JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of a Monoclonal Immunoassay Selective for Chlorinated Cyclodiene Insecticides

JUAN J. MANCLÚS,[†] ANTONIO ABAD,[†,⊥] MIJAIL Y. LEBEDEV,[†,§] FATEMEH MOJARRAD,[‡] BARBORA MICKOVÁ,[†,#] JOSEP V. MERCADER,[†,||] JAIME PRIMO,[‡] MIGUEL A. MIRANDA,[‡] AND ANGEL MONTOYA*[,†]

Centro de Investigación e Innovación en Bioingeniería and Instituto de Tecnología Química, Universidad Politécnica de Valencia, 46022 Valencia, Spain

Organochlorine pesticides still generate public health concerns because of their unresolved health impact and their persistence in living beings, which is demanding appropriate analytical techniques for their monitoring. In this study, an enzyme-linked immunosorbent assay based on monoclonal antibodies (MAbs) for the detection of an important group of organochlorine pesticides, the cyclodiene group, has been developed. With this aim, several hapten−protein conjugates, characterized by exposure of the common hexachlorinated bicyclic (norbornene) moiety and differing in the linking structure to the carrier protein, were prepared. From mice immunized with these conjugates, several MAbs with the ability to sensitively bind the majority of cyclodienes were obtained. Among them CCD2.2 MAb displaying the broadest recognition to cyclodiene compounds (endosulfan, dieldrin, endrin, chlordane, heptachlor, aldrin, toxaphene: I_{50} values in the 6−25 nM range) was selected for the assay. Interestingly, this MAb showed certain stereospecificity toward other polychlorinated cycloalkanes because the γ-isomer of hexachlorocyclohexane (lindane) was also very well recognized (I_{50} value of 22 nM). This immunoassay is potentially a very valuable analytical tool for the rapid and sensitive determination of cyclodiene insecticides and related compounds, which in turn may contribute to the understanding of the biological activities and of the overall environmental impact of these persistent organic pollutants.

KEYWORDS: Organochlorine pesticides; cyclodiene insecticides; endosulfan; lindane; monoclonal antibodies; immunoassay; ELISA

INTRODUCTION

Beginning with their irruption in the 1940s, organochlorine pesticides (OCPs) were widely used in agriculture and malarial control programs with dramatic beneficial effects, but they have fallen into disuse because of their persistence in the environment. Chlorinated cyclodiene (CCD) insecticides are a group of OCPs that includes compounds such as endosulfan, heptachlor, chlordane, aldrin, endrin, and dieldrin. Their mode of action, toxicology, metabolism, and distribution have been extensively reviewed (*1*, *2*). Although to a different extent for each compound, CCD residues and their metabolites are prone to bioaccumulation and biomagnification (*3*−*6*). Moreover, some of these insecticides have been shown to have estrogenic activity (*7*, *8*), whereas synergistic estrogenic effects among them and with other environmentally relevant OCPs have also been observed (*9*). In other studies, the estrogenic activity of CCDs has not been demonstrated (*10*), nor can a direct relationship be established between environmental pesticide pollution, especially OCPs, and cancer (*11*). Due to their environmental impact, the use of CCDs on agriculture has been almost banned since the 1970s, but their residues are continuously detected in food and in the environment (*12*−*15*). Endosulfan is one of the few organochlorine insecticides remaining in widespread use, and its residues have been found throughout the world environment (*16*) and with a high frequency of occurrence in foods (*15*, *17*). The ubiquitous presence of CCD residues in the environment and the controversy related to their health impact on humans and wildlife pointed out the need for an adequate methodology for their environmental monitoring, as a tool to complement the great effort in epidemiologic and biological studies currently carried out.

Traditional methods for determining OCPs involve solvent extraction, liquid−liquid partitioning, and cleanup of the extract prior to identification and quantitative determination by chro-

* Author to whom correspondence should be addressed (telephone +34 96 3877093; fax +34 96 3877093; e-mail amontoya@eln.upv.es).
[†] Centro de Investigación e Innovación en Bioingeniería.
[‡] Instituto de Tecnología Química.
[⊥] Present address: Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Apartado 73, 46100 Burjassot, Valencia, Spain.
[§] Present address: M. V. Lomonosov State University, Faculty of Chemistry, Department of Chemical Enzymology, 119899 Moscow, Russia.
[#] Present address: Institute of Chemical Technology, Department of Biochemistry and Microbiology, 16628 Prague 6, Czech Republic.
[||] Present address: UCB Pharma, S.A., Santiago Ramón y Cajal 6, 08750 Molins de Rei, Barcelona, Spain.

10.1021/jf035382h CCC: $27.50
Published on Web 04/20/2004

matography (*18*, *19*). Consequently, these methods are time-consuming, labor-intensive, and costly in terms of solvent use and disposal and require sophisticated equipment available only in well-equipped centralized laboratories. Therefore, chromatographic techniques are not suitable for the analysis of the large number of samples required for comprehensive monitoring studies. Immunochemical techniques have lately gained a position as alternative and/or complementary methods for the analysis of agrochemicals because of their simplicity, cost-effectiveness, and high sample throughput. Moreover, immunoassays (IAs) are field-portable and do not require sophisticated instrumentation. All of these features make IAs very valuable methods for large monitoring programs (*20*−*23*).

Immunoassay development requires the production of antibodies to the analytes and their incorporation into adequate assay formats, usually enzyme-linked immunosorbent assays (ELISAs). The successful generation of specific antibodies and sensitive assays to a small molecule is greatly dependent upon a proper design of immunizing and assay haptens. In this respect, it is still unpredictable how haptens are presented to the immune system, making it advisable to examine several haptenic structures (*24*, *25*). Particularly, detection of a group of compounds of similar structure can be often accomplished by judicious synthesis of hapten−protein immunogens to expose common features to all of the members of the group to the maximum while minimizing the presentation of structural differences to the immune system (*26*, *27*). Once analyte immunogens are prepared, the debate arises whether polyclonal or monoclonal antibodies are obtained. If an unlimited supply of a single and homogeneous type of antibody is required, the choice is monoclonal technology. Additionally, standardized immunoreagents may facilitate acceptance of immunoassays in the analytical laboratory by ensuring a long-term supply of kits with a defined performance (*21*, *28*).

Since the pioneering work of Langone and Van Vunakis (*29*), who designed a radio-immunoassay for dieldrin and aldrin, several works have been published reporting on the immunochemical detection of cyclodienes. Thus, using the aldrin derivative hapten described by these authors, an ELISA based on rabbit polyclonal antiserum was developed for detecting dieldrin/aldrin in dairy products (*30*). Polyclonal antibodies were also obtained from a haptenic structure of the endosulfan metabolite endosulfan diol and used in an immunoassay with a limit of detection for endosulfan of 3 ppb in environmental samples (*31*). The first monoclonal antibodies (MAbs) recognizing cyclodienes with different specificities were produced using an ether derivative of aldrin (*32*) and were used in pharmacological studies (*33*). Stanker et al. (*34*) obtained two MAbs derived from a hydroxyl−chlordene hapten, which bound all CCDs tested. Later, enzyme immunoassays for endosulfan and its metabolites based on rabbit polyclonal antibodies was reported (*35*). These authors explored several haptens to obtain appropriate antibodies and to develop immunoassays. Recently, a fiber optic immunosensor, which detects cyclodiene insecticides at the parts per billion level, has been described using rabbit polyclonal antibodies raised from a chlorendic caproic acid hapten (*36*). Several applications to food and environmental samples of commercial ELISA kits for cyclodiene insecticides have been reported (*37*−*39*).

In previous work carried out in our laboratory, IAs based on MAbs for DDT and related compounds were developed (*40*). In the present study, the development of IAs to another important persistent organochlorine pollutant group, CCD insecticides, was undertaken. As mentioned above, MAbs recognizing several of these insecticides have already been obtained, although they showed moderate affinity to the target compounds. Therefore, our main goal was to produce MAbs showing high affinity and selectivity to CCD insecticides. This work comprised the synthesis of several haptens sharing common structures of cyclodiene insecticides and differing in the linker moiety to carrier proteins, the production and characterization of MAbs, and the evaluation and optimization of different assay formats and conditions.

