Development of an Enzyme-Linked Immunosorbent Assay for 3,5,6-Trichloro-2-pyridinol. 1. Production and Characterization of Monoclonal Antibodies

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3,5,6-Trichloro-2-pyridinol (TCP) is the major degradation product of both the widely used chlorpyrifos and chlorpyrifos-methyl insecticides and the herbicide triclopyr. In this paper we describe the development of immunoassays for the detection of this metabolite. With this aim, two types of haptens were synthesized, with spacer attachment either through the 6-position of the pyridyl ring or through the hydroxyl group. Protein conjugates of these haptens were used for the immunization of mice to produce monoclonal antibodies (MAbs). From haptens of the former type, a panel of MAbs displaying 50% inhibition (I_{50}) for TCP in the range 1−10 nM was obtained. Characterization of these MAbs showed their high affinity and specificity for TCP. The best MAbs were used in various assay formats in combination with homologous and heterologous assay haptens. Several immunoassays with I_{50} lower than 1 nM were developed. These immunoassays are potentially very valuable analytical tools for the determination of TCP in food and in the environment, as well as for monitoring human exposure to chlorpyrifos.

Keywords: *3,5,6-Trichloro-2-pyridinol (TCP); chlorpyrifos; monoclonal antibodies; ELISA*

INTRODUCTION

As a consequence of the widespread use of pesticides, the presence of their residues in food and in the environment has become an important issue in analytical science. There is a growing concern regarding the potential toxicity and/or ecotoxicity of the transformation products associated with these residues, which is demanding the development of appropriate analytical techniques for their monitoring (Somasundaran and Coats, 1991).

3,5,6-Trichloro-2-pyridinol (TCP) is the major degradation product of the insecticide chlorpyrifos [*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] and chlorpyrifos-methyl [*O*,*O*-dimethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] and of the herbicide triclopyr [[(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid] (Woodburn *et al*., 1993; Racke, 1993). Both biotic and abiotic transformation processes participate in the formation of TCP from parent pesticides within environmental and biological compartments. TCP is a polar compound that displays very different physical and chemical properties than the chlorpyrifos insecticides, and therefore exhibits different mobility and persistence in the environment (Racke, 1993). The presence of TCP in human urine is considered as a biomarker of exposure to chlorpyrifos insecticides (Nolan *et al*., 1984).

Chlorpyrifos insecticides are widely used for agricultural and nonagricultural purposes (Worthing and Hance, 1991; Racke, 1993), which could explain their high residue occurrence in food as reported in recent comprehensive survey programs (U.S. FDA, 1994; Neidert *et al*., 1994). Concerning TCP, scarce data on its determination in real food or environmental samples have been reported (Cohen *et al*., 1990). However, a study of human exposure to organophosphates, carried out during 1976−1980, showed the presence of detectable amounts of TCP in 5.8% of urine samples (Kutz *et al*., 1992), which is consistent with the high occurrence of the parent pesticides in food.

Current routine multiresidue methods applied by regulatory or control agencies for the measurement of parent chlorpyrifos insecticides are not amenable for TCP determinations because of the different physicochemical properties of TCP. TCP determinations have been carried out mainly by gas chromatography (GC) involving specific analytical procedures. TCP has been extracted from solid matrices with water-miscible solvents in alkaline conditions, acidified, and subjected to liquid-liquid partitioning. A variable number of cleanup steps have been applied depending on the matrix, but in all the cases a derivatization step of TCP to silane derivatives or methyl ethers was required for sample preparation prior to GC analysis. This methodology has been applied to determine TCP in several matrices, such as citrus (Iwata *et al*., 1983), bananas (U.S. FDA, 1977), urine (Kutz *et al*., 1992; Bartels and Kastl, 1992), and dates (Mourer *et al*., 1990), with limits of detection varying from 0.5 to 50 ng/mL (2.5 to 250 nM). Such cumbersome analytical procedures are not appropriate for the rapid and cost-effective determination of TCP in large sample loads. As an alternative method, immunochemical techniques are gaining a position within the analysis of agrochemicals because of their simplicity, cost-effectiveness, and high sample throughput (Sherry, 1992). In this respect, several immunoassays have been developed for chlorpyrifos pesticides (Skerritt *et al*., 1992; Manclús *et al*., 1994; Hill *et al*., 1994), and all of them show low cross-reactivity with TCP. Concerning the degradation products of organophosphorous pesticides, immunoassays for substituted phenolic compounds have also been developed (Li *et al*., 1991), but none has been reported for pyridyl compounds such as TCP. The objective of this work was to

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develop immunoassays for TCP as an alternative to chromatographic techniques.

