthun, Universität Zürich, Switzerland), and then electroporated into *E. coli* XL1-Blue [*rec*A1 *end*A1 *gyr*A96 *th*i-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F′ *pro*AB *lac*I^q ZΔ*M15* Tn*10* (Tet^r)]^c (Stratagene Ltd., Cambridge, U.K.) as previously described (Krebber et al., 1997). Recombinant bacteria were grown on 2xTY agar plates containing 1% glucose and 25 μg/mL chloramphenicol overnight at 37 °C. Screening was performed by expressing the individual transformed colonies (1 mL) in the presence of 1 mM IPTG for 4 h at 25 °C with shaking. After centrifugation, the periplasmic extracts were produced by resuspending the *E. coli* pellets in 300 μL of ice-cold TES buffer as previously described (Harrison et al., 1996). In-frame scFv expression was monitored by the detection by ELISA of the c-Myc peptide fused at the C-terminal end (Cravchick and Matus, 1993) using the commercial available 9E10 mAb (4 μg/mL in MPBST; Sigma Chemical Co., Poole, U.K.). Functional scFvs were detected in an indirect ELISA as previously described for parathion (Garrett et al., 1997), using 10 μg/mL chlorpyriphos-ethyl dextran for microtitration plate coating, chlorpyriphos-ethyl as competitor, and the 9E10 mAb for c-Myc detection. Sequencing was performed on an automated DNA sequencer using the Big Dye terminator cycle sequencing kit (Applied Biosystems).

Cloning into pAK400 and pAK600. Purified plasmids from the scFvs were *Sfi*I digested (Promega) and gel-purified (Quiaquick Kit, Qiagen Ltd., Dorking, U.K.), and the scFv fragments were ligated into the previously digested pAK400 and pAK600 vectors (kindly given by Dr. A. Plückthun) and transformed into the nonsupressor strain *E. coli* HB2151 [K12, *ara*, D(*lac-pro*), *thi*/F′*pro*A$^+$B$^+$, *lac*I^{q}ZDM15]. The scFv fragments cloned into the pAK600 vectors resulted in an "in frame" fusion of the carboxy-terminal domain of the heavy chain variable region with the N-terminal domain of the *E. coli* alkaline phosphatase, whereas the pAK400 vector resulted in an "in frame" fusion with a 6 histidine tail as described by Krebber et al. (1997). Chloramphenicol was used to select the recombinant bacteria as described above. Recombinant clones were analyzed by PCR using the outer primers and confirmed by restriction analyses in agarose gel (Sambrook et al., 1989).

Soluble Expression of scFv Fragments. Fresh 50 mL 2xTY broths containing 25 μg/mL chloramphenicol were inoculated with an overnight culture of the bacteria and incubated at 25 °C with agitation until an OD_{600} of 0.5 was reached. Expression was induced with 1 mM IPTG and allowed to proceed on an orbital shaker for 4 h at 25 °C (24 h for pAK600 and pAK-GFP). After collection by centrifugation at 3000*g* for 30 min at 4 °C, the bacteria were disrupted three times by a French press and separated into soluble and insoluble fractions by centrifugation (3000*g* for 30 min).

Standard Enzyme-Linked Immunosorbent Assay (ELISA). Microtitration plates (Nunc Immunoplate MaxiSorp, Gibco) were coated with either chlorpyrifos−dextran (10 μg/mL) or chlorpyrifos−ovalbumin (1 μg/mL; 100 μL/well) in PBS for 16

h at 4 °C. After three washes with PBST, the plates were blocked with 3% (w/v) of nonfat dried milk in PBST (MPBST) at room temperature for 1 h, and washed twice again with PBST. A volume of 100 μL per well was used throughout all assay steps, and all of the incubations were carried out at room temperature. The antibodies were incubated with pesticide on the microtitration plate in the presence of MPBST 1% (w/v) for 2 h. After incubation, the plates were washed four times with PBST. The polyclonal and monoclonal antibodies were detected by incubation for 1.5 h with an anti-rabbit IgG−horseradish peroxidase (HRP) labeled antibody (1:1000 in PBST; Sigma Chemical Co.) and an anti-mouse IgG HRP-labeled antibody (1:1500 in PBST; Sigma Chemical Co.), respectively. The scFvs were first detected by incubation for 1.5 h with an anti-c-Myc peptide monoclonal antibody [clone 9E10, 4 μg/mL in MPBST 2% (w/v); Sigma Chemical Co.]. After four washes with PBST, an anti-mouse IgG HRP-labeled antibody (1:1500 in PBST; Sigma Chemical Co.) was added, and the microtitration plates were incubated for 1.5 h. After five more washes with PBST, the substrate (3,3′,5,5′-tetramethylbenzidine solution; Vetoquinol, Bicester, U.K.) was added. The reaction was stopped by the addition of 50 μL/well of 2 M sulfuric acid, and the optical densities were recorded at 450 nm on a plate reader (Dynatech MR5000, Dynatech Laboratories Ltd., Billingshurst, U.K.).