MATERIALS AND METHODS

Reagents and Instruments. Pesticide and metabolite standards were from Riedel-de Haën (Seelze, Germany) and Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions were prepared in *N*,*N*-dimethylformamide (DMF, dried) and stored at −20 °C. Starting products for hapten synthesis and hapten−protein coupling reagents were from Fluka-Aldrich Química (Madrid, Spain). Analytical grade solvents and $CDCl_3$ were from Scharlau (Barcelona, Spain). Preparative layer chromatography was performed on 2 mm precoated silica gel 60 F_{254} from Merck (Darmstadt, Germany). Ovalbumin (OVA), Freund's adjuvants, Sephadex G-25, and *o*-phenylenediamine (OPD) were obtained from Sigma Química (Madrid, Spain). Bovine serum albumin (BSA) fraction V, enzyme-immunoassay grade horseradish peroxidase (HRP), Hybridoma Fusion and Cloning Supplement (HFCS), and poly(ethylene glycol) (PEG) 1500 were from Roche Applied Science (Heidelberg, Germany). Peroxidase-labeled rabbit anti-mouse immunoglobulins and goat anti-mouse immunoglobulins were obtained from Dako (Glostrup, Denmark). Culture media (high-glucose Dulbecco's modified Eagle's medium with Glutamax I and sodium pyruvate, DMEM), fetal calf serum (Myoclone Super Plus), and supplements were from Gibco (Paisley, Scotland). Culture plasticware was from Bibby Sterilin Ltd. (Stone, U.K.). Flat-bottom polystyrene ELISA plates (high-binding plates, catalog no. 3590) were from Costar (Cambridge, MA).

^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a Varian Gemini 330 spectrometer (Sunnyvale, CA), operating at 300 and 75 MHz, respectively. Chemical shifts are given relative to tetramethylsilane. Ultraviolet−visible spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan). ELISA plates were washed with a 96PW microplate washer from SLT Labinstruments GmbH (Salzburg, Austria), and absorbances were read in dual-wavelength mode (490−650 nm) with an Emax microplate reader from Molecular Devices (Sunnyvale, CA).

Hapten Synthesis. Most of the compounds used in this study present only minor safety concerns. However, it is advisable to work in a well-ventilated fume hood during synthesis work. CCD haptens synthesized for this study are depicted in **Figure 1**. The first reaction of hapten synthesis was, in all of the cases, a Diels−Alder addition of hexachlorocyclopentadiene and a particular alkene for each hapten. For haptens **CCD1** and **CCD4**, the alkene already contained a terminal carboxylic group used for protein coupling, whereas for haptens **CCD2** and **CCD3** the carboxylic group was introduced by succinylation of intermediate hydroxyl compounds.

*4,5,6,7,8,8-Hexachloro-3a,4,7,7a-tetrahydro-4,7-methanoindan-1-yl Acetic Acid (**CCD1**).* Hexachlorocyclopentadiene (2.72 g, 0.01 mol) and 2-cyclopentene-1-acetic acid (1.2 g, 0.01 mol) were placed in a round-bottom flask, heated to 130 °C, and kept at this temperature for 5 h. The crude product was left to reach room temperature, whereby white crystals appeared. They were filtered and recrystallized from dichloromethane: mp 165 °C; ^1H NMR ($CDCl_3$) δ 3.32 (q, 1H), 3.0−2.88 (dd, 1H), 2.67 (dd, 1H), 2.34 (dd, 1H), 2.26−2.14 (m, 1H), 2.04−1.88 (m, 2H), 1.50−1.34 (m, 2H); ^{13}C NMR δ 178.2, 131.7, 131.4, 105.1, 81.5, 81.3, 58.3, 53.9, 39.2, 37.0, 34.2, 25.3; elemental analysis for $C_{12}H_{10}C_{16}O_2$: C, 36.13; H, 2.52; Cl, 53.32; found: C, 36.07; H, 2.56; Cl, 53.30.

*4-(4,5,6,7,8,8-Hexachloro-3a,4,7,7a-tetrahydro-4,7-methano-1H-indenyl-1-oxy) 4-Oxobutanoic Acid (**CCD2**).* This hapten was synthesized as described by Stanker et al. (*34*). Briefly, the adduct obtained after Diels−Alder addition of hexachlorocyclopentadiene and cyclopentadiene was oxidized with SeO_2 to render 1-hydroxychlordene, which was converted into the hemisuccinate **CCD2** by reaction with succinic anhydride.

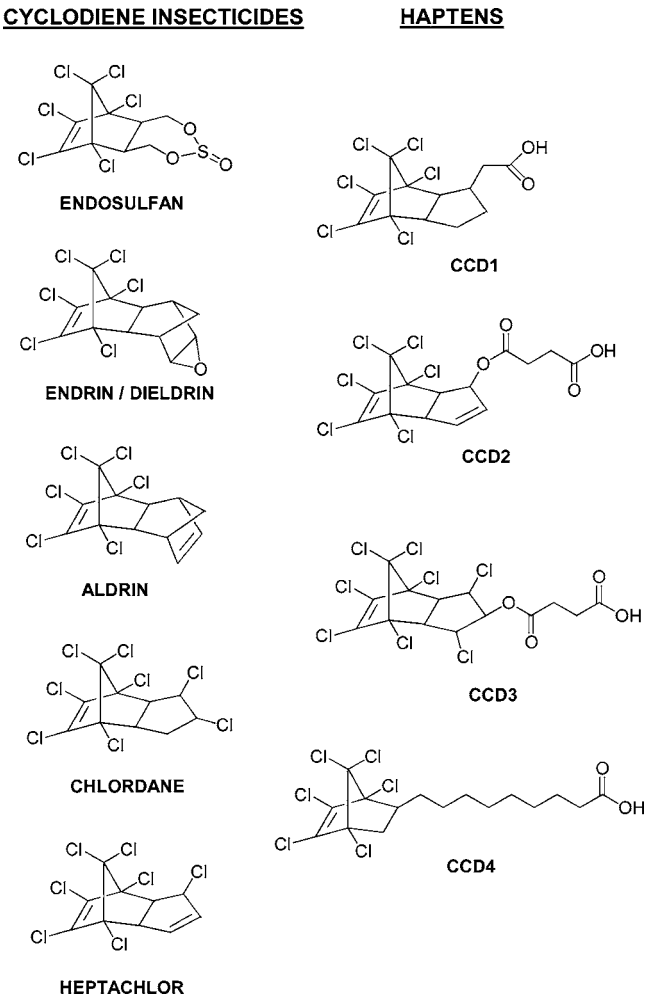

Figure 1. Structures of the chlorinated cyclodiene insecticides and of the haptens synthesized.

*4-(1,3,4,5,6,7,8,8-Octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindanyl-2-oxy) 4-Oxobutanoic Acid (**CCD3**).* The synthetic procedure reported by Lee et al. (*35*) was followed. Briefly, the adduct 1-chlorochlordene was converted into the chlorohydrin of 1-chlorochlordane, which was succinylated.

*1,2,3,4,7,7-Hexachloronorbornen-5-yl Nonanoic Acid (**CCD4**).* Hexachlorocyclopentadiene (2.72 g, 0.01 mol) and 10-undecylenic acid (1.84 g, 0.01 mols) were placed in a round-bottom flask, heated to 130−140 °C, and kept at this range of temperature for 5 h. The crude was purified on silica gel preparative chromatography plates, using dichloromethane as eluant. The adduct was obtained as an oil: ^{1}H NMR ($CDCl_3$) δ 11.51 (bs, 1H), 2.78−2.70 (m, 1H), 2.62 (dd, 1H), 2.34 (t, 2H), 1.78−1.58 (m, 4H), 1.28 (bs, 10H), 0.94 (bs, 1H); ^{13}C NMR δ 180.5, 131.3, 130.4, 102.5, 82.6, 78.7, 47.4, 40.7, 34.0, 30.4, 29.3, 29.1, 29.0, 28.9, 27.3, 24.5; elemental analysis for $C_{16}H_{20}Cl_6O_2$: C, 42.04; H, 4.40; Cl, 46.54; found: C, 42.24; H, 4.41; Cl, 47.0.