Hapten design is a key step in the development of immunoassays for small molecules like TCP because the hapten is primarily responsible for determining antibody recognition properties. To explore the maximum number of possibilities to obtain good quality antibodies, as well as for further assay improvement, the synthesis of a number of haptens with different spacer arms attached through different molecule sites is generally recommended (Harrison *et al.*, 1990). Herein, the synthesis of several haptens and their use to produce monoclonal antibodies (MAbs) to TCP is described. The characterization of the MAbs for sensitivity and specificity is presented, and the influence of heterologous haptens in different assay formats is evaluated for the development of immunoassays to this pesticide degradation product.

MATERIALS AND METHODS

Reagents and Instruments. Chlorpyrifos, chlorpyrifos-methyl, TCP standards, and technical grade chlorpyrifos were generously provided by DowElanco (Midland, MI). Starting products for the synthesis of haptens, compounds used in the cross-reactivity studies, and hapten-protein coupling reagents were obtained from Fluka-Aldrich Química (Madrid, Spain). Analytical grade solvents were from Scharlau (Barcelona, Spain). Thin-layer chromatography (TLC) was performed on 0.2-mm pre-coated silica gel 60 F_{254} on aluminum sheets from Merck (Darmstadt, Germany). Column chromatography was carried out in silica gel (0.063−0.2-mm particle size, 70−230 mesh), also from Merck.

Ovalbumin (OVA), Freund's adjuvants, pristane, and *o*-phenylenediamine (OPD) were obtained from Sigma Química (Madrid, Spain). Bovine serum albumin fraction V (BSA), enzyme-immunoassay-grade horseradish peroxidase (HRP), and polyethyleneglycol (PEG) 1500 were purchased from Boehringer Mannheim (Germany). Culture media and supplements were from GibcoBRL (Paisley, Scotland).

Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained with a Varian VR-400S spectrometer (Sunnyvale, CA) that was operated at 400 MHz. Chemical shifts are reported relative to tetramethylsilane. Ultraviolet−visible (UV−vis) spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan). Enzyme-linked immunosorbent assay (ELISA) plates were washed with an Ultrawash II plate washer, and well absorbances were read with a MR 700 plate reader, both from Dynatech (Sussex, U.K.).

Hapten Synthesis. TCP derivatives that were used as immunizing haptens were prepared by introduction of different alkyl chain spacers, ending in a carboxylic acid, in two sites of the pyridyl ring. In the first type of haptens, the spacer was attached through a thioether linkage to the 6-position of the ring (Figure 1, haptens **1** and **2**). The second type of haptens involved *O*-alkylation of TCP (Figure 1, haptens **3** and **4**). Compound **5**, with both attachment site and spacer modifications, was synthesized to be used as heterologous hapten.

*3-(3,5-Dichloro-6-hydroxy-2-pyridyl)thiopropanoic Acid (**1**).* This hapten came from a chlorpyrifos hapten (*O*,*O*-diethyl *O*-[3,5-dichloro-6-[(2-carboxyethyl)thio]-2-pyridyl]phosphorothioate) previously synthesized by direct substitution of the chlorine in the 6-position of the pyridyl ring of chlorpyrifos by a 3-mercaptopropanoic acid spacer arm (Manclús *et al.*, 1994). Hapten **1** was then prepared by hydrolysis of the thiophosphate ester as follows. To a solution of *O*,*O*-diethyl *O*-[3,5-dichloro-6-[(2-carboxyethyl)thio]-2-pyridyl] phosphorothioate (250 mg) in tetrahydrofuran (THF, 3 mL) was added 1 M NaOH (5 mL), and the mixture was refluxed for 1 h. After adding distilled water (25 mL), the solution was acidified to pH 3 with 2 M HCl and extracted with ethyl acetate (EtAc; 3 × 30 mL). The combined organic extracts were dried over anhydrous sodium sulfate, and the solvent evaporated to give

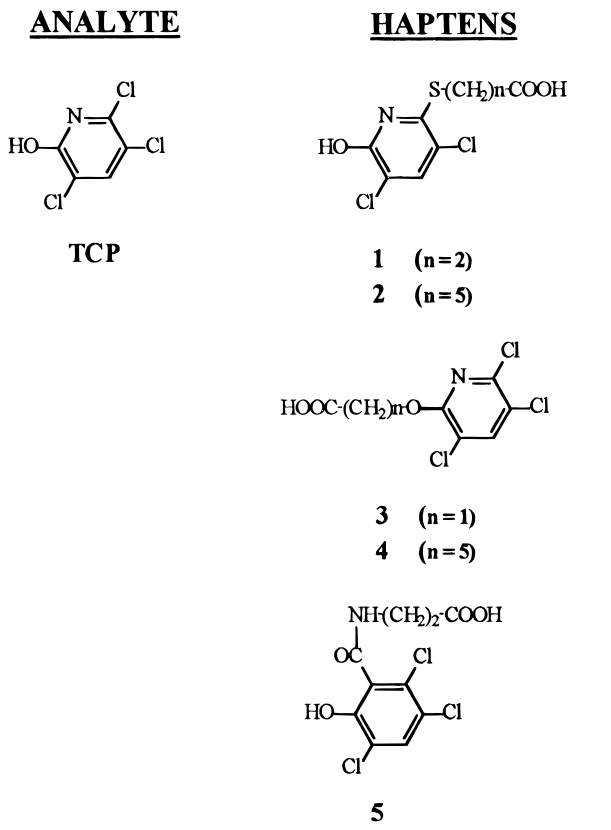


Figure 1. Structures of TCP and the haptens synthesized for this study.