Cross-Reactivity. The cross-reactivity analyses were performed using the ELISA procedure described above with microtitration plates coated with chlorpyrifos−dextran (10 μg/mL, 100 μL/well) and the antibodies incubated with serial dilutions of individual standard grade organophosphates (OPs). The cross-reaction was calculated as the ratio of the mass of pesticide giving 50% inhibition of the maximum response to the concentration of chlorpyrifos-ethyl as standard.

ELISA Using a Directly Labeled Alkaline Phosphatase. Microtitration plates (Nunc Immunoplate MaxiSorp, Gibco) were coated with chlorpyrifos−dextran and blocked as described above. scFv soluble extracts from clones bearing the alkaline phosphatase gene were diluted in MPBST and incubated for 2 h at room temperature against increasing concentrations of the pesticide. The microtitration plates were then washed five times with PBST, and 100 μL/well *p*-nitrophenyl phosphate substrate (Sigma Chemical Co.) was added. The microtitration plates were incubated for 3 h at 37 °C, and the optical densities were recorded at 400 nm using a plate reader (Dynatech MR5000, Dynatech Laboratories Ltd.).

RESULTS AND DISCUSSION

Hapten Synthesis. With the aim of exploring the use of antibodies and antibody fragments as analytical tools in pesticide analysis, we produced polyclonal, monoclonal, and recombinant scFv antibodies using a simple hapten design. Two synthetic approaches for the immunogen were available: (i) to attach a spacer arm to the thiophosphate group (Hill et al., 1994; Manclús et al., 1994), which requires several protection/deprotection steps of the active group during the chemical synthesis; (ii) to substitute directly the activated chlorine in the 6-position of the aromatic ring with alkyl acids (Manclús et al., 1994; Lawruk et al., 1996). Manclús et al. (1996) showed that both approaches could produce anti-chlorpyrifos antibodies with high specificity and sensitivity. Therefore, we chose the simplest synthesis method consisting of the substitution of a chlorine in the aromatic ring by a mercaptopropionic acid arm (Figure 1). The product purified by HPLC and obtained in low yields (<1%) had its identity confirmed by mass spectroscopy and was subsequently conjugated to KLH and ovalbumin. To improve the assay sensitivity, the chlorpyrifos−propionic acid derivative was also coupled to amino dextran as suggested by Xiao et al. (1995) and used throughout the experiments. The dextran conjugate was very soluble, easy to synthesize and purify, and, although the assay format did not show the same sensitivity as the different heterologous formats discussed below, it was reliable and resulted in a very low background.