Preparation of Protein−Hapten Conjugates. All haptens used in this study contained a free carboxylic group suitable to react with amine groups of proteins. Hapten−protein conjugations were carried out by the *N*-hydroxysuccinimide (NHS)-active ester method of Langone and Van Vunakis (*29*), with slight modifications.

Immunogenic and Coating Conjugates. Typically, haptens (~25 μmol in the appropriate volume of DMF to bring the final concentration of hapten to 100−200 mM) were activated during 2 h at room temperature with a 50% molar excess (molar ratio 1:1.5) of NHS and *N*,*N*′-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 10 min and with vigorous 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 ratios in the mixture were 50:1 for immunogens and 20:1 for coating conjugates. The mixture was stirred at room temperature for 2 h. Finally, conjugates were separated from uncoupled haptens 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. The degree of hapten conjugation to proteins, that is, the number of amino groups substituted with haptens in carrier molecules, was estimated by the determination of the number of free amino groups before and after conjugation, using trinitrobenzenesulfonic acid as the titration reagent (*41*). Apparent molar ratios in the range of 14−21 and 5−8, for BSA and OVA conjugates, respectively, were estimated.

Enzyme Conjugates. HRP was used to prepare the enzyme tracers. Following the same procedure as before, haptens were first activated and then conjugated to HRP (5 mg/mL) using 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 were estimated spectrophotometrically.

Production of Monoclonal Antibodies. *Immunization.* BALB/c female mice (8−10 weeks old) were immunized with BSA−**CCD1**, −**CCD2**, −**CCD3**, and −**CCD4** conjugates. The first dose consisted of 100 μg of conjugate intraperitoneally injected as an emulsion of PBS and complete Freund's adjuvant. At 2 and 4 weeks after the initial dose, mice received booster injections with the same amount of immunogen emulsified in incomplete Freund's adjuvant. One week after the last injection, mice were tail-bled and sera tested for anti-hapten antibody titer by indirect ELISA and for analyte recognition properties by competitive indirect ELISA. After a resting period of at least 3 weeks from the last injection in adjuvant, mice selected to be spleen donors for hybridoma production received a final soluble intraperitoneal injection of 100 μg of conjugate in PBS, 4 days prior to cell fusion.

Cell Fusion. P3-X63/Ag 8.653 murine myeloma cells (ATCC, Rockville, MD) were cultured in high-glucose DMEM 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. (*42*). Mouse spleen lymphocytes were fused with myeloma cells at a 5:1 ratio using PEG 1500 as the fusing agent. The fused cells were distributed in 96-well culture plates at an approximate density of 2×10^5 cells/100 μL of s-DMEM per well. Twenty-four hours after plating, 100 μL of HAT selection medium (s-DMEM supplemented with 100 μM hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine) containing 2% HFCS (v/v) was added to each well. Half the medium of the wells was replaced by fresh HAT medium on days 4 and 7 postfusion. 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 10 days after cell fusion, culture supernatants were screened for the presence of antibodies that recognized the analyte. 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 the analyte, respectively. Optimum conditions were pursued for the screenings. Thus, the coating conjugate concentrations were those selected when the analyte recognition by mouse sera was evaluated, and culture supernatants were appropriately diluted to obtain ELISA absorbance below 2.0. Selected hybridomas were cloned by limiting dilution using HT medium supplemented with 2% HFCS (v/v). Stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen.

Purification of Monoclonal Antibodies. MAbs, all being of IgG class, were purified on a small scale directly from late stationary phase culture supernatants by saline precipitation with saturated ammonium sulfate followed by affinity chromatography on protein G−Sepharose 4 Fast Flow (Amersham Biosciences, Barcelona, Spain). Purified MAbs were stored at 4 °C as ammonium sulfate precipitates.

Enzyme-Linked Immunosorbent Assays. ELISA plates were coated overnight with conjugate or antibody solutions in 50 mM carbonate buffer, pH 9.6. A volume of 100 μL per well was used throughout all assay steps, and all incubations were carried out at room

temperature. After each incubation, 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, an 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 analytes. In the antibody-coated format, the specific antibody was coated directly or by using a capture auxiliary antibody, and competitive ELISAs were followed to evaluate the assay properties using different enzyme tracers. For competition assays, the concentrations of antibodies, hapten conjugates, or enzyme tracers were optimized by checkerboard titration. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four-parameter logistic equation (*43*), using Molecular Devices (Sunnyvale, CA) and Sigmaplot (Jandel Scientific, Weinheim, Germany) software packages.

Preparation of Standards. From a 100 mM stock in DMF, daily serial dilutions (factor 5, 10 dilutions) from 2.5 mM in organic solvent were made. From eight of these dilutions, eight standards were prepared by diluting 1/200 in PBS (6250, 250, 50, 10, 2, 0.4, 0.08, and 0.0032 nM in assay), using borosilicate glass tubes. Initially, 1,4-dioxane was the organic solvent used, but after assay conditions were optimized, DMF was finally selected.

Conjugate-Coated Format. Plates were coated with the selected concentrations of OVA−hapten conjugates. Then, serum, culture supernatant, or antibody dilutions in PBS were added and incubated for 1 h. Next, plates were incubated for 1 h with peroxidase-labeled rabbit anti-mouse immunoglobulins diluted 1/2000 in PBST (PBS containing 0.05% Tween 20). 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 and 62 mM sodium phosphate, pH 5.35). After 10 min, the reaction was stopped with 2.5 M sulfuric acid, and the 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 followed by 50 μL of the appropriate concentration of antibody (serum, culture supernatant, or purified MAb).

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

Indirect Antibody-Coated Format. The difference with the previous format was that plates were first coated with goat anti-mouse immunoglobulins at 2 μg/mL in carbonate buffer, followed by an incubation for 2 h with the specific antibodies at appropriate concentrations in PBST.

RESULTS AND DISCUSSION

Synthesis of CCD Haptens. It is well-known that antibodies elicited to haptenic conjugates show a preferential recognition to the part of the molecule that is furthest from the attachment site of the hapten to the carrier protein (*24*, *25*, *44*). As the final goal of this study was the production of MAbs capable of recognizing the majority of the cyclodiene insecticides, a hapten design addressed to expose a structure common to these compounds was followed. In this sense, all of these organochlorine insecticides share a characteristic hexachlorobicyclic (norbornene) moiety, which prompted us to synthesize a series of haptens bearing this bicyclic structure and differing in the spacer arm, containing suitable reactive groups for covalent linking to proteins. As can be seen in **Figure 1**, the linker moiety introduced for haptens **CCD1**, **CCD2**, and **CCD3** was a cyclopentane ring with a carboxymethyl substituent (hapten **CCD1**), two chlorines and a hemisuccinate spacer arm (hapten **CCD2**), or a double bond and a hemisuccinate group (hapten **CCD3**). The design of a simple hapten corresponding to a common substructure of the compounds may constitute a valuable approach to class-specific antibody generation (*27*). Thus, a distinctive possibility was provided by hapten **CCD4**, which contains a more flexible, simple spacer arm consisting of a long hydrocarbonate chain ($[-CH_2-]_{10}$). Theoretically, protein conjugates of these haptens should present the characteristic norbornene structure to the immune system.

Table 1. Properties of the Sera of Mice Immunized with Cyclodiene Haptens

	immunizing hapten[a]			
	CCD1	**CCD2**	**CCD3**	**CCD4**
serum titer[b]	1×10^5	5×10^4	1×10^4	
endosulfan I_{50}[c]	ni[d]	0.7 μM	ni	

[a] Representative sera obtained 1 week after the third booster injection of the respective BSA–hapten conjugate. [b] Serum dilution giving 3 times background absorbance in ELISA using homologous haptens. [c] Data obtained from competitive ELISAs performed with optimum concentrations of homologous OVA–hapten conjugates and serum dilutions giving absorbances around 1.0. [d] No inhibition up to 10 μM endosulfan.