1 (140 mg, 87%) as a white solid: ^{1}H NMR (dimethylsulfoxide-d_6) δ 7.92 (s, 1H, ArH), 3.26 (t, 2H, SCH_2), 2.66 (t, 2H, CH_2-COO).

*3-(3,5-Dichloro-6-hydroxy-2-pyridyl)thiohexanoic Acid (**2**).* Hapten **2** was prepared following the same synthesis strategy as for **1**, but starting from a chlorpyrifos hapten bearing a 6-mercaptohexanoic acid spacer. First, this acid was prepared according to Gee *et al.* (1988). Then, to a solution of 6-mercaptohexanoic acid (1 g, 6.75 mmol) in absolute ethanol (30 mL), KOH (0.756 g, 13.5 mmol) was added and heated until dissolved. Next, technical grade chlorpyrifos (2.37 g, 6.75 mmol) dissolved in absolute ethanol (30 mL) was added. After refluxing for 1 h, the reaction mixture was filtered, and the solvent was evaporated. To the residue was added 5% $NaHCO_3$ (50 mL) followed by washing with hexane (Hx; 2 × 50 mL). The aqueous layer was acidified to pH 3 and extracted with dichloromethane (DCM; 3 × 50 mL). The extract was dried over sodium sulfate and concentrated, and the residue was subjected to column chromatography (Hx:THF:acetic acid, 75:25:1). Combination of the fractions showing only one spot on TLC (R_f 0.37, same solvent) provided the chlorpyrifos hapten, *O*,*O*-diethyl *O*-[3,5-dichloro-6-[(5-carboxypenthyl)thio]-2-pyridyl] phosphorothioate (220 mg, 17%): ^{1}H NMR ($CDCl_3$) δ 7.63 (s, 1H, ArH), 4.33 (q+q, 4H, 2 CH_2O), 3.18 (t, 2H, SCH_2), 2.38 (t, 2H, CH_2COO), 1.6 (3m, 6H, 3 CH_2), 1.41 (t, 6H, 2 CH_3). Then, to a solution of this compound (150 mg) in THF (3 mL) was added 1 M NaOH (5 mL), and the mixture was refluxed for 1 h. Subsequent acidification, extraction with EtAc, and evaporation of the solvent gave **2** (75 mg, 75%) as a white solid: ^{1}H NMR (dimethylsulfoxide-d_6) δ 7.90 (s, 1H, ArH), 3.11 (t, 2H, SCH_2), 2.20 (t, 2H, CH_2COO), 1.51 (m, 6H, $3CH_2$).

*[(3,5,6-Trichloro-2-pyridyl)oxy]acetic Acid (**3**).* Hapten **3** is the herbicide triclopyr and was purchased from Riedel-de Haën (Germany).

*[(3,5,6-Trichloro-2-pyridyl)oxy]hexanoic Acid (**4**).* A mixture of ethyl 6-bromo-hexanoate (1.02 g, 4.6 mmol) and sodium 3,5,6-trichloro-2-pyridinolate (750 mg, 3.4 mmol) in acetonitrile (12 mL) was refluxed for 5 h. Filtration of the mixture, concentration of the filtrate, and column chromatography (Hx: EtAc, 80:20) gave the ethyl ester of **4** (1030 mg, 88%); TLC R_f

0.73 (same solvent). The ester was dissolved in THF (2 mL), and 2 M NaOH (25 mL) was added. After refluxing for 1 h, the mixture was acidified to pH 3 and extracted with DCM (2 × 50 mL). The extract was dried over anhydrous sodium sulfate and concentrated. Column chromatography (Hx:EtAc: acetic acid, 65:35:1) gave **4** (561 mg, 60%) as a white solid: TLC R_f 0.63 (same solvent); ^{1}H NMR ($CDCl_3$) δ 8.11 (s, 1H, ArH), 4.42 (t, 2H, CH_2O), 2.38 (t, 2H, CH_2COO), 1.72 (3m, 6H, 2 CH_2).

*3-[(2-Hydroxy-3,5,6-trichlorophenyl)methanamido]propanoic Acid (**5**).* The *tert*-butyl ester of this acid was synthesized as described previously (Manclús *et al.*, 1994). Then, the ester (100 mg) was treated with 50% trifluoroacetic acid in DCM (2 mL) for 1 h. After evaporation of the solvent, column chromatography (Hx:EtAc:acetic acid, 65:35:1) gave **5** (76 mg): TLC R_f 0.38 (same solvent mixture); ^{1}H NMR (acetone-d_6) δ 7.65 (s, 1H, ArH), 3.67 (t, 2H, NCH_2), 2.68 (t, 2H, CH_2-COO).

