Hapten Synthesis and Production of Monoclonal Antibodies to DDT and Related Compounds

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This work describes the production and characterization of monoclonal antibodies (MAbs) to the organochlorine insecticide DDT and their incorporation into several ELISA configurations. A collection of DDT haptens was synthesized by introducing appropriate spacers at two sites of the analyte molecular structure. From mice immunized with hapten−protein conjugates, MAbs with I_{50} values to *p,p′*-DDT in the 2−11 nM range in homologous conjugate-coated assays were obtained. According to their cross-reactivity pattern with DDT isomers and metabolites, MAbs can be classified as *class-specific* or *DDT-specific* antibodies. Both types of MAbs were obtained from mice immunized with the same hapten−protein conjugate simply by applying a different selection criterion in the screening of fusion supernatants. These immunoassays are potentially very valuable analytical tools for the rapid and sensitive determination of DDT and congeners in food and the environment and for monitoring human exposure to these ubiquitous and toxic compounds.

Keywords: *ELISA; insecticide; organochlorine pesticide; monoclonal antibodies; DDT; DDE; DDD; hapten design; hapten heterology; ELISA format*

INTRODUCTION

Since the discovery in the 1940s of the insecticidal properties of the chlorinated compound DDT [dichlorodiphenyltrichloroethane or 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane], more than 1 million tons of this pesticide have been used worldwide to control insect pests in agriculture, forestry, and human health (Buser and Müller, 1995). DDT is a highly hydrophobic compound with a great stability to physical, chemical, and biological degradation, which has resulted in the accumulation of its residues in the adipose tissues of animals and man, as well as in the environment (Kennish and Ruppel, 1996). The widespread occurrence of DDT, together with the fact that its elimination from the environment may take decades due to its persistence, evoked a great concern regarding the potential toxicological effects of its long-term residues. As a consequence, the use of DDT was totally banned in developed countries in the 1970s. Nevertheless, DDT is still extensively used in developing countries, where it is very valuable in malaria control programs (Mukherjee and Gopal, 1993; López-Carrillo *et al.*, 1996; Waliszewski *et al.*, 1996).

In technical formulations, DDT exists in two isomeric forms, *p,p′*-DDT and *o,p′*-DDT, even though the first one is the only isomer with insecticidal properties. The main DDT metabolites DDE (dichlorodiphenyldichloroethene) and DDD (dichlorodiphenyltrichloroethane), which are also lipophilic and even more stable than DDT, can also exist in the two corresponding isomeric forms (Buser and Müller, 1995; Guardino *et al.*, 1996; Kennish and Ruppel, 1996). Therefore, an analytical method for DDT should be able to determine the six isomers, although method efficiency is normally assessed by the recovery of the most abundant and frequently encountered *p,p′*-isomers.

Monitoring human and animal exposure to DDT residues through the analysis of biological tissues and fluids, such as milk, urine, and blood, is of great toxicological significance, particularly for those countries in which DDT is still in use. Furthermore, contamination of food and the environment by persistent organochlorine pesticides is a serious concern in tropical countries (Waliszewski *et al.*, 1996). Traditional methods for determination of chlorinated pesticides in tissue samples involve solvent extraction, partitioning of the pesticide residues from fat into another liquid, and cleanup of the extract to remove coextractives prior to gas chromatography. Consequently, the methods are lengthy and labor-intensive and are therefore not suitable for the analysis of the large number of samples required for comprehensive studies (Ahmad and Marolt, 1986; Hernández *et al.*, 1993; Bucholski *et al.*, 1996; Menezes and Felix, 1996).

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 are field-portable and do not require sophisticated instrumentation. All of these features convert immunoassays into very valuable methods for large monitoring programs (Sherry, 1992; Van Emon and López-Ávila, 1992; Meulenberg *et al.*, 1995; Aga and Thurman, 1997).

The key component of an immunoassay is the antibody, since it is responsible for the sensitive and specific recognition of the analyte. Several attempts to obtain antibodies for DDT and related compounds have been previously carried out. Initial work using protein conjugates of DDA [bis(*p*-chlorophenyl)acetic acid] as

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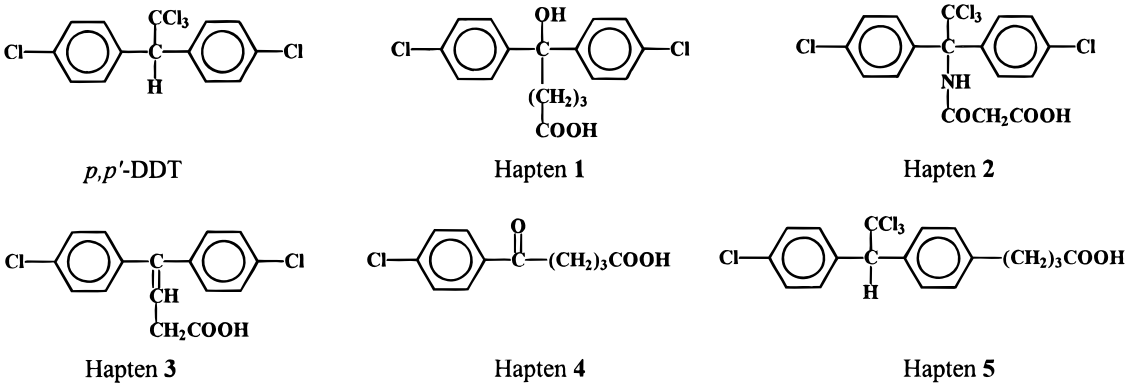