Production of Antibodies to CCD Insecticides. *Mouse Polyclonal Response.* To examine the suitability of the synthesized haptens to raise anti-CCD antibodies, mice were immunized with BSA conjugates of haptens **CCD1**, **-2**, **-3**, and **-4**. After the third injection, mouse sera were characterized for the presence of antibodies recognizing the conjugated immunizing haptens (serum titer) and for their ability to bind endosulfan (a representative of the cyclodiene insecticide family), by estimating their I_{50} value. Results of the characterization are summarized in **Table 1**. Serum titers (serum dilution giving 3 times the background absorbance) were estimated by indirect ELISA using the homologous OVA−hapten conjugates. Mouse sera obtained from BSA−**CCD1**, **-2**, and **-3** showed high levels of polyclonal antibodies recognizing each respective homologous hapten conjugate, with titers ranging from $1/10^4$ to $1/10^5$. Surprisingly, the immunization with BSA−**CCD4** did not raise an anti-hapten response. Next, the ability to recognize endosulfan was evaluated by competitive indirect ELISA. Only sera from mice immunized with BSA−**CCD2** bound competitively endosulfan, with an I_{50} of 0.7 μM, but curves showed shallow slopes as well as no complete inhibition of antibody binding (data not shown). No inhibition was found for the rest of the sera with endosulfan up to 10 μM.

Previous works to raise antibodies to members of the cyclodiene family have been carried out using haptens contemplated in this study. Lee et al. (*35*) obtained a high-titer polyclonal response in rabbits, by immunizing with protein conjugates of haptens **CCD2** and **CCD3**. These antibodies were capable of recognizing endosulfan with high affinity. Not surprisingly, considerable differences in the polyclonal response to the same immunogen between rabbits and mice has been previously found (*45*, *46*). In terms of affinity, rabbit polyclonal antibodies are undoubtedly superior to those of mice, but the reason for immunizing mice is mostly for applying the monoclonal antibody technology, as in this work. On the other hand, despite the fact that hapten **CCD4** appeared to be an ideal candidate to obtain antibodies recognizing the cyclodiene compounds because it contains just the common norbornene structure and a simple spacer arm, its protein conjugate was not able to raise anti-hapten antibodies. As suggested by Fasciglione et al. (*47*), this haptenic structure may be not exposed but rather hidden in the three-dimensional structure of the carrier protein.

Table 2. Summary of the Results of Cell Fusions and Hybridoma Selection

immunizing hapten	fusion	no. of wells seeded	positive[a] (hapten)	competitive[b] (analyte)	no. of cloned hybridomas[c]
CCD1	1	192	5	1	
	2	192	20	2	
	3	672	8	1	
	4	672	24	7	3
CCD2	5	864	78	63	4
	6	960	25	1	
	7	768	307	9	3
CCD3	8	768	4		
	9	768	18		
	10	768	15		

[a] Wells with antibodies that recognized the OVA–hapten conjugates (homologous assays) by indirect ELISA (absorbance > 0.5). [b] Wells with antibodies that recognized free endosulfan (inhibition > 50% by 1 μM endosulfan for fusions 1–5 and by 100 nM endosulfan for fusions 6–10). Homologous competitive ELISAs were carried out with the OVA–hapten concentrations previously selected for evaluating mouse sera. Culture supernatants giving absorbances out of range were diluted until absorbance < 2.0. [c] Hybridomas secreting antibodies with the lowest I_{50} for endosulfan were stabilized and cloned.

Production of MAbs. Fusions were undertaken from all mice that responded with an adequate level of anti-hapten antibodies, that is, mice immunized with BSA−**CCD1**, **-2**, and **-3**. Screenings of fusion cultures were performed using optimum coating concentrations (homologous conjugate-coated ELISA format) in simultaneous noncompetitive and competitive assays. Endosulfan was selected as competitor because it is still in use, and therefore it has a particular environmental and food relevance. Results of hybridoma selection are summarized in **Table 2**. As shown, all of the fusions rendered wells with antibodies recognizing the corresponding homologous conjugated haptens (positive wells), although with a wide range of yields, and a few of them were found to be competitive (inhibition > 50% by 1 μM endosulfan). Thus, four fusions were needed to clone and stabilize competitive hybridomas from hapten **CCD1**. The first fusion from mice immunized with hapten **CCD2** rendered a high yield of competitive wells, from which four were selected on the basis of their highest recognition of endosulfan. Thereafter, the endosulfan concentration cutoff used to screen culture supernatants was lowered to 0.1 μM to look for higher affinity monoclonal antibodies. Finally, after two additional fusions, three new cell lines were obtained that fulfilled the latter condition. With regard to hapten **CCD3**, no competitive well (cutoff, 0.1 μM endosulfan) was found after three fusions.

With the hybridoma production and selection procedure performed from haptens **CCD1**, **-2**, and **-3**, best results were obtained with hapten **CCD2**, which is in accordance with its best polyclonal response. Using this hapten, one MAb with moderate affinity was obtained by Stanker et al. (*34*) after five independent fusions.

Characterization of the MAbs. MAbs produced by each of the 10 selected hybridomas were small-scale purified from culture supernatants and were subsequently characterized for affinity and selectivity to the members of CCDs using homologous and heterologous haptens in different ELISA formats.

Affinity. The ability to recognize endosulfan was first estimated using homologous conjugate-coated ELISA format. After adequate assay concentrations were selected, I_{50} values for endosulfan in the 72−250 and 8−22 nM range were obtained for MAbs derived from haptens **CCD1** and **CCD2**, respectively (**Table 3**). All of the MAbs derived from hapten **CCD2** displayed a higher affinity to endosulfan than those from **CCD1**. Next, antibodies were assayed in heterologous assays using all of the haptens synthesized in this study. Haptens **CCD1** and **CCD2** were appropriately recognized, although heterologous competitive assays gave similar I_{50} values rather than homologous ones. In contrast, hapten **CCD3** was poorly recognized, and competitive curves were obtained only with half the MAbs derived from hapten **CCD2**. In any case, I_{50} values of heterologous assays did not get any affinity improvement. As for **CCD4**, there was no antibody recognition at all, confirming the folding-back of this haptenic structure in the carrier protein.

Table 3. I_{50} Values (Nanomolar) for Endosulfan Obtained with Different Haptens and Formats[a]

MAb	conjugate-coated ELISA (OVA–hapten) CCD1	CCD2	CCD3	antibody-coated ELISA (HRP–hapten) CCD1	CCD2	CCD3
CCD1.1	78	pr[b]	nr[c]	84	81	nr
CCD1.2	250	244	pr	241	650	341
CCD1.3	72	122	pr	300	168	nr
CCD2.1	16	18	pr	pr	45	nr
CCD2.2	9	10	nr	nr	nr	nr
CCD2.3	11	9	12	nr	nr	nr
CCD2.4	11	13	14	pr	54	nr
CCD2.5	10	8	ni[d]	13	29	nr
CCD2.6	17	18	nr	nr	nr	nr
CCD2.8	20	22	nr	133	46	pr

[a] Competitive ELISAs were performed in optimum conditions, i.e., limiting concentrations of immunoreagents giving maximum absorbance around 1.0. **CCD4** hapten conjugates were not recognized. [b] Poor recognition. [c] No recognition. [d] No inhibition up to 0.4 μM endosulfan.

The competitive behavior of MAbs in the direct and indirect antibody-coated formats was also evaluated. As shown in **Table 3**, the recognition pattern of immobilized antibodies (antibody-coated format) is clearly different from that of antibodies in solution (conjugate-coated format) and is characterized by a much lower degree of HRP−hapten recognition. Indeed, HRP tracers of haptens **CCD1** and **CCD2** were recognized by all of the CCD1 MAbs and by approximately half of those derived from **CCD2**, whereas HRP−**CCD3** and −**CCD4** were almost unrecognized. In all of the cases, competitive curves obtained with immobilized antibodies provided higher I_{50} values (less sensitive assays) than those in the conjugate-coated format. Moreover, MAbs indirectly immobilized using goat anti-mouse IgG precoated plates did not significantly modify either the recognition pattern or the assay sensitivity (data not shown).