Preparation of Protein−Hapten Conjugates. All haptens used in this study contained a carboxylic group and were conjugated to proteins by the *N*-hydroxysuccinimide (NHS)-active ester method according to Langone and Van Vunakis (1975), with slight modifications.

Immunogenic and Coating Conjugates. Typically, haptens [~25 μmol in the appropriate volume of *N*,*N*-dimethylformamide (DMF) to bring the final concentration of hapten to 100−200 mM] were activated during 2 h of incubation at room temperature with a 50% molar excess of NHS and dicyclohexylcarbodiimide. Next, the mixture was centrifuged, and the supernatant collected. To a solution of 10 mg/mL protein (BSA for immunogens, OVA for coating conjugates) in 0.2 M borate buffer (pH 9.0) was added, over a 10 min period and with vigorously stirring, the activated ester mixture diluted in the volume of DMF necessary to bring the solution to 20% DMF. The initial hapten-to-protein molar ratio in the mixture was 50:1 for immunogens and 30:1 for coating conjugates. The mixture was stirred at room temperature for 2 h. Finally, the conjugates were separated from uncoupled hapten by gel filtration on Sephadex G-25, using PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4) as eluant. Conjugate formation was confirmed spectrophotometrically. UV−vis spectra showed qualitative differences between carrier proteins and conjugates in the region of maximum absorbance of haptens (320−330 nm for haptens **1** and **2**; 295−300 nm for haptens **3** and **4**). The hapten-to-protein molar ratio of conjugates was estimated from the spectral data of the hapten, the protein, and the corresponding conjugate. Assuming that the molar absorptivity of haptens was the same for the free and conjugated forms, the apparent molar ratios of the conjugates were 25, 17, 35, 22 for BSA-**1**, **2**, **3**, **4**, respectively, and 12, 6, 15, 9, 10 for OVA-**1**, **2**, **3**, **4**, **5**, respectively.

Enzyme Conjugates. HRP was used to prepare the enzyme tracers. Following the same procedure as before, haptens **1**, **2**, and **5** were activated and then conjugated to HRP (5 mg/mL) with a 20-molar excess of activated hapten. Enzyme tracers were purified by gel filtration and stored at 4 °C in a 1:1 mixture of saturated ammonium sulfate and PBS containing 0.1% BSA. HRP conjugate concentrations and molar ratios were estimated spectrophotometrically. With the same assumptions as before, the estimated molar ratios were ~2 for HRP-**1**, **2**, and **5**.

Production of Monoclonal Antibodies to TCP. *Immunization.* Three BALB/c female mice (8−10 weeks old) were immunized with each BSA conjugate of the haptens **1**−**4**. The first dose consisted of 100 μg of conjugate injected intraperitoneally (ip) as an emultion of PBS and complete Freund's adjuvant. Two and four weeks after the initial dose, mice received a booster injection with the same amount of immunogen emulsified in incomplete Freund's adjuvant. One week after the last injection, mice were tail-bled, and sera was tested for anti-hapten antibody titer by indirect ELISA and for analyte recognition properties by competitive indirect ELISA. Four days prior to cell fusion, mice selected to be spleen donors for hybridoma production received a final soluble ip injection of 100 μg of conjugate in PBS.

Cell Fusion. P3-X63/Ag 8.653 murine myeloma cells (ATCC; Rockville, MD) were cultured in high-glucose Dulbecco's Modified Eagle's Medium supplemented with 2 mM L-glutamine, 1 mM nonessential amino acids, 25 μg/mL gentamicin, and 15% fetal bovine serum (referred to as s-DMEM). Cell fusion procedures were carried out essentially as described by Nowinski *et al.* (1979). Mouse spleen lymphocytes were fused with myeloma cells at a 5:1 ratio, with PEG 1500 as the fusing agent. The fused cells were distributed in 96-well culture plates at an approximate density of 4×10^5 cells/100 μL of s-DMEM per well. Twenty-four hours after plating, 100 μL of HAT selection medium (s-DMEM suplemented with 100 μM hypoxanthine, 0.4 μM aminopterine, 16 μM thymidine) was added to each well. Half the medium of the wells was replaced with fresh HAT medium on days 4 and 7 post-fusion. Cells were grown in HAT medium for 2 weeks and then HAT was substituted by HT medium (HAT medium without aminopterine).