Figure 1. Chemical structures of *p,p′*-DDT and the haptens synthesized for this study.

immunogens resulted in low-affinity antisera that mainly recognized DDA, the chief urinary metabolite of DDT (Haas and Guardia, 1968; Centeno *et al.*, 1970; Furuya and Urasawa, 1981; Banerjee, 1987). Further work by Bürgisser *et al.* (1990) using a new immunogenic dicofol-derived hapten allowed them to obtain a monoclonal antibody (MAb) to DDT that exhibited an I_{50} around 100 nM in a competitive solid-phase radioimmunoassay. However, this assay has not found application for residue analysis probably due to its low sensitivity and the use of a radioactive label. Immunosystems also included in its catalog a qualitative/semiquantitative immunoassay for DDT, but the company has ceased operation. Therefore, to our knowledge no suitable enzyme immunoassay for this important and ubiquitous chemical has been reported. This situation greatly contrasts with the large number of pesticides for which enzyme-linked immunosorbent assays (ELISA) have been developed (Meulenberg, 1995). The aim of this work was to obtain high-affinity monoclonal antibodies to DDT and to develop enzyme immunoassays as an alternative to chromatographic techniques for the screening of large numbers of biological and environmental samples.

Hapten design is a key step in the development of immunoassays for small molecules because the hapten is primarily responsible for determining antibody recognition properties (Hammock *et al.*, 1990; Harrison *et al.*, 1991). 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 molecular sites is generally recommended (Harrison *et al.*, 1990; Goodrow *et al.*, 1995). Herein, the synthesis of several haptens and their use to produce MAbs to DDT 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 and its derivatives.

MATERIALS AND METHODS

Chemicals, Immunoreagents, and Instruments. Standards of *p,p′*-DDT, *o,p′*-DDT, *p,p′*-DDE, *o,p′*-DDE, *p,p′*-DDD, *o,p′*-DDD, and dicofol were from Riedel-de Haën (Seelze, Germany). Stock solutions were prepared in dry DMF and stored at 4 °C. Starting products for hapten synthesis and hapten−protein coupling reagents were obtained from Fluka-Aldrich Química (Madrid, Spain).

Freund's adjuvants, OVA, and OPD were obtained from Sigma Química (Madrid, Spain). BSA fraction V, enzyme immunoassay grade HRP, Hybridoma Fusion and Cloning Supplement (HFCS), and PEG 1500 were purchased from Boehringer Mannheim (Germany). Peroxidase-labeled rabbit anti-mouse Igs and affinity-isolated goat anti-mouse Igs were obtained from Dako (Glostrup, Denmark). DMEM culture medium, Myoclone Super Plus fetal calf serum, and supplements were from Gibco BRL (Paisley, Scotland). Culture plasticware was from Bibby Sterilin Ltd. (Stone, U.K.). P3-X63-Ag8.653 mouse plasmacytoma line was from American Type Culture Collection (Rockville, MD).

Polystyrene culture plates (high-binding plates, catalog no. 3590) were from Costar (Cambridge, MA). ELISA plates were washed with an Ultrawash II microplate washer from Dynatech (Sussex, U.K.), and absorbances were read in dual-wavelength mode (490−630 nm) with an Emax microplate reader from Molecular Devices (Sunnyvale, CA). ^{1}H and ^{13}C NMR spectra were obtained with a Varian VXR-400S spectrometer (Sunnyvale, CA), operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. Chemical shifts are reported relative to tetramethylsilane. UV−vis spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan).

Hapten Synthesis. DDT haptens used in this work (Figure 1) were prepared by introduction of alkyl chain spacers, ending in a carboxylic acid, in either the central carbon atom (haptens **1**−**3**) or the aromatic ring (hapten **5**) of the DDT structure. Hapten **4** is only half the complete DDT molecule, and it was a byproduct in the synthesis of hapten **1**.

*5,5-Bis(4-chlorophenyl)-5-hydroxypentanoic Acid (**1**) and 5-(4-Chlorophenyl)-5-oxopentanoic Acid (**4**, Figure 2).* Glutaric anhydride (1.14 g, 0.01 mol) was placed in dry ether (40 mL) in the presence of argon. After warming to dissolve the anhydride, *p*-chlorophenylmagnesium bromide (0.03 mol, 30 mL of a 1 M ether solution) was added from a dropping funnel. The reaction mixture was boiled under reflux for 2 h and finally was stirred at room temperature for 14 h. After this time, the reaction mixture was poured over a saturated solution of ammonium chloride. The aqueous phase was washed with ether, acidified with 1 M HCl (1:1 by volume), and extracted with ether. The organic phase was washed with water, dried ($MgSO_4$), and concentrated. The weight of residue was 2.5 g. Hapten **4**, previously described by Masse *et al.*(1989), was obtained upon recrystallization from water (1.3 g, 50%), while hapten **1** remained undissolved (1 g, 40%).