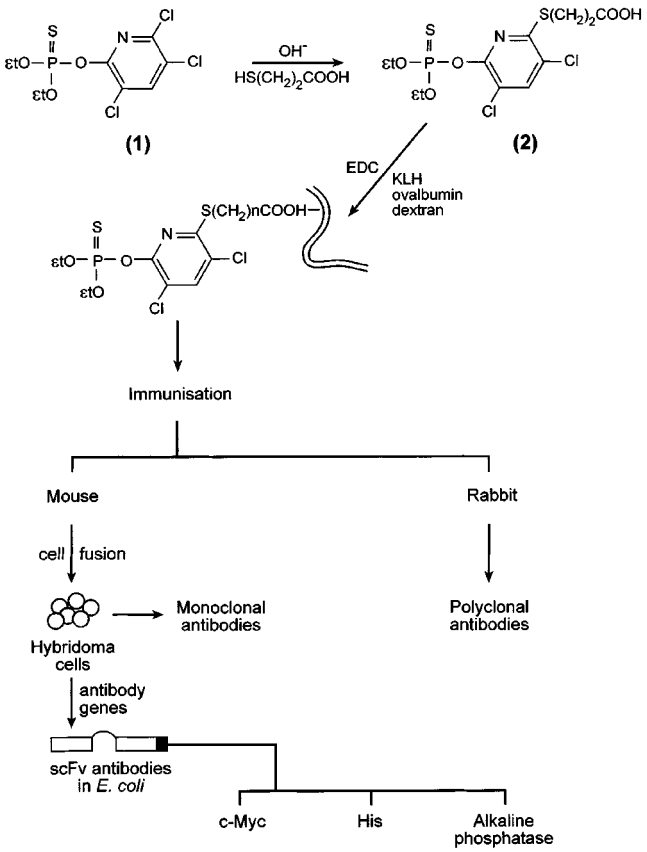

Figure 1. Schematic diagram showing the steps followed in the production of the polyclonal, monoclonal, and recombinant antibodies: (1) chemical structure of chlorpyrifos and (2) the chlorpyrifos propionic acid derivative used to immobilize the pesticide to the carrier molecule.

Polyclonal Antisera. In agreement with published results, the chlorpyrifos−propionic acid derivative proved to be a very effective immunogen as the mouse and rabbit antisera produced possessed high and constant titers after few injections. In general, the immune sera reached a high titer (1:100000) after three immunizations and stayed at this level in subsequent immunizations. The polyclonal antibodies recognized chlorpyrifos (Figure 2) and, for comparative studies in different formats, were affinity purified on a protein G column. Table 1 displays the limit of detection and I_{50} (concentration giving 50% inhibition of the maximum response).

Monoclonal Antibodies. The mouse hybridoma cell lines designated IFRN 1301 and IFRN 1302 were produced from two distinct animals and fusion procedures separated by a time interval of 6 months. Both mAbs were selected for their ability to recognize chlorpyrifos by ELISA. The mAb IFRN 1302 was isotyped as IgG1-kappa light chain and was successfully purified on a protein A column. The mAb IFRN 1301 carried a lambda-type light chain. Despite being recognized by anti-IgG antibodies, the precise heavy chain isotype of IFRN 1301 could not be established using commercially available kits. The mAb did not bind to protein A, whereas its binding properties were lost in the acidic conditions required to elute from protein G columns. mAb 1301 was partially purified from the hybridoma

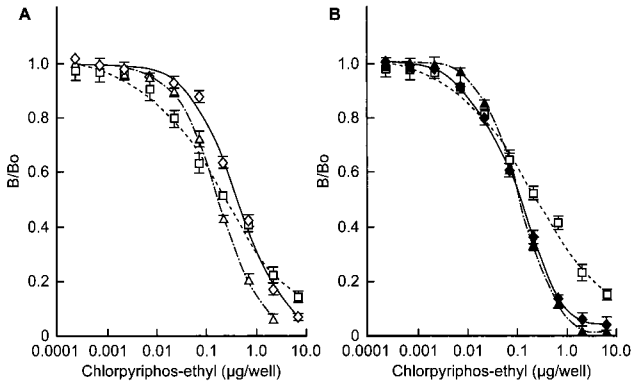

Figure 2. ELISA using chlorpyrifos as standard: (A) □, purified rabbit polyclonal antibody diluted 1:2000 in PBST; ◇, mAb IFRN 1301 diluted 1:2000 in PBST; △, scFv IFRN AB01 diluted 1:100 in PBST; (B) □, purified rabbit polyclonal antibody diluted 1:2000 in PBST; ◆, mAb IFRN 1302 diluted 1:2000 in PBST; ▲, scFv IFRN AB02 diluted 1:100 in PBST. Plates were coated with chlorpyrifos−dextran as described under Materials and Methods. Each point represents the average of four determinations, and the error bars indicate one standard deviation.

Table 1. Binding Properties of the Chlorpyrifos Antibodies

antibody	I_{50} (ng/mL)	LOD (ng/well)
rabbit polyclonal	2200	6
mAb IFRN 1301	4200	6
mAb IFRN 1302	1000	20
scFv AB01[a]	1600	7
scFv AB02[a]	1000	7
scFv 1302−AP[b]	500	1

[a] c-Myc-tag derivative. [b] *E. coli* alkaline phosphatase derivative.

culture supernatant by ammonium sulfate precipitation. In a competitive ELISA assay, both mABs showed higher sensitivity than the polyclonal antibodies (Figure 2), whereas, in agreement with published results (Manclus et al., 1996), the rabbit polyclonal antisera showed a shallower slope as well as a higher background than the monoclonal antibodies. Table 1 displays the I_{50} and limit of detection values of the assay. The I_{50} values of 11.4 and 2.8 μM for IFRN 1301 and 1302, respectively, are comparable to those of antibodies raised against the same hapten derivative (Manclús et al., 1996).