Hybridoma Selection and Cloning. Eight to ten days after cell fusion, culture supernatants were screened for the presence of antibodies that recognized TCP. The screening consisted of the simultaneous performance of a noncompetitive and a competitive indirect ELISA, to test the ability of antibodies to bind the OVA conjugate of the immunizing hapten and to recognize TCP, respectively. Coating conjugate concentrations used in the screenings were those selected when evaluating the analyte recognition by mouse sera, and culture supernatants were appropriately diluted to obtain ELISA absorbance below 2.0. The analyte concentration for competition assays was established at convenience. For the first screenings the cutoff concentration was in the micromolar order, and thereafter it was lowered to search for the highest affinity antibodies. Selected hybridomas were cloned by limiting dilution on a feeder layer of BALB/c thymocytes (~10^6 cells/well) and peritoneal macrophages (~5000 cells/well). Stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen.

Purification of Monoclonal Antibodies. Antibodies were purified on a small scale directly from late stationary phase culture supernatants by affinity chromatography on Protein A-agarose (Pharmacia, Sweden) according to the procedure of Schuler and Reinacher (1991). For scaling-up, ascitic fluid was obtained from pristane-primed mice. Antibodies were purified from clarified ascites by saline precipitation followed by anion-exchange chromatography on DEAE-Sepharose (Sigma).

Enzyme-Linked Immunosorbent Assays. Flat-bottomed polystyrene ELISA plates (Corning #25805-Easy Wash 96, Corning, NY) were coated overnight with conjugate or antibody solutions in 50 mM carbonate buffer (pH 9.6). Standards were prepared in PBS by serial dilutions from a stock solution in DMF. A volume of 100 μL per well was used throughout all assay steps. All incubations were carried out at room temperature. After each step, plates were washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20). Two basic formats were used depending on the assay component immobilized into the ELISA plates. In the conjugate-coated format, a noncompetitive indirect ELISA was used to estimate mouse serum antibody titers and for the screening of culture supernatants, and a competitive indirect ELISA was used for the study of antibody sensitivity and specificity to TCP. In the antibody-coated format, a competitive direct ELISA was followed to evaluate the assay properties with different enzyme tracers. For competition assays, the concentrations of antibodies, hapten conjugates, or enzyme tracers were optimized by checkerboard titration. Usually, several combinations of the immunoreagents, under subsaturating conditions giving absorbances around 1.0, were evaluated to select those providing the highest sensitivity. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four-parameter logistic equation (Raab, 1983) with the Sigmaplot software package (Jandel Scientific, Germany).

Conjugate-Coated Format (Indirect ELISA). Plates were coated with the selected concentrations of hapten-OVA conjugates. Then, serum, culture supernatant, or antibody dilutions in PBS containing 0.05% Tween 20 (PBST) were added

and incubated for 1 h. Next, plates were incubated for 1 h with peroxidase-labeled rabbit anti-mouse immunoglobulins (Dako, Denmark) diluted 1/2000 in PBST. Finally, peroxidase activity bound to the wells was determined by adding the substrate solution (2 mg/mL OPD and 0.012% H_2O_2 in 25 mM citrate, 62 mM sodium phosphate, pH 5.35). After 10 min, the reaction was stopped with 2.5 M sulfuric acid, and absorbance at 490 nm was read and recorded. For competitive assays, the procedure was the same except that after coating, a competition step was introduced by adding 50 μL of the competitor (TCP or related compounds) followed by 50 μL of the appropriate concentration of antibody (serum, culture supernatant, or purified MAb).

Antibody-Coated Format (Direct ELISA). In this format, plates were coated with antibodies at the selected concentrations. Next, the competition was established for 1 h between TCP standards and the selected dilutions of enzyme tracers (hapten-HRP conjugates). Peroxidase activity was measured as just described.

RESULTS AND DISCUSSION

Synthesis of Immunizing Haptens. Regarding its immunogenic structure, TCP is a small, simple analyte, consisting of an aromatic ring with substituents (Figure 1). An appropriate hapten design should preserve as many ring substituents as possible and produce minor modifications in the ring electronic distribution as a consequence of spacer attachment. Chemical properties of the TCP molecule are determined by the ring substituents and the heteroatom, being the ionizable hydroxyl group, and the activated chlorine in the 6-position of the ring putative sites for spacer attachment. Consequently, two types of TCP haptens were prepared. The first type was prepared by removing the thiophosphate group of haptens previously synthesized for the parent chlorpyrifos pesticide (Manclús *et al*., 1994). For these haptens, derivatization was accomplished by substitution of the chlorine in the 6-position with appropriate mercaptoalkyl acids (2 and 5 methylene groups for haptens **1** and **2**, respectively; Figure 1). The only structural modification introduced in these haptens was the spacer coupling as a thioether linkage instead of one of the chlorine substituents. This way, the chemical properties of TCP may be well preserved, as suggested by the successful use of similar thioether linkage for spacer coupling to synthesize triazine haptens (Goodrow *et al*., 1990).

In the second class of haptens, the spacer arm was attached through the hydroxyl group by *O*-alkylation. Hapten **3** with a 2-carbon spacer is the commercially available herbicide triclopyr. Hapten **4** with a longer spacer (6-carbon) was synthesized from TCP and the suitable alkyl bromide. In these cases, the hydroxyl group was clearly modified, whereas all chlorine substituents remained present (Figure 1).