Hapten **4** formed white crystals: mp 122−124 °C (water); ^{1}H NMR ($CDCl_3$) δ 7.9 (d, J = 8 Hz, 2H, 2,6-ArH), 7.43 (d, J = 8 Hz, 2H, 3,5-Ar-H), 3.05 (t, J = 7 Hz, 2H, $ArCOCH_2$), 2.50 (t, 2H, J = 7 Hz, $C\underline{H}_2COOH$), 2.07 (t, J = 7 Hz, 2H, $CH_2C\underline{H}_2$-CH_2); ^{13}C NMR 198.2 (CO, ketone), 179.1 (CO, acid), 139.7 (CCl), 135.1 (CCO), 129.5 and 129 (aromatic CH), 37.2, 32.8, and 18.8 (CH_2).

Hapten **1** was an oily yellowish compound: ^{1}H NMR ($CDCl_3$) δ 7.24−7.22 (m, 4H aromatic), 2.40 (t, J = 7 Hz, 2H, $C\underline{H}_2COOH$), 2.27 [m, 2H, $C(OH)C\underline{H}_2$], 1.57−1.52 (m, 2H, $C\underline{H}_2C\underline{H}_2CH_2$); ^{13}C NMR 170.7 (CO), 141.9 and 133.9 (aromatic C), 128.9 and 127.0 (aromatic CH), 86.9 (COH), 33.1, 29.0, and 16.5 (CH_2).

*1,1-Bis(4-chlorophenyl)-2,2,2-trichloroethylaminocarbonyl-acetic Acid (**2**, Figure 3).* Dicofol (250 mg, 0.67 mmol), ethyl cyanoacetate (1 mL), and acetic acid (1 mL) were placed together in a round-bottom flask, and 96% sulfuric acid (4 mL) was added slowly. After the addition was complete, the mixture was warmed over a hot water bath at 50 °C for 15 min. The reaction mixture became dark red, and then it was

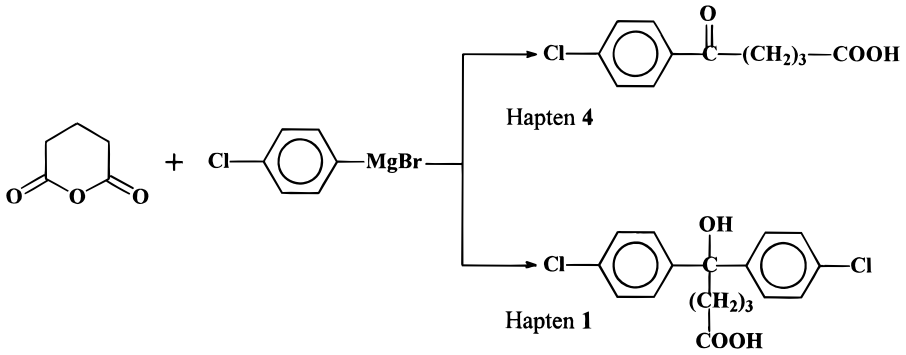


Figure 2. Reaction scheme of the synthesis of haptens **1** and **4**.

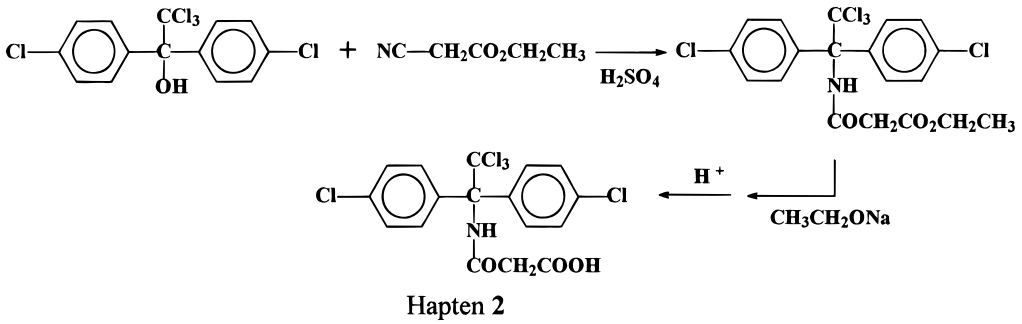


Figure 3. Reaction scheme of the synthesis of hapten **2**.