Selectivity. MAbs showing the highest affinity for endosulfan (I_{50} around 10 nM, MAbs CCD2.2, -2.3, -2.4, and -2.5) were further characterized by performing competitive curves using other members of the cyclodiene insecticide family and other organochlorine compounds as competitors. Relative cross-reactivity (CR) data for each compound are shown in **Table 4**. With regard to endosulfan derivatives, all of the MAbs showed stereospecificity toward β-endosulfan, and endosulfan sulfate and endosulfan diol were predominantly better and less recognized than endosulfan, respectively. As expected, all of the CCDs assayed were recognized, although to different degrees for each MAb. Thus, whereas endosulfan, endrin, and dieldrin were recognized to a similar extent by MAbs CCD2.2 and CCD2.4, the recognition of chlordane, heptachlor, and aldrin was lower for all MAbs. Likewise, toxaphene, a complex mixture of compounds including many chlorinated norbornanes, showed a high CR (>100%). In contrast, the caged structure of the hexachloropentadiene dimer, mirex, was not recognized

Table 4. Selectivity of the MAbs to CCD Insecticides

compound	cross-reactivity[a] (%) CCD2.2 MAb	CCD2.3 MAb	CCD2.4 MAb	CCD2.5 MAb
endosulfan (mix)	100	100	100	100
α-endosulfan	82	55	84	18
β-endosulfan	114	168	114	211
endosulfan sulfate	219	181	63	222
endosulfan diol	54	9	104	18
dieldrin	109	34	109	72
endrin	113	59	133	19
chlordane	28	22	34	20
heptachlor	54	24	53	15
aldrin	47	21	39	14
toxaphene	112	101	120	110
mirex	<0.01	<0.01	<0.01	<0.01
γ-HCH (lindane)	30	2	12	10
α-HCH	14	3	4	3
pentachlorophenol	0.19	0.30	0.06	0.04
2,4,5-trichlorophenol	0.03	0.02	<0.01	<0.01
p,*p*-DDT	<0.01	<0.01	<0.01	<0.01
4,4′-PCB	<0.01	<0.01	<0.01	<0.01

[a] Percentage of cross-reactivity = (IC_{50} of endosulfan/IC_{50} of other compound) × 100. Values correspond to the average of three estimations. Competitive ELISAs in the conjugated-coated format were performed in the following conditions: plates were coated with OVA–**CCD1** at 1 μg/mL for MAb CCD2.2 and at 0.5 μg/mL for the rest; MAb concentrations were CCD2.2 (100 ng/mL), CCD2.3 (320 ng/mL), CCD2.4 (140 ng/mL), and CCD2.5 (350 ng/mL).

(CR < 0.01%). The selectivity pattern exhibited by these antibodies partially resembles that previously reported for antibodies raised from the same haptenic structure (*34*, *35*). Concerning the cross-reactivity toward other organochlorine compounds, the most remarkable finding is the notorious recognition of γ-hexachlorocyclohexane (γ-HCH, lindane) shown especially by MAb CCD2.2 (I_{50} = 25 nM for lindane, 30% CR). Again, the recognition of HCH compounds showed certain stereospecificity because the α-isomer CR was lower than that of the γ-isomer for MAbs CCD2.2, -2.4, and -2.5. On the other hand, the recognition of chlorinated compounds bearing aromatic rings diminished considerably. Thus, CR for polychlorinated phenols was <0.3%, whereas it was negligible for important organochlorine pollutants such as *p*,*p*-DDT and 4,4′-PCB (<0.01%). Considering all of this, it seems that MAbs recognition is directed to polychlorinated cyclic, not aromatic, hydrocarbons. Among them the CCD insecticides that contain the original hexachlorobicyclic immunizing structure are the best recognized compounds, but interestingly the γ-isomer of hexachlorocyclohexane is also fairly well recognized. Similar behavior in terms of polychlorocycloalkane functionality as minimum requirement has been found for MAbs derived from an aldrin derivative hapten, which also exposed the norbornene structure (*33*), but in this study I_{50} values were at least 10-fold higher.

Selection of Immunoassay Conditions. *Immunoreagents and Format.* From the characterization study, MAbs derived from hapten **CCD2** showed the highest sensitivity to endosulfan, and among them MAbs CCD2.2, -2.3, -2.4, and -2.5 gave I_{50} values around 10 nM endosulfan in both homologous and heterologous assays (**Table 3**). Hapten **CCD1** was selected as assay hapten because, giving similar behavior, it is easier to synthesize. On the basis of the selectivity data shown in **Table 4**, MAbs CCD2.2 and CCD2.4 recognized the highest number of members of the chlorinated cyclodiene insecticide family, and between them MAb CCD2.2, giving slightly higher sensitive assays, was selected. Moreover, this MAb had the added value of recognizing sensitively lindane, a pollutant for which an efficient analytical methodology in the environment had been long demanded. Referring to the assay format, as MAbs immobilized did not provide useful immunoassays, the conjugate-coated format was hereinafter followed.

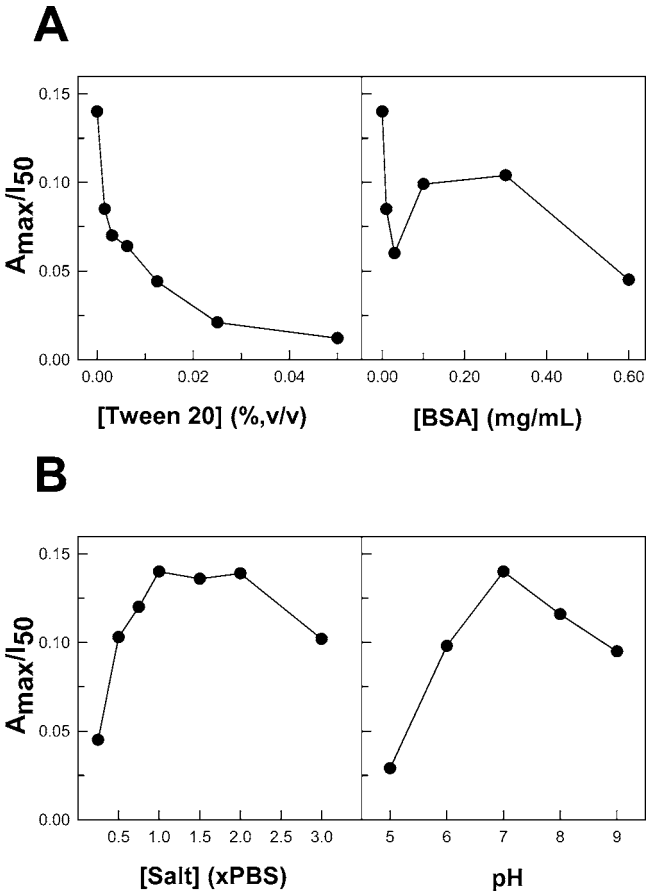

Figure 2. Influence of the assay buffer composition on immunoassay parameters: (**A**) buffer additives, BSA and Tween 20; (**B**) physicochemical conditions, salt concentration, and pH.

Assay Buffer Composition. Once the specific components of the immunoassay were selected (MAb CCD2.2; OVA−**CCD1** as assay conjugate), the influence of several physicochemical properties of the medium on assay characteristics was investigated, to optimize the buffer components. First, the nonionic surfactant Tween 20 and BSA are two additives commonly used in ELISA to reduce nonspecific interactions, but IA characteristics can greatly change as a function of their concentration (*35*, *48*, *49*). To study their influence, competitive curves were obtained in the presence of different additive concentrations. Then, curve parameters were plotted as the A_{max}/I_{50} ratio, which is a convenient estimate of the effect studied on ELISA sensitivity, the higher ratio indicating the higher sensitivity (*48*). **Figure 2A** shows the variation of this ratio as a function of additive concentration. The addition of Tween 20 to the assay buffer of the competitive step affected notoriously the curve parameters—the lower its concentration, the higher the assay sensitivity. A similar tendency was observed for BSA addition. Therefore, optimum assay sensitivity required not to add any of the additives studied.