Production of Antibodies to TCP. *Mouse Polyclonal Response.* To evaluate the suitability of the synthesized haptens to raise anti-TCP antibodies, mice were immunized with BSA conjugates of haptens **1**, **2**, **3**, and **4**. After three injections, the titer (serum dilution giving three times background absorbance) of antibodies recognizing conjugated haptens was estimated by indirect ELISA using the respective homologous OVA-hapten as coating conjugate. All immunogens showed high anti-hapten reactivity with titers in the $1/3 \times 10^5 - 1/10^6$ range. Sera were subsequently tested for their ability to recognize TCP by indirect competitive ELISA. Only sera from mice immunized with BSA-**1** bound competitively with TCP, with a concentration producing 50% inhibition of antibody binding (I_{50}) of 15 μM TCP. No inhibition was found for the rest of the sera with TCP up to 50 μM.

Table 1. Summary of Cell Fusion and Hybridoma Selection Results

immunizing hapten	fusion no.	no. of wells seeded	no. of wells positive[a] (hapten)	no. of wells competitive[b] (analyte)	no. of cloned hybridomas[c]
1	1	480	121	3	1
	2	384	56	–[d]	–
	3	384	251	8	4
2	4	480	260	–	–
3	5	384	292	–	–
	6	480	98	–	–
4	7	384	175	–	–

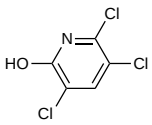
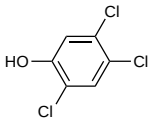
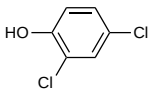
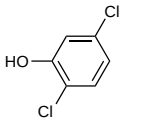
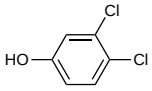
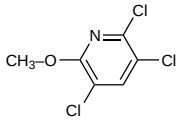
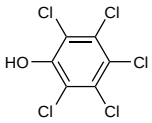
[a] Wells with antibodies that recognized the OVA-hapten conjugates (homologous assays) by indirect ELISA (absorbance > 1.0). [b] Wells with antibodies that recognized free TCP (inhibition > 50% by 1 μM TCP for fusions no. 1 and 4–7, and 100 nM TCP for fusions no. 2 and 3). Competitive ELISAs were carried out with the OVA-hapten concentrations previously selected for evaluating mouse sera, and supernatants giving absorbances out of range were diluted until entering the range (absorbance < 2). [c] Only hybridomas secreting antibodies with the lowest I_{50} for TCP were stabilized and cloned. [d] Indicates that none was found.

Production of MAbs. The immunological properties of mouse polyclonal sera and MAbs raised to the same haptenic structure are often fairly different. In fact, MAbs with an affinity to the analyte up to four orders of magnitude better than that of the mouse sera have been obtained (Abad and Montoya, 1994). Moreover, absence of analyte recognition by the heterogeneous polyclonal mouse serum does not preclude that MAbs with such ability may be found. Then, although an anti-TCP polyclonal response was demonstrated only with the BSA-**1** immunogen, at least one cell fusion was undertaken for each immunizing hapten. As the objective of this work was to obtain high affinity antibodies to TCP, additional fusion effort was focused mainly on mice immunized with haptens that gave positive results in the first fusion.

Cell fusion and hybridoma selection results are summarized in Table 1. In general, all fusions rendered a high yield of wells with antibodies recognizing the corresponding homologous conjugated haptens, but only a few of these wells showed reactivity towards TCP. In fact, with the concentration cutoff of 1 μM TCP selected for initial screenings, competitive wells were found only in the fusion carried out from the immunizing hapten **1**. The first fusion rendered three wells with antibodies recognizing TCP. One of them, showing the highest affinity for TCP (I_{50} < 100 nM), was cloned by limiting dilutions. For fusions 2 and 3, the TCP concentration used to screen culture supernatants was lowered to 100 nM. In fusion 2, none of the wells contained antibodies showing >50% inhibition by this cutoff concentration. However, fusion 3 was especially successful, because of the 251 wells considered positive by indirect ELISA using OVA-**1** as coating conjugate, eight showed >50% inhibiton of binding by 100 nM TCP in the competitive assay. Antibodies from these supernatants were subsequently tested for inhibition by lower TCP concentrations. Finally, four hybridoma cell lines showing I_{50} values ranging from 1 to 10 nM TCP were cloned and stabilized.