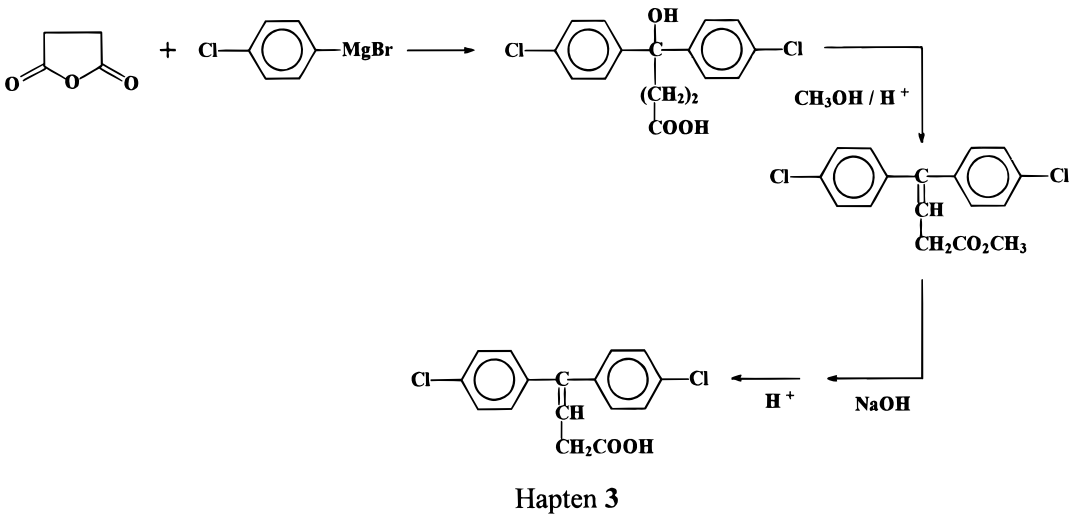


Figure 4. Reaction scheme of the synthesis of hapten **3**.

stirred at room temperature for 20 h. The mixture was poured over water and extracted with ether. The ether phase was washed with water and sodium bicarbonate solution, dried, and concentrated. The crude product (200 mg) was purified on a silica gel preparative plate using hexane/CH_2Cl_2 (1:1) as eluant. Three fractions were separated. Dicofol was recovered (60 mg), the second compound was a dimeric ether of dicofol (70 mg), and the least polar compound was the ethyl ester of hapten **2** (30 mg): ^{1}H NMR ($CDCl_3$) δ 9.15 (m, 1H, NH), 7.55 (d, J = 8 Hz, 4H aromatic), 7.29 (d, J = 8 Hz, 4H aromatic), 4.29 (q, J = 7 Hz, 2H, $C\underline{H}_2CH_3$), 3.38 (s, 2H, $COCH_2$), 1.33 (t, J = 7 Hz, 3H, $CH_2C\underline{H}_3$).

The ester was hydrolyzed to acid using sodium ethanolate. Sodium (6 mg) was dropped into ethyl alcohol (1 mL) in a small flask provided with a condenser; the mixture was heated until all of the sodium was dissolved and then cooled in an ice−salt bath. Then the ester (30 mg) was added and the reaction mixture was stirred for 15 min. Thereafter, the solvent was evaporated. Water was added, and the mixture was acidified with dilute HCl and extracted with ether, dried, and concentrated to afford 15 mg of hapten **2**: ^{1}H NMR ($CDCl_3$) δ 9 (bs, 1H, NH), 7.55 (d, J = 10 Hz, 4H aromatic), 7.29 (d, J = 10 Hz, 4H aromatic), 3.43 (s, 2H, $C\underline{H}2$); ^{13}C NMR 171.2 (COOH), 163.6 (CONH), 136.3 and 134.7 (aromatic C), 131.5 and 127.6 (aromatic CH), 104.9 (CCl_3), 77.4 ($\underline{C}NHCO$), 40.55 (CH_2).

*4,4-Bis(4-chlorophenyl)-3-butenoic Acid (**3**, Figure 4).* This hapten was synthesized by an alternative route to that described by Himmele *et al.* (1985). Succinic anhydride (1.0 g, 0.01 mol) was placed in dry ether (40 mL) in the presence of argon. After warming to dissolve the anhydride, *p*-chlorophenylmagnesium bromide (0.03 mol, 30 mL of a 1 M ether solution) was added from a dropping funnel. The reaction mixture was boiled under reflux for 2 h and finally stirred at room temperature for 14 h. After this time, the mixture was poured over a saturated solution of ammonium chloride. The aqueous phase was washed with ether, acidified with 1 M HCl (1:1 by volume), and extracted with ether. The ethereal extract was washed with water, dried ($MgSO_4$), and concentrated to afford an oily residue (2.6 g). Purification of 400 mg of this crude was achieved by esterification with methanol/sulfuric acid, separation by silica gel preparative chromatography (hexane/CH_2Cl_2, 1:1), and hydrolysis. This led to 140 mg of hapten **3**: ^{1}H NMR ($CDCl_3$) δ 7.40−7.09 (m, 8H, aromatic), 6.22 (t, J = 8 Hz, 1H, C=CH), 3.19 (d, J = 8 Hz, 2H, CH_2).