Recombinant scFv Antibodies. When mAbs had been produced, the two stable hybridoma cell lines were used as separate sources of immunoglobulin genes for generation of scFv antibodies. The scFv antibodies present in *E. coli* periplasmic extracts were screened for their ability to bind free chlorpyrifos by ELISA. Functional scFvs were found without any prior selection. A significant difference in numbers of functional scFvs was observed between the two cell lines, suggesting a bias toward the mAb IFRN 1301 gene family during PCR amplification (Table 2). To understand the origin of these results, we sequenced nonfunctional scFvs from both cell lines. The scFvs derived from IFRN 1301 and 1302 were designated IFRN AB01 and AB02, respectively. The nucleic acid sequences for scFvs IFRN AB01 and AB02 have been filed in Genbank as AF132309 and AF132308, respectively. The functionality of the scFvs was found to be linked to the specific pair of heavy and light chains. In gene shuffling experiments (data not shown), neither the chimeras of the two functional scFvs (heavy 2 × light d or heavy 1 × light c) nor the combination of any of the heavy chains with a library of light chains from other hybridoma cell lines (data not shown) was able to generate scFvs able to bind the pesticide.

As expected, the nonfunctional pairs contained a significant level of the light chain "a" pseudogene derived from the myeloma cell line P3-X63-Ag8.653 (Genbank M35669). In a few cases, the primer used mutated artificially the stop codon at position 105 to a serine in the light chain sequence producing full-length constructs. In both cell lines we also found a second pseudogene, light chain "b" (Genbank AF106674), that has not been described before. The presence of the same two heavy chains in the scFvs from both cell lines was unexpected. Cross-contamination cannot be excluded, although the occurrence of the two heavy chains in the same proportions in both cell lines and the absence of contaminant functional light chains in either group suggest that the heavy chains could have been present initially in both cell lines.

As shown in Table 2, the kappa light chain "d" (Genbank AF132308) is the limiting factor in the generation of functional scFvs from the hybridoma line producing mAb IFRN 1302. The successful amplification of light chain "c" (Genbank AF132309) from the less variable lambda gene family explains the higher levels of functional scFvs derived from hybridomas producing mAb IFRN 1301. The heavy chain from scFv AB01 was classified from the amino acid sequence as a member of the family XIX subgroup IB and possessed a lambda light chain from family I subgroup II (Kabat, 1991), whereas the heavy chain from AB02 belonged to the family VIII subgroup IIA and possessed a kappa light chain from family II subgroup I. These results taken together emphasize the importance of the use of an extended primer mix as discussed by Krebber et al. (1997). As shown in Figure 2, similar inhibition curves were obtained for scFv AB02 and mAb 1302, from which it was derived, in an ELISA. Recombinant antibody AB01 gave a lower limit of detection than both the mAb 1301, from which it was derived, and the purified polyclonal antibody. In general, the scFvs gave almost identical inhibition curves, with I_{50} of 1.6 μg/mL for IFRN AB01 and 1.0 μg/mL for AB02 (Table 1).

Cross-Reactivity. The antibodies and scFvs were further characterized by testing their cross-reactivity with a group of pesticides. As shown in Table 3 and as would be predicted from the immunogen structure, the scFvs AB01 and 02 and mAb 1302 recognized preferentially the ethyl ester of chlorpyrifos of the 28 organophosphorus structures tested by ELISA. In contrast, the polyclonal antibodies, although recognizing the ethyl ester, showed higher recognition of the methyl chlorpyrifos ester, as did mAb IFRN 1301, which also recognized tetrachlorvinphos strongly. The cross-reactivity studies showed that the antibodies were very specific toward the chlorinated structure in the aromatic ring. However, the thiophosphate ester part of the structure is also important, for none of the antibodies was able to bind to the breakdown product 3,5,6-trichloro-2-pyridinol. Unexpectedly, both the I_{50} results (Table 1) and the cross-reactivity study (Table 3) suggested that the scFv IFRN AB01 had a higher affinity for the free chlorpyrifos-ethyl than the mAb from which it had been derived. Assuming that the variable region sequence of scFv AB01 exactly matches the sequence of mAb 1301, then the different properties could be due to factors that would affect the protein folding in a prokaryotic expres-