Next, the influence of buffer composition, ionic strength, and pH on ELISA characteristics was examined. Thus, competitive curves were obtained using several dilutions of 10-fold-concentrated PBS as assay buffer. Likewise, the representation of the A_{max}/I_{50} ratio against the salt concentration was helpful

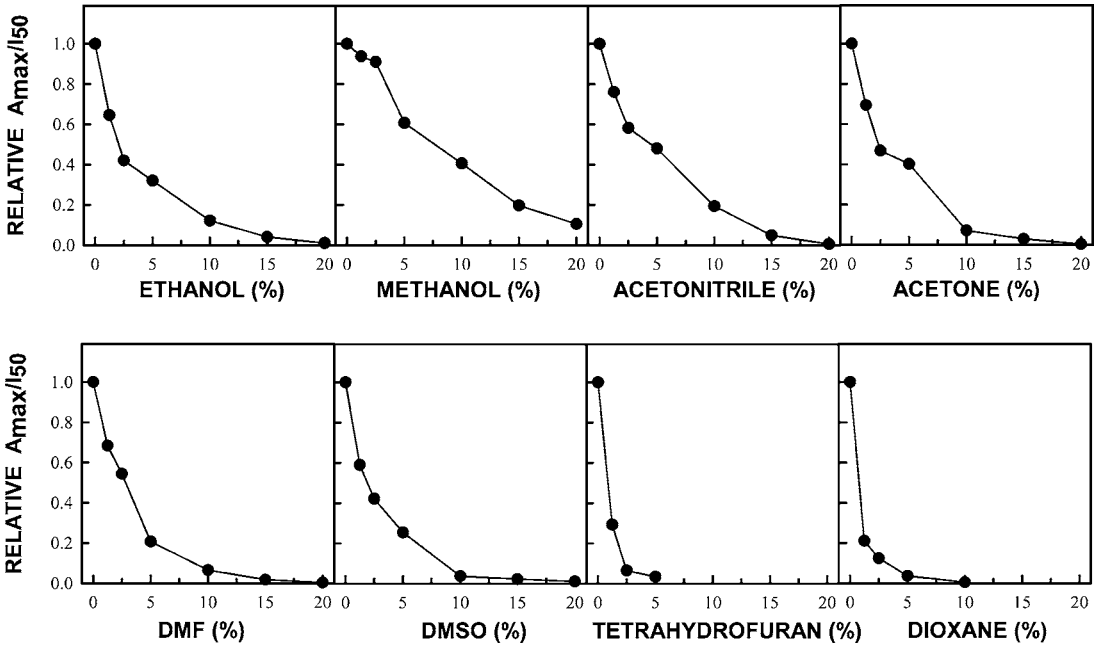

Figure 3. Effect of organic solvent concentrations in immunoassay parameters. Data were obtained from standard curves performed in triplicate.

in the selection of the optimum buffer. Immunoassay did not work in distilled water. As shown in **Figure 2B**, the ratio increased markedly as the salt concentration increased, reaching a plateau between 1 and 2 × PBS, and from here it decreased smoothly. A similar behavior has been found in our laboratory for ELISAs to other nonpolar analytes (*48*−*50*). Among the buffer conditions tested, the lowest salt concentration affording the highest A_{max}/I_{50} ratio was selected (1 × PBS). Finally, the variation of ELISA parameters within a range of assay buffer pH is also depicted in **Figure 2B**. The highest assay sensitivity was achieved at neutral pH, which is the pH routinely used for PBS.

Solvent Tolerance. Solvents are often used to extract analytes from samples, and then extract dilution is a simple, common practice to detect target analytes by ELISA. Moreover, in this work, with the aim of reducing the handling of aqueous solutions of nonpolar analytes to a minimum, intermediate standard dilutions were made in water miscible organic solvents. Therefore, it was imperative to examine how the immunoassay performed in the presence of solvents, to select the most appropriate ones to be used in extraction and in standard preparation. To carry out the experiments, competitive curves were performed by adding different solvent proportions to the assay buffer. Solvents evaluated were those that are water miscible, namely, methanol, ethanol, acetonitrile, acetone, DMF, dimethyl sulfoxide (DMSO), tetrahydrofuran, and 1,4-dioxane. In general, this immunoassay can be considered of low tolerance to the presence of solvents, because assay sensitivity, depicted in **Figure 3** as relative A_{max}/I_{50} ratio, dropped significantly even with solvent proportions as low as 1.25%. As often reported for pesticide immunoassays (*49*, *50*), methanol was the best-tolerated solvent, which might be the choice for analyte extraction from samples when required. For standard preparation, a solvent with a boiling point above 100 °C (DMF, DMSO, dioxane), thereby easier to pipet, was preferred. Initially, dioxane was used for this purpose, but as revealed in this study, this solvent was unfortunately the less tolerated one. Consequently, DMF and DMSO were evaluated as alternatives to dioxane. Standard curves obtained using DMF and DMSO yield assays slightly more sensitive (I_{50} = 7 nM to endosulfan), mainly because assay signal (A_{max}) increased in the presence of 0.25% of these solvents, thus allowing the reduction of MAb concentration necessary to give appropriate signals (absorbance ∼1). Finally, DMF was selected because it required a smaller amount of MAb in the assay (60 ng/mL).

A typical standard curve performed in optimum conditions is shown in **Figure 4**. Endosulfan can be determined in the competitive assay from 2 to 50 nM (20−80% inhibition), with an I_{50} value of 7 nM and a limit of detection (10% inhibition) of 1 nM. Taking advantage of the immunoassay selectivity, chlorinated cyclodiene insecticides can be analyzed with I_{50} values ranging from 6 to 25 nM and, very interestingly, lindane has an I_{50} value of 22 nM.

Conclusions. The goal of this work was the production of class-specific, highly sensitive MAbs and the development of IAs to chlorinated cyclodiene insecticides. This was accomplished by immunizing mice with BSA conjugates of several haptens characterized by presenting the hexachlorinated bicyclic moiety common to all of the cyclodienes and differing in the linking structure to the carrier protein.

After application of the hybridoma technology, four MAbs were selected and characterized in terms of affinity to endosulfan using several assay haptens and formats. In the conjugate-coated format, homologous haptens provided assays with I_{50} values of ∼10 nM, whereas the heterologous haptens used did not afford significant sensitivity improvement. The antibody-coated format was useless because most of the HRP−haptens were unrecognized. With regard to specificity, MAbs showed a broad recognition pattern of CCD insecticides. Among them, CCD2.2 MAb, displaying the highest recognition of these compounds, was selected for the assay. Furthermore, the selectivity of this MAb could be widened to hexachlorinated cyclic hydrocarbons because the γ-isomer of HCH was also very well recognized. This is particularly important because lindane, which is one of the few OCPs still used for animal husbandry and agricultural treatments, is one of the most frequently detected OCPs in total diet studies (*15*, *51*), and an efficient means of environmental monitoring for this pollutant has long been demanded.