*4-{4-[1-(4-Chlorophenyl)-2,2,2-trichloroethyl]phenyl}butanoic Acid (**5**, Figure 5).* Chloral hydrate crystals (2.3 g, 0.002

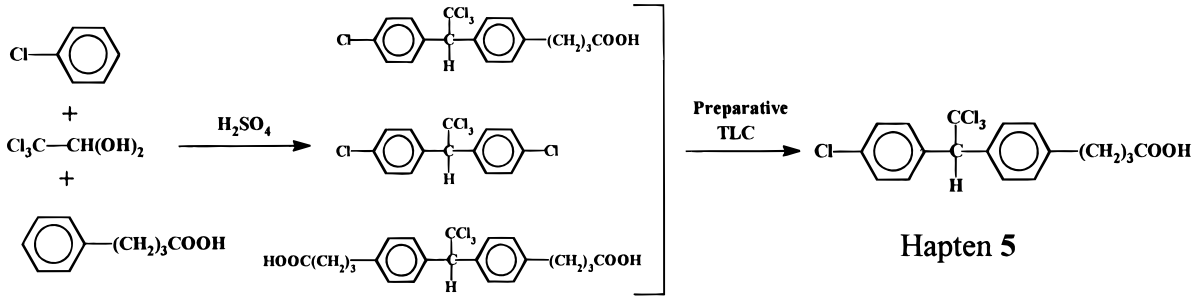


Figure 5. Reaction scheme of the synthesis of hapten **5**.

mol), chlorobenzene (2 mL, 0.002 mol), and 4-phenylbutanoic acid (1.6 g, 0.001 mol) were placed together in a round-bottom flask and warmed on a water bath, with occasional shaking, until all crystals dissolved. The reaction mixture was cooled in an ice bath, and 96% sulfuric acid (7 mL, 6.4 g) was added slowly. The mixture was shaken mechanically for 1.5 h. After this time, it was poured slowly with stirring into water (100 mL). Then it was extracted with ether, dried, and concentrated. The crude product (2.5 g) was purified on silica gel preparative chromatography plates (200 mg per plate) using CH_2Cl_2 as eluant. This way, 1 g of hapten **5** was obtained: ^{1}H NMR ($CDCl_3$) δ 7.49−7.04 (m, 8H, aromatic), 2.47 (bs, 2H, CH_2), 2.25 (bs, 2H, CH_2), 1.79 (bs, 2H, CH_2); ^{13}C NMR 173 (COOH), 142, 136.7, 135.4 and 133.9 (aromatic C), 131.3, 129.9, 128.4 and 128.2 (aromatic CH), 101.3 (CCl_3), 70,34 (CHCCl3), 35, 35.2, and 27 (CH2); IR (CH_2Cl_2) ν^{-1} cm^{-1} 1704 (CO).

Preparation of Immunizing Conjugates. Haptens **1**, **2**, and **5** were covalently attached to BSA using the modified active ester method (Langone and Van Vunakis, 1982). Twenty-five micromoles of the hapten was incubated overnight at room temperature with stoichiometric amounts of *N*-hydroxysuccinimide and dicyclohexylcarbodiimide in 0.5 mL of DMF. After centrifuging, 400 μL of the clear supernatant containing the active ester was slowly added to 2 mL of a 15 mg/mL BSA solution in 50 mM carbonate buffer, pH 9.6. The mixture was allowed to react at room temperature for 4 h with stirring, and finally the conjugate was purified by gel filtration on Sephadex G-50 using 100 mM sodium phosphate buffer, 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. The hapten to protein molar ratio of conjugates was then estimated from the spectral data of the hapten, the protein, and the corresponding conjugate. By assuming that the molar absorptivity of haptens was the same for the free and conjugated forms, apparent molar ratios were estimated as 16, 20, and 23 for haptens **1**, **2**, and **5**, respectively.

Preparation of Coating Conjugates. All of the haptens were covalently attached to OVA using the mixed-anhydride method (Rajkowski *et al.*, 1977). Eighteen micromoles of the hapten was allowed to react at room temperature for 1 h with stoichiometric amounts of tri-*n*-butylamine and isobutyl chloroformate in 200 μL of DMF. One hundred microliters of the resulting activated hapten was added to 30 mg of OVA in 2 mL of 50 mM carbonate buffer, pH 9.6. The coupling reaction was incubated at room temperature for 2−3 h with stirring, and the conjugates obtained were purified as described for the immunogens. The extent of coupling of each hapten to OVA was determined by UV−vis spectrophotometry. By assuming additive absorbance values, hapten to protein molar ratios were evaluated as 4, 21, 3, 9, and 11 for haptens **1**, **2**, **3**, **4**, and **5**, respectively.

Preparation of Enzyme Conjugates. The mixed-anhydride method was also used for covalent coupling of haptens to HRP. Typically, 2.9 μL of tri-*n*-butylamine and 1.6 μL of isobutyl chloroformate were added to 13.3 μmol of the hapten in 200 μL of DMF. The mixture was stirred for 1 h at room temperature. After 1.8 mL of DMF was added, 100 μL of this diluted solution of activated hapten was incubated for 2 h at room temperature with 1 mL of a 2.2 mg/mL solution of HRP in 50 mM carbonate buffer, pH 9.6. HRP−hapten conjugates were purified as described for the immunogens. HRP conjugate concentrations and molar ratios were estimated spectrophotometrically. With the same assumptions as before, the estimated hapten to enzyme molar ratios were 3, 4, 1, 1, and 10 for haptens **1**, **2**, **3**, **4**, and **5**, respectively.