Table 2. Variable Chain Content of the scFvs Produced from Hybridoma Cell Lines Secreting mAbs IFRN 1301 and IFRN 1302 Screened for the Presence of c-Myc and for Binding to Chlorpyrifos in the ELISA

	IFRN AB01[a]					IFRN AB02[b]				
ELISA[c]	V_H	%	V_L	%	total %	V_H	%	V_L	%	total %
c-Myc +/Chl +	heavy 2	100	light c	100	18	heavy 1	100	light d	100	1
c-Myc+/Chl −	heavy 1	100	light c	67	42					0
			light a[d]	33						
c-Myc −	heavy 1	75	light a	75	40	heavy 1	80	light a	60	99
	heavy 2	25	light b	25		heavy 2	20	light b	40	

[a] Ninety-six colonies were screened. [b] Seventy-two colonies were screened. [c] c-Myc peptide detection and chlorpyrifos-ethyl binding. [d] Mis-sense mutation S105*.

Table 3. Antibody Cross-Reactions (Percent)[a]

OP	polyclonal	mAb IFRN 1301	scFv IFRN AB01	mAb IFRN 1302	scFv IFRN AB02
chlorpyrifos-ethyl	100	100	100	100	100
chlorpyrifos-methyl	234	410	48	40	53
tetrachlorvinphos	53	350	3	4	4
others[b]	<1	<1	<1	<1	<1

[a] The polyclonal, monoclonal, and recombinant scFv antibodies' cross-reactivity as determined by the I_{50} in a competitive ELISA against 28 related prganophosphorus molecules. [b] The following OPs and derivatives were used: malathion, dichlorvos, edifenphos, vanidothion, mevinphos, pyrimiphos-ethyl, fenitrothion, dicrotophos, parathion-methyl, propetamphos, carbonofenothion, azamethiphos, etrimphos, metacriphos, cyanofenphos, chlorpyrifos-ethyl, methamidophos, pyrimiphos-methyl, chlorpyrifos-methyl, trichloropyridinol, diazinon, parathion-ethyl, tetrachlorvimphos, paraoxon-ethyl, paraoxon-methyl, fenamiphos, monocrotophos, and chlorfenvinphos.

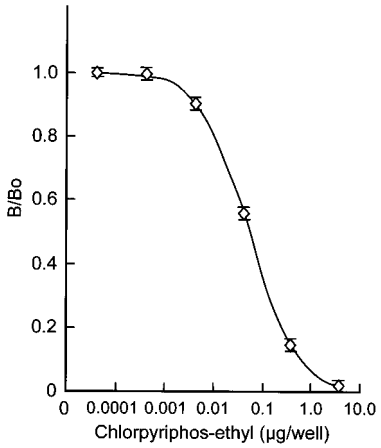

Figure 3. Chlorpyrifos ELISA using recombinant scFv−alkaline phosphatase. Plates were coated with chlorpyrifos−dextran as described under Materials and Methods. Each point represents the average of four determinations, and the error bars indicate one standard deviation. Crude *E. coli* soluble extract of scFv IFRN AB02−alkaline phosphatase derivative was diluted 5 times in PBST.

sion system (such as the failure of specialized chaperones to help in the folding process) or factors that directly affect the conformation of the scFv binding site (such as the length or structure of the artificial linker or disulfide cross-linking).

Limit of Detection. Although the results presented in Table 1 are compatible with the homologous ELISA system employed, lower detection limits have been described by other groups. Performance has been improved by using a heterologous system (Manclús et al., 1996) and the limit of detection further improved (by a factor of 10) by optimizing the assay conditions (Manclús and Montoya, 1996a,b). Lawruk et al. (1996), using a hapten design for antibody production similar to that of the Manclus group, presented an optimized magnetic particle based enzyme immunoassay format with an I_{50} of 0.94 ng/mL. Hill et al. (1994), choosing chlorpyriphos-methyl and conjugating the protein through the phosphate group, developed an ELISA based on polyclonal antibodies with an I_{50} of 0.2−0.6 ng/mL (Hill et al., 1994) but could not obtain mAbs against chlorpyrifos using this approach (Skerritt et al., 1992; Edward et al., 1993). High sensitivity in an ELISA format, although desirable, is not an intrinsic antibody characteristic in formats that do not use a "steady state" equilibrium but rather a dynamic on/off state. Comparison of the affinities of our antibodies and their optimal sensitivity by ELISA, and in different biosensor formats, are ongoing work.