Once assay immunoreagents were selected and characterized, the optimum buffer composition for the assay competition step

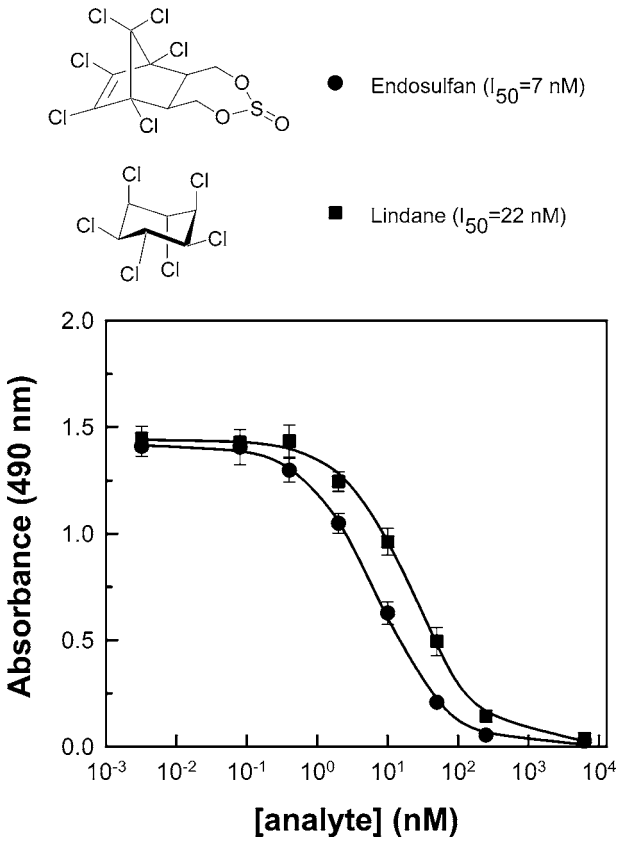

Figure 4. Representative standard curve for endosulfan and lindane obtained under optimized conditions: assay hapten, OVA–**CCD1** (1 μg/mL); CCD2.2 MAb (60 ng/mL); assay buffer, PBS containing 0.25% DMF. Each point represents the mean ± SD of three replicates.

was determined. The optimized immunoassay allows the sensitive detection of not only chlorinated cyclodiene insecticides (I_{50} values in the 6−25 nM range) but also lindane (I_{50} value of 22 nM). To the best of our knowledge, this is the immunochemical detection method of lindane with the highest sensitivity so far reported. In fact, lindane was not recognized in the highly selective polyclonal antibody-based immunoassay described by Lee et al. (*35*), and it was only weakly recognized (I_{50} value around 1 μM) in the monoclonal ELISA reported by Stanker et al. (*34*).

Organochlorine pesticides continue to be a constant source of concern because of their unresolved health impact and their persistence in living beings. Recently, debate has heightened concerning the link of these compounds to certain types of cancers or to endocrine-disrupting activity. As a complement of previous work in this laboratory with IAs to the DDT group, the selective and highly sensitive IAs to cyclodiene insecticides herein described will enable a more comprehensive monitoring of OCPs, which may be very helpful for understanding the biological activities and the overall environmental impact of these persistent organic pollutants.

ABBREVIATIONS USED

A_{max}, maximum absorbance; BSA, bovine serum albumin; CCDs, chlorinated cyclodienes; CR, cross-reactivity; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; HCH, hexachlorohexane; HFCS, hybridoma fusion and cloning supplement; HRP, horseradish peroxidase; IAs, immunoassays; MAb, monoclonal antibody; I_{50}, concentration giving 50% inhibition of maximum response; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; OCPs, organochlorine pesticides; OPD, *o*-phenylenediamine; OVA,ovalbumin; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20; PEG, poly(ethylene glycol).

LITERATURE CITED

(1) Smith, A. G. Chlorinated hydrocarbon insecticides. In *Handbook of Pesticide Toxicology*; Hayes, W. J., Laws, E. R., Eds.; Academic Press: London, U.K., 1991; Vol. 2, pp 731−865.

(2) Tomlin, C., Ed. *The Pesticide Manual: A World Compendium*, 11th ed.; British Crop Protection Council publications: Surrey, U.K., 1997.

(3) WHO (World Health Organization). *Environmental Health Criteria 38: Heptachlor*; World Health Organization: Geneva, Switzerland, 1984.

(4) Hargrave, B. T.; Harding, G. C.; Vass, W. P.; Erickson, P. E.; Fowler, B. R.; Scott, V. Organochlorine pesticides and polychlorinated-biphenyls in the Artic-ocean food web. *Arch. Environ. Contam. Toxicol.* **1992**, *22*, 41−54.

(5) Murphy, D. L.; Gooch, J. W. Accumulation of cis and trans chlordane by channel catfish during dietary exposure. *Arch. Environ. Contam. Toxicol.* **1995**, *29*, 297−301.

(6) Strandberg, B.; Bandh, C.; Vanbavel, B.; Bergqvist, P. A.; Broman, D.; Ishaq, R.; Naf, C.; Rappe, C. Organochlorine compounds in the gulf-of-Bothnia−Sediment and benthic species. *Chemosphere* **2000**, *40*, 1205−1211.

(7) McLachlan, J. A. Functional toxicology−A new approach to detect biologically-active xenobiotics. *Environ. Health Perspect.* **1993**, *101*, 386−387.

(8) Soto, A. M.; Soonenschein, C.; Chung, K. L.; Fernández, M. F.; Olea, N.; Serrano, F. O. The E-Screen assay as a tool to identify estrogens−An update on estrogenic environmental-pollutants. *Environ. Health Perspect.* **1995**, *103*, 113−122.

(9) Arnold, S. F.; Klotz, D. M.; Collins, B. M.; Vonier, P. M.; Guillette, L. J., Jr.; McLachlan, J. A. Synergistic activation of estrogen receptor with combinations of environmental chemicals. *Science* **1996**, *272*, 1489−1492.

(10) Shelby, M. D.; Newbold, R. R.; Tully, D. B.; Chae, K.; Davis, V. L. Assessing environmental chemicals for estrogenic using a combination of in vitro and in vivo assays. *Environ. Health Perspect.* **1996**, *104*, 1296−1300.

(11) Ames, B. N.; Gold, L. S. Environmental pollution, pesticides, and the prevention of cancer: Misconceptions. *FASEB J.* **1997**, *11*, 1041−1052.

(12) Kleivane, L.; Skaare, J. U.; Bjorge, A.; Deruiter, E.; Reijnders, P. J. Organochlorine pesticide-residue and PCBs in harbor porpoise (*Phocoena-Phocoena*) incidentally caught in Scandinavian waters. *Environ. Pollut.* **1995**, *89*, 137−146.

(13) Femandez, M. A.; Alonso, C.; Gonzalez, M. J.; Hemandez, L. M. Occurrence of organochlorine insecticides, PCB and PCB congeners in waters and sediments of the Ebro river (Spain). *Chemosphere* **1999**, *38*, 33−43.

(14) Doong, R. A.; Lee, C. Y.; Sun, Y. C. Dietary-intake and residues of organochlorine pesticides in foods from Hsinchu, Taiwan. *J. AOAC Int.* **1999**, *82*, 677−682.

(15) FDA (U.S. Food and Drug Administration). *Food and Drug Administration Pesticide Program: Residue Monitoring 1999*; http://vm.cfsan.fda.gov/~dms/pesrpts.html.

(16) Simonich, S. L.; Hites, R. A. Global distribution of persistent organochlorine compounds. *Science* **1995**, *269*, 1851−1854.

(17) Neidert, E.; Trotman, R. B.; Saschenbrecker, P. W. Levels and incidences of pesticide residues in selected agricultural food comrnodities available in Canada. *J. AOAC Int.* **1994**, *77*, 18−33.

(18) FDA (U.S.Food and Drug Administration). *Pesticide Analytical Manual*, 3rd ed.; U.S. Food and Drug Administration: Washington, DC, 1994; Vol. I, Chapter 3.

(19) Mukherjee, I.; Gopal, M. Chromatographic techniques in the analysis of organochlorine pesticide residues. *J. Chromatogr.* **1996**, *754*, 33−42.