Production of MAbs to DDT. *Immunization.* BALB/c female mice (8−10 weeks old) were immunized with BSA−**1**, −**2**, and −**5** conjugates. First dose consisted of 30 μg of conjugate intraperitoneally injected as an emulsion of PBS (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and complete Freund's adjuvant. Two subsequent injections with immunogens emulsified in incomplete Freund's adjuvant were given at 3 week intervals. One week after the last injection, mice were tail-bled and sera tested for antihapten 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-Ag8.653 murine myeloma cells (ATCC, Rockville, MD) were cultured in high-glucose DMEM supplemented with 2 mM GLUTAMAX I, 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 using 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/well in 100 μL of s-DMEM. 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) 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 1 week, and then HAT was substituted by HT medium (HAT medium without aminopterine).

Hybridoma Selection and Cloning. Eight to eleven days after cell fusion, culture supernatants were screened for the presence of antibodies that recognized *p,p′*-DDT. 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 *p,p′*-DDT, respectively. For each culture supernatant, the signal obtained in noncompetitive conditions was compared with the competitive one, and the ratio of both absorbances was used as the criterion for selecting high-affinity antibody-secreting clones. Selected hybridomas were cloned by limiting dilution on a feeder layer of BALB/c thymocytes (*ca.* 10^6 cells/well) and peritoneal macrophages (*ca.* 5000 cells/well). Stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen.

Purification of MAbs. Antibodies were purified directly from late stationary phase culture supernatants by saline precipitation followed by anion-exchange chromatography on DEAE-Sepharose (Sigma). Most culture supernatants were able to provide enough MAb (3−10 mg/100 mL) for characterization studies and further work. Purified MAbs were stored at 4 °C as ammonium sulfate precipitates.

ELISAs. Flat-bottom polystyrene ELISA plates 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, using borosilicate glass tubes. 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 for the study of antibody sensitivity and specificity to DDT. 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 (HRP−hapten conjugates).

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 (Raab, 1983), using Sigmaplot software package (Jandel Scientific, Germany).

Conjugate-Coated Format. Plates were coated with 1 μg/mL of OVA−hapten conjugates. Then, different antibody concentrations in PBS containing 0.1% BSA were added and incubated for 2 h. Next, plates were incubated for 1 h with peroxidase-labeled rabbit anti-mouse Igs 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, 62 mM sodium phosphate, pH 5.4). 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 (*p,p′*-DDT or related compounds) in PBS followed by 50 μL of the appropriate concentration of antibody.

Antibody-Coated Format. In this format, plates were coated with antibodies at 1 μg/mL. Next, the competition was established for 1 h between DDT standards diluted in PBS (50 μL/well) and the selected concentrations of enzyme tracers (50 μL/well). 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 1 μg/mL in PBST.

RESULTS AND DISCUSSION

Synthesis of Haptens. Regarding its immunogenic structure, DDT is a small, highly hydrophobic, and simple analyte, consisting of two aromatic rings substituted with a chlorine atom and connected to each other by a carbon atom (Figure 1). An appropriate hapten design should preserve as many substituents as possible and produce minor modifications in the ring electronic distribution as a consequence of spacer attachment (Manclús and Montoya, 1996). Consequently, two approaches were followed to prepare haptens resembling the DDT chemical structure: (I) attachment of a spacer arm through the carbon atom that joins the two aromatic rings and (II) introduction of a spacer arm in the aromatic ring by substitution of the chlorine atom. The first strategy led to the synthesis of haptens **1**−**3**, whereas hapten **5** belongs to the second chemical approach. Consistent with their highest similarity to the analyte, compounds **1**, **2**, and **5** were used as immunizing and assay haptens, whereas haptens **3** and **4** were used to prepare heterologous conjugates. Heterologous conjugates based on haptens consisting of only a part of the complete analyte structure, such as compound **4**, have been previously used to improve the sensitivity of immunoassays for PCBs (Chiu *et al.*, 1995) and chlorpyrifos (Manclús *et al.*, 1996).

Table 1. Summary of Cell Fusion and Hybridoma Selection Results

immunizing hapten	fusion no.	no. of wells: seeded	positive[a] (conjugate)	competitive[b] (analyte)	no. of cloned hybridomas[c]
1	1	288	29	1	1
	2	152	1	0	0
2	1	744[e]	20	0	0
5	1	384	5	3	−[d]
	2	384	36	36	4
	3	288	5	0[g]	0
	4	384	8	4[g]	−
	5	1632[f]	41	32[g]	3

[a] Wells with antibodies that recognized the OVA−hapten conjugates (homologous assays) by indirect ELISA (absorbance > 0.5). [b] Wells with antibodies that recognized 1 μM *p,p′*-DDT in solution (inhibition > 50%). [c] Only hybridomas secreting antibodies with the lowest I_{50} for *p,p′*-DDT were stabilized and cloned. [d] Indicates that competitive antibodies were initially cloned, but they were rejected during the cloning proccess because better antibodies were obtained in further fusions. [e] This cell fusion was carried out with lymphocytes obtained from two spleens. [f] The number of seeded wells was significantly higher in this fusion than in the rest because hybridomas were seeded at a lower cell density by using HFCS. [g] In the screening of these cell fusions, wells were considered competitive if the signal was inhibited >50% by 0.1 μM *p,p′*-DDT and <25% by 0.1 μM *p,p′*-DDE.