scFv Antibody Derivatives. The detection system of ELISA relies on an enzymatic reaction after the unbound phase is removed. To produce directly labeled enzyme−antibody conjugates, the carboxy-terminal c-Myc sequences of scFvs IFRN AB01 and AB02 were replaced by the wild-type *E. coli* alkaline phosphatase. The crude scFv preparations were tested by competitive ELISA. Both clones showed high total alkaline phosphatase activity (data not shown). However, only the IFRN AB01 derivative (Figure 3) was able to bind free chlorpyrifos in an ELISA. The most likely explanation for this result might relate to the different features within the sequence that could affect the periplasmic folding process. Folding in vivo can be the limiting process in the production of many recombinant proteins, and it has been shown that the single most important factor affecting folding is the primary sequence of the antibody variable region because single amino acid substitutions can lead to aggregation of folding intermediates during in vivo and in vitro folding (Knappik and Pluckthun, 1995). Point mutations of particular amino acid residues at appropriate positions within scFv IFRN 1302 might improve expression without interfering with its binding properties.

Due to the low specific activity of the wild-type alkaline phosphatase used, a 3 h substrate incubation time was necessary to generate signals. Nevertheless, when compared with the scFv IFRN AB01 c-Myc derivative (Figure 2B), the alkaline phosphatase derivative showed a slight shift toward lower chlorpyrifos concentrations with an I_{50} of 500 ng/mL of chlorpyrifos. Long substrate incubation times can be a problem, and ways of shortening the incubation time by using higher specific activity alkaline phosphatases have been described (Kerschbaumer et al., 1997). In their construct, an alkaline phosphatase with up to 35-fold higher specific activity was obtained by the substitution of aspartate to serine in the active site. Furthermore, a polyhistidine tail was engineered into the alkaline phosphatase gene to facilitate the immobilized metal-

ion affinity chromatography (IMAC) purification, which would be an obvious starting point for future work.

The His-tag, consisting of four to six consecutive histidine residues genetically fused to recombinant proteins, has been in use for several years to purify proteins by IMAC (Müller et al., 1998). To enhance expression and facilitate scFv purification, His-tag derivatives carrying a much stronger Shine−Dalgarno sequence (pAK400; Krebber et al., 1997) were produced. Although higher levels of expression were achieved and the purification by IMAC simplified the purification procedure, the detection of this derivative with commercially available anti-His tag antibodies was not straightforward. PAK400 derivatives produced only faint bands in an immunoblot and no signal in the ELISA with the commercially available anti-His-tag antibodies (data not shown) but were easily detected by immunoblot using the FLAG N-terminal sequence. Nevertheless, utilization of the His-tagged scFvs will only be fully exploited by using direct immobilization to a metal surface or to an immobilized antibody in a biosensor surface as described by Müller et al. (1998).

Conclusion. Recombinant scFv antibodies have been produced from mAb-producing hybridoma cell lines and compared to the mAbs and to polyclonal antibodies produced with the same immunogen. One of the scFvs (IFRN AB02) showed characteristics very similar to those of its parent mAb; however, the other scFv (IFRN AB01) had a different pattern of cross-reactivity and a lower limit of detection when compared with its parent mAb. Although the two scFvs had very similar characteristics, sequencing results showed that they had different heavy and light chains. Sequencing of nonfunctional scFvs from both mAbs revealed aberrant light chains and highlighted the problems associated with producing scFvs from hybridomas and the need for a comprehensive mix of primers.

Production of a recombinant scFv fused to alkaline phosphatase produced an ELISA with a lower limit of detection compared to the usual format using a second labeled antibody. The construct could be useful in a chlorpyrifos ELISA once the specific activity of the enzyme is increased to allow a shorter detection time.

ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assay; Fv, fragment of antibody variable region; IPTG, isopropyl β-D-thiogalactopyranoside; LOD, limit of detection; mAb, monoclonal antibody; PCR, Polymerase Chain Reaction; scFv, single-chain Fv fragment; SOE-PCR, splicing by overlap extension PCR; V_H, heavy chain variable domain; V_L, light chain variable domain.

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