With the hybridoma production and selection procedures performed from TCP haptens **1**, **2**, **3**, and **4**, high-affinity anti-TCP MAbs were obtained solely from hapten **1**. The main structural difference between hapten **1** and haptens **3** and **4** was that the ionizable

Table 2. MAb Recognition of Compounds Structurally Related with TCP[a]

compound	structure	LIB-MC2		LIB-MC3	
		I_{50} (nM)	CR[b] (%)	I_{50} (nM)	CR[b] (%)
3,5,6-trichloro-2-pyridinol (TCP)		1	100	1.3	100
2,4,5-trichlorophenol		50	2	65	2
2,4-dichlorophenol		960	0.1	1300	0.1
2,5-dichlorophenol		1240	0.08	1820	0.07
3,4-dichlorophenol		17400	<0.01	18100	<0.01
2-methoxy-3,5,6-trichloropyridine		38400	<0.01	64000	<0.01
pentachlorophenol		3730	0.02	4080	0.03

[a] Data were obtained from competitive indirect ELISAs; the coating conjugate was OVA-**1** (10 ng/mL) for both MAbs, LIB-MC2 (30 ng/mL) and LIB-MC3 (50 ng/mL). [b] Percentage of cross-reactivity = (I_{50} of TCP/I_{50} of other compound) × 100.

hydroxyl group was exposed and masked, respectively. These results suggest that the negatively charged oxygen is a strong determinant of the immune response. Moreover, in this case, the spacer length also seems to exert a marked effect on the hapten immunogenic properties, because hapten **1** with a shorter methylene spacer (two carbons) gave better results than hapten **2** with a longer spacer arm (five carbons).

Characterization of the MAbs. The four MAbs selected were further characterized regarding their affinity and specificity to TCP, using the homologous competitive indirect ELISA. LIB-MC2 and LIB-MC3 MAbs, both derived from fusion 3, showed the highest affinity to TCP, with I_{50} values of 1 and 1.3 nM, respectively.

Specificity. The specificity of these MAbs was evaluated with compounds of closely related molecular structure as competitors, their respective I_{50} were obtained, and these data were compared with TCP I_{50} values. Cross-reactivity (CR) values for each compound are given in Table 2. LIB-MC2 and LIB-MC3 MAbs showed an almost identical CR pattern, both being highly specific for TCP; that is, none of the compounds tested had CR values >2%. A simple change of the pyridyl analyte to the phenyl compound markedly reduced the recognition (2% CR with 2,4,5-trichlorophenol), indicating the importance of the nitrogen in the immunological interaction. The recognition diminished even more for the three related dichlorophenol compounds, especially if the chlorine adjacent to the hydroxyl group was not present. The CR of 2-methoxy-3,5,6-trichloropyridine, a minor metabolite of chlorpyrifos and chlorpyrifos-methyl, was negligible. This finding indicates that the modification of the hydroxyl group, in this case by methylation, drastically hinders the MAb binding to the compound, which again supports the strong participation of the charged oxygen in the analyte−antibody interaction. Pentachlorophenol was also very poorly recognized by the MAbs, and no significant cross-reactivity was found for other related agrochemicals (triclopyr, 4-nitrophenol, 3-methyl-4-nitrophenol) or chemicals (phenol). The parent insecticides chlorpyrifos and chlorpyrifos-methyl cross-reacted weakly (0.2% CR), although these apparent cross-reactions may have actually been caused by minimum amounts of TCP present in the chlorpyrifos stock solution or produced in the assay medium in the course of the experiment.

Evaluation of Hapten Heterology and Assay Format. Once antibodies to a particular analyte have been obtained, it is well-known that the immunoassay properties, primarily the sensitivity, can be affected by the conjugated hapten used for competition and by the assay format (Marco *et al*., 1995). Therefore, to design the most suitable ELISA for TCP, LIB-MC2 and LIB-MC3 MAbs were incorporated into different formats, in combination with homologous and heterologous haptens. Two basic formats were evaluated: the conjugate-coated and the antibody-coated formats. Hapten **2**, with a longer spacer arm, haptens **3** and **4**, with spacer attachment through the hydroxyl group, and hapten **5**, with a different spacer attached in an adjacent position, were used for heterology evaluation.

Using the conjugate-coated format, both MAbs recognized the OVA conjugates of the heterologous haptens

Table 3. I_{50} (nM) Data for TCP Obtained with Different Haptens and Formats[a]

	indirect ELISA (OVA-hapten)			direct ELISA (HRP-hapten)	
MAb	**1**	**2**	**5**	**1**	**2**
LIB-MC2	0.98	1.61	3.40	0.93	1.41
LIB-MC3	1.34	1.60	0.60	1.55	1.91

[a] Competitive ELISAs were performed in optimum conditions (*i.e.*, limiting concentrations of immunoreagents giving maximum absorbance ~1.0).

2 and **5**. On the contrary, and not surprisingly, OVA-**3** and OVA-**4** were not recognized by the MAbs. After selecting optimum conditions, high-affinity assays were obtained with all recognized haptens, as shown in Table 3. For LIB-MC2, the I_{50} value of the homologous assay (0.98 nM) was not improved by the heterologous assays. For LIB-MC3, the OVA-**5** conjugate provided the lowest I_{50} for TCP (0.60 nM), although much higher immunoreagent concentrations were required. This different behavior in the recognition of hapten **5** represented the unique significant difference found between LIB-MC2 and LIB-MC3, because both showed an identical specificity pattern and similar I_{50} values for the rest of haptens.