Production of Antibodies to DDT. *Mouse Polyclonal Response.* To evaluate the suitability of the synthesized haptens to raise anti-DDT antibodies, mice were immunized with BSA conjugates of haptens **1**, **2**, and **5**. After three injections, the titer (serum dilution giving an absorbance around 1.0 in the established conditions) of antibodies recognizing conjugated haptens was estimated by indirect ELISA using the respective homologous OVA−hapten as coating conjugate. Sera from all of the immunogens showed antihapten reactivity with titers in the $(1-5) \times 10^4$ range and were subsequently tested for their ability to recognize *p,p′*-DDT by indirect competitive ELISA. Only sera from mice immunized with BSA−**5** bound competitively *p,p′*-DDT, with a concentration producing 50% inhibition of antibody binding (I_{50}) of 1 μM *p,p′*-DDT. No inhibition was found for the rest of the sera.

Production of MAbs. The immunological properties of mouse polyclonal and monoclonal antibodies raised to the same haptenic structure are often fairly different. In fact, MAbs with an affinity to the analyte up to 4 orders of magnitude better than that of the mouse sera have been obtained (Abad and Montoya, 1994). Then, although an anti-DDT polyclonal response was demonstrated only with the BSA−**5** immunogen, at least one cell fusion was undertaken for each immunizing hapten.

Cell fusion and hybridoma selection results are summarized in Table 1. The yield of wells with antibodies recognizing the corresponding homologous conjugated haptens (positive clones) was highly variable from one fusion to another. Moreover, not all of the positive wells showed reactivity to *p,p′*-DDT (competitive wells). In the first fusion carried out from each immunizing hapten, wells showing inhibition by 1 μM *p,p′*-DDT were found only from mice immunized with haptens **1** and **5** (one and three hybridomas, respectively). As the objective of this work was to obtain high-affinity antibodies to DDT, additional fusion effort was focused on mice immunized with haptens that gave positive results in the first fusion.

In fusion 2 with hapten **1**, the only positive well did not contain antibodies showing 50% inhibition by a concentration cutoff of 1 μM *p,p′*-DDT. However, the second fusion from hapten **5** was especially successful, since all positive wells behaved competitively. In fact,

Table 2. Affinity to *p,p′*-DDT of MAbs Using Homologous Haptens in the Conjugate-Coated (CC) and in the Indirect Antibody-Coated (IAC) Formats

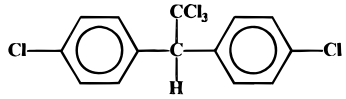
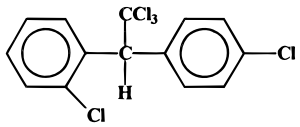
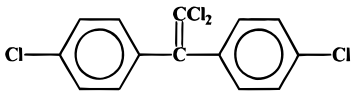
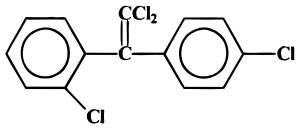
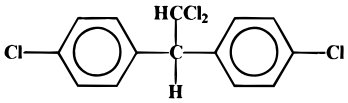
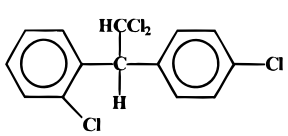
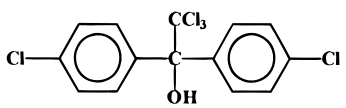
	I_{50} (nM)	
MAb	CC format	IAC format
LIB**1**-11	11.1	90.8
LIB**5**-21	2.1	13.0
LIB**5**-25	2.1	16.0
LIB**5**-28	2.3	13.4
LIB**5**-212	2.9	14.1
LIB**5**-51	4.6	9.3
LIB**5**-52	6.4	11.4
LIB**5**-53	6.5	9.4

most of them displayed an affinity to *p,p′*-DDT higher than that of the antibodies raised in the first fusion. Therefore, the three antibodies raised from fusion 1 were discarded and the four hybridomas from fusion 2 showing the strongest recognition of *p,p′*-DDT were stabilized and cloned. Preliminary characterization of these antibodies from culture supernatants indicated that all of them recognized *p,p′*-DDE and *p,p′*-DDT almost equally. At this point we reasoned that it would also be of analytical interest to obtain antibodies able to discriminate between both compounds. With this idea in mind, new cell fusions were undertaken with hapten **5**. In the screening of fusion supernatants, both *p,p′*-DDE and *p,p′*-DDT were used as competitors, so only those wells showing an inhibition of the noncompetitive signal >50% by 0.1 μM *p,p′*-DDT and <25% by 0.1 μM *p,p′*-DDE were considered competitive. Among those wells that fulfilled this criterion, the three hybridomas displaying the highest inhibition by *p,p′*-DDT and the lowest inhibition by *p,p′*-DDE were selected and cloned.