(20) Van Emon, J. M.; Gerlach, C. L. A status report on field-portable immunoassay. *Environ. Sci. Technol.* **1995**, *29*, 312A−317A.
(21) Meulenberg, E. P. Immunochemical detection of environmental and food contaminants: Development, validation and application. *Food Technol. Biotechnol.* **1997**, *35*, 153−163.
(22) Sherry, J. Environmental immunoassays and other bioanalytical methods: Overview and update. *Chemosphere* **1997**, *34*, 1011−1025.
(23) Lee, N. A.; Kennedy, I. R. Environmental monitoring of pesticides by immunochemical techniques: Validation, current status, and future perspectives. *J. AOAC Int.* **2001**, *84*, 1393−1406.
(24) Goodrow, M. H.; Sanbom, J. R.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. Strategies for immunoassay hapten design. In *Immunoanalysis of Agrochemicals*; Nelson, J. O., Karu, A. E., Wong, R. B., Eds.; ACS Symposium Series 586; American Chemical Society: Washington, DC, 1995; pp 119−139.
(25) Marco, M. P.; Gee, S.; Hammock, B. D. Immunochemical techniques for enviromental analysis II. Antibody production and immunoassay development. *Trends Anal. Chem.* **1995**, *14*, 415−425.
(26) Johnson, J. C.; Van Emon, J. M.; Pullman, D. R.; Keeper, K. R. Development and evaluation of antisera for detection of the *O,O*-diethyl phosphorothionate and phosphorothionothiolate organophosphorus pesticides by immunoassay. *J. Agric. Food Chem.* **1998**, *46*, 3116−3123.
(27) Haasnoot, W.; DuPre, J.; Cazemier, G.; Kemmers-Voncken, A.; Verheijen, R.; Jansen, B. J. M. Monoclonal antibodies against a sulfathiazole derivative for the immunochemical detection of sulfonamides. *Food Agric. lmmunol.* **2000**, *12*, 127−138.
(28) Stanker, L. H.; Beier, R. C. Introduction to immunoassays for residue analysis. In *lmmunoassays for Residue Analysis*; Beier, R. C., Stanker, L. H., Eds.; ACS Symposium Series 621; American Chemical Society: Washington, DC, 1996; pp 2−16.
(29) Langone, J. J.; Van Vunakis, H. Radioimmunoassay for dieldrin and aldrin. *Res. Commun. Chem. Pathol. Pharmacol.* **1975**, *10*, 163−171.
(30) Morsy, M. A.; Ibrahim, A. A.; Hewedi, M. M. Detection of dieldrin by enzyme-linked immunosorbent assay in some dairy products. In *Immunoassays for Residue Analysis*; Beier, R. C., Stanker, L. H., Eds.; ACS Symposium Series 621; American Chemical Society: Washington, DC, 1996; pp 177−186.
(31) Dreher, R. M.; Podratzki, B. Development of an enzyme immunoassay for endosulfan and its degradation products. *J. Agric. Food Chem.* **1988**, *36*, 1072−1075.
(32) Karu, A. E.; Liliental, J. E.; Schmidt, D. J.; Lim, A. K.; Carlson, R. E.; Swanson, T. A.; Buirke, A. W.; Charmerlik, M. Monoclonal antibody-based immunoassay of cyclodienes. *Proceedings, 6th Annual EPA Waste Testing and Quality Assurance Symposium*; U.S. GPO: Washington, DC, 1990; Vol. I, pp 237−245.
(33) Esser, T.; Karu, A. E.; Toia, R. F.; Casida, J. E. Recognition of tetremethylenedisulfotetramine and related sulfamides by the GABA-gated chloride channel and a cyclodiene-sensitive monoclonal antibody. *Chem. Res. Toxicol.* **1991**, *4*, 162−167.
(34) Stanker, L. H.; Watkins, B.; Vanderlaan, M.; Ellis, R.; Rajan, J. Analysis of heptachlor and related cyclodiene insecticides in food. In *Immunoassays for Trace Chemical Analysis*; Vanderlaan, M., Stanker, L., Watkins, B., Roberts, D., Eds.; ACS Symposium Series 451; American Chemical Society: Washington, DC, 1991; pp 108−123.
(35) Lee, N.; Skerritt, J.; McAdam, D. P. Hapten synthesis and development of ELISAs for detection of endosulfan in water and soil. *J. Agric. Food Chem.* **1995**, *43*, 1730−1739.
(36) Brummel, K. E.; Wright, J.; Eldefrawi, M. E. Fiber optic biosensor for cyclodiene insecticides. *J. Agric. Food Chem.* **1997**, *45*, 3292−3298.
(37) Wigfield, Y. Y.; Grant, R. Evaluation of an immunoassay kit for the detection of certain organochlorine (cyclodiene) pesticide-residues in apple, tomato, and lettuce. *Bull. Environ. Contam. Toxicol.* **1992**, *49*, 342−347.
(38) Dombrowski, T. R.; Thurman, E. M.; Mohrman, G. B. A first application of enzyme-linked-immunosorbent-assay for screening cyclodiene insecticides in ground water. In *Environmental Immunochemical Methods: Perspectives and Applications*; Van Emon, J. M., Gerlach, C. L., Johnson, J. C., Eds.; ACS Symposium Series 646; American Chemical Society: Washington, DC, 1996; pp 148−154.
(39) Meulenberg, E. P.; Devree, L. G.; Dogterom, J. Investigation of indicative methods in The Netherlands−Validation of several commercial ELISAs for pesticides. *Anal. Chim. Acta* **1999**, *399*, 143−149.
(40) Abad, A.; Manclús, J. J.; Mojarrad, F.; Mercader, J. V.; Miranda, M. A.; Primo, J.; Guardiola, V.; Montoya, A. Hapten synthesis and production of monoclonal antibodies to DDT and related compounds. *J. Agric. Food Chem.* **1997**, *45*, 3694−3702.
(41) Habeeb, A. F. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem.* **1966**, *14*, 328−336.
(42) Nowinski, R. C.; Lostrom, M. E.; Tam, M. R.; Stone, M. R.; Burnette, W. N. The isolation of hybrid cell lines producing monoclonal antibodies against the P15(e) protein of ecotropic murine leukemia viruses. *Virology* **1979**, *93*, 111−126.
(43) Raab, G. M. Comparison of a logistic and a mass-action curve for radioimmunoassay data. *Clin. Chem.* **1983**, *29*, 1757−1761.
(44) Manclús, J. J.; Primo, J.; Montoya, A. Development of enzyme-linked immunosorbent assay for the insecticide chlorpyrifos. 1. Monoclonal antibody production and immunoassay design. *J. Agric. Food Chem.* **1996**, *44*, 4052−4062.
(45) Hahnau, S.; Weiler, E. W. Monoclonal antibodies for the enzyme immunoassay of the mycotoxin cyclopiazonic acid. *J. Agric. Food Chem.* **1993**, *41*, 1076−1080.
(46) Abad, A.; Primo, J.; Montoya, A. Development of an enzyme-linked immunosorbent assay to carbaryl. 1. Antibody production from several haptens and characterization in different immunoassay formats. *J. Agric. Food Chem.* **1997**, *45*, 1486−1494.
(47) Fasciglione, G. F.; Marini, S.; Bannister, J. V.; Giardinam, B. Hapten-carrier interactions and their role in the production of monoclonal antibodies against hydrophobic haptens. *Hybridoma* **1996**, *15*, 1−9.
(48) Manclús, J. J.; Montoya, A. Development of enzyme-linked immunosorbent assay for the insecticide chlorpyrifos. 2. Assay optimization and application to environmental waters. *J. Agric. Food Chem.* **1996**, *44*, 4063−4070.
(49) Mercader, J. V.; Montoya, A. Development of monoclonal ELISAs for azinphos-methyl. 2. Assay optimization and water sample analysis. *J. Agric. Food Chem.* **1999**, *47*, 1285−1293.
(50) Abad, A.; Montoya, A. Development of an enzyme-linked immunosorbent assay to carbaryl. 2. Assay optimization and application to the analysis of water samples. *J. Agric. Food Chem.* **1997**, *45*, 1495−1501.
(51) Lázaro, R.; Herrera, A.; Ariño, A.; Conchello, M. P.; Bayarri, S. J. Organochlorine pesticide residues in total diet samples from Aragón (mortheastern Spain). *J. Agric. Food Chem.* **1996**, *44*, 2742−2747.

Received for review November 24, 2003. Revised manuscript received March 10, 2004. Accepted March 10, 2004. Part of this work was supported by IMPIVA (Generalitat Valenciana, Spain), Project 89.93/1035.

JF035382H