To evaluate the antibody-coated format, HRP tracers of haptens **1**, **2**, and **5** were prepared. The HRP-**5** tracer did not yield a useful assay because of the high tracer concentration necessary to provide an appropriate signal in the noncompetitive assay. As shown in Table 3, competitive assays performed with enzyme tracers of haptens **1** and **2** gave I_{50} values similar to those obtained in the conjugate-coated format. A modification of the antibody-coated format, consisting of the indirect coating of MAbs on goat anti-mouse IgG precoated plates, was also evaluated. This format afforded a reduction of the MAb concentration required to give similar maximum absorbance than that obtained with the direct coating, but it did not significantly modify the assay sensitivity.

Competitive curves for LIB-MC2 and LIB-MC3 MAbs, in the two formats evaluated and with the assay hapten giving the lowest I_{50} for TCP, are presented in Figure 2. As shown, a variety of immunoassays for TCP can be designed, displaying similar standard curves. For a particular application, the choice among these immunoassays should be based on their performance on the matrix in which the determination of TCP is intended to be carried out.

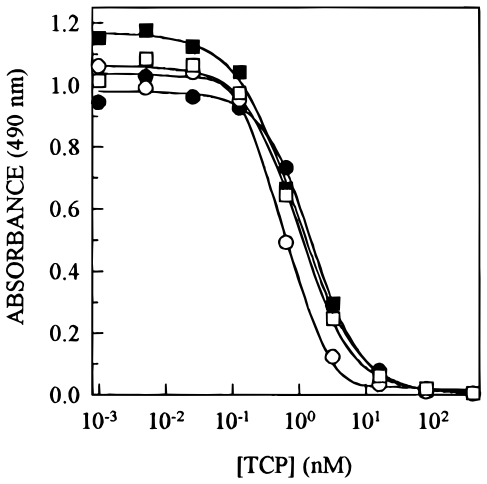


Figure 2. TCP competitive curves obtained with LIB-MC2 (squares) and LIB-MC3 (circles) MAbs in the indirect (clear symbols) and direct (solid symbols) ELISA formats, using the assay hapten showing the lowest I_{50} in each case. (□) OVA-**1** (10 ng/mL) and LIB-MC2 (30 ng/mL); (○) OVA-**5** (500 ng/mL) and LIB-MC3 (100 ng/mL); (■) LIB-MC2 (1 μg/mL) and HRP-**1** (5 ng/mL); (●) LIB-MC3 (1 μg/mL) and HRP-**1** (13.3 ng/mL).

CONCLUSIONS

Following general guidelines about hapten design, several TCP haptens were synthesized. Immunogens from all haptens elicited a high titer of antibodies recognizing the immunizing hapten conjugate, but only the polyclonal response from hapten **1** showed the presence of anti-TCP antibodies. Similarly, high-affinity MAbs to TCP (I_{50} values in the nanomolar range) were solely obtained from mice immunized with hapten **1** immunogens. For designing haptens to TCP, the hydroxyl group, which is negatively charged at neutral pH, has been demonstrated to be an important immunological determinant. Therefore, leaving the hydroxyl group free along with coupling of an appropriate spacer through another ring position are essential requirements to obtain suitable mimics of the TCP molecular structure. From cross-reactivity studies with structurally related compounds, MAbs proved to be highly specific to TCP. The negligible cross-reaction with the *O*-methylated TCP compound confirmed the strong participation of the negative charge of the deprotonated hydroxyl group in the analyte−antibody interaction.

When evaluating the analytical properties of immunoassays performed by combining several assay haptens and formats, no noticeable improvement was found. Thus, TCP could be determined with different ELISA systems with similar characteristics. To choose the one that best fulfills the analytical requirements of a given application, the performance of these immunoassays in the corresponding matrix should therefore be investigated.

TCP immunoassays offer considerable advantages in sample preparation over chromatographic techniques, because this hydrophilic compound would be readily available in aqueous buffers for the immunochemical determination without further treatment. In an accompanying paper, the assay optimization and its applicability to analyze TCP in environmental water samples is reported.

ABBREVIATIONS USED

BSA, bovine serum albumin; CR, cross-reactivity; DCM, dichloromethane; ELISA, enzyme-linked immunosorbent assay; EtAc, ethyl acetate; GC, gas chromatography; HRP, horseradish peroxidase; Hx, hexane; MAb, monoclonal antibody; I_{50}, concentration giving 50% inhibition of maximum response; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; OPD, *o*-phenylenediamine; OVA, ovalbumin; PBS, 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4; PBST, PBS containing 0.05% Tween 20; PEG, polyethyleneglycol; TCP, 3,5,6-trichloro-2-pyridinol; THF, tetrahydrofuran; TLC, thin-layer chromatography; UV−vis, ultraviolet−visible.

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