Characterization of the MAbs. MAbs obtained from haptens **1** (LIB**1**-11) and **5** (LIB**5**-*) were characterized for affinity and specificity to DDT using homologous and heterologous haptens in different ELISA formats.

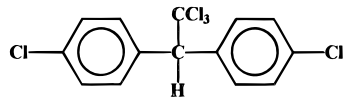
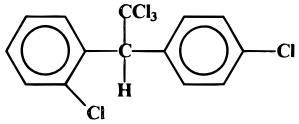
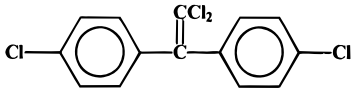
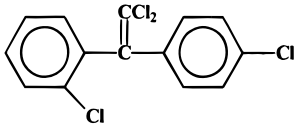
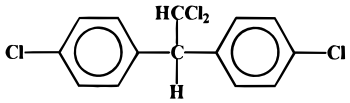
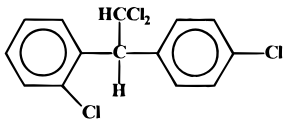
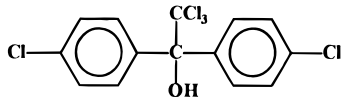
Affinity. The ability of the eight MAbs to recognize *p,p′*-DDT was first determined using the homologous conjugate-coated ELISA format. I_{50} values ranged from 2.1 to 11.1 nM (Table 2). All MAbs derived from hapten **5** displayed a higher affinity to *p,p′*-DDT than the MAb derived from hapten **1**. When the antibodies were assayed in the same ELISA format but using heterologous conjugates, LIB**1**-11 MAb recognized coating conjugates of haptens **3** and **4**, although competitive assays with these heterologous conjugates did not improve the high sensitivity of the homologous assay (I_{50} values of 39.8 and 12.4 nM with the conjugates OVA−**3** and OVA−**4**, respectively). None of the MAbs derived from hapten **5** (LIB**5**-* MAbs) recognized heterologous conjugates based on haptens **1**−**4**, so no competitive heterologous assays could be performed. It should be noted that MAbs derived from hapten **5**, with the spacer arm

Table 3. MAb Recognition of DDT Isomers in the Homologous Conjugate-Coated ELISA Format[a]

		monoclonal antibody cross-reactivity[b] (%)							
compound	structure	LIB**1**-11	LIB**5**-21	LIB**5**-25	LIB**5**-28	LIB**5**-212	LIB**5**-51	LIB**5**-52	LIB**5**-53
p,p′-DDT		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
o,p′-DDT		8.5	46.0	61.3	51.2	45.1	127.4	124.3	109.7
p,p′-DDE		13.9	83.8	92.4	72.8	90.0	5.2	3.4	3.8
o,p′-DDE		11.3	22.5	26.8	23.7	20.5	4.4	2.4	3.2
p,p′-DDD		56.2	305.6	223.9	202.1	475.1	10.2	4.9	3.8
o,p′-DDD		5.6	66.4	81.2	73.1	73.7	11.4	4.4	3.5
dicofol		8.2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[a] Coating conjugates were OVA-**1** for the LIB**1**-11 MAb and OVA-**5** for the rest of the antibodies. Both conjugates were used at 1 μg/mL. Each antibody was used at a concentration previously determined as optimal. [b] Percentage of cross-reactivity = (I_{50} of *p,p′*-DDT/I_{50} of other compound) × 100.

Table 4. MAb Recognition of DDT Isomers in the Homologous Indirect Antibody-Coated ELISA Format[a]

		monoclonal antibody cross-reactivity[b] (%)						
compound	structure	LIB**5**-21	LIB**5**-25	LIB**5**-28	LIB**5**-212	LIB**5**-51	LIB**5**-52	LIB**5**-53
p,p′-DDT		100.0	100.0	100.0	100.0	100.0	100.0	100.0
o,p′-DDT		44.4	48.2	46.8	42.8	117.4	113.3	100.3
p,p′-DDE		86.8	83.1	88.6	87.5	5.7	3.8	2.5
o,p′-DDE		19.6	22.5	20.2	19.4	3.9	2.7	1.8
p,p′-DDD		131.0	135.8	119.2	134.8	12.2	7.4	4.9
o,p′-DDD		38.1	52.7	37.1	41.6	8.2	5.7	3.9
dicofol		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[a] The goat anti-mouse capture antibody was used at 2 μg/mL, and the MAbs at 1 μg/mL. The enzyme tracer was HRP-**5** for all MAbs, and it was used at the concentration previously determined as optimal for each MAb. [b] Percentage of cross-reactivity = (I_{50} of *p,p′*-DDT/I_{50} of other compound) × 100.

attached to the aromatic ring, did not recognize conjugates from hapten **1**, derivatized at the central carbon atom, and *vice versa*. Therefore, although hapten heterology has been demonstrated as a good approach to improve the sensitivity of pesticide immunoassays (Wie and Hammock, 1984; Marco *et al.*, 1995), the heterologous hapten structure and/or the way it is presented to the antibody should not involve great differences with respect to the immunizing structure. Otherwise, haptens could become unrecognized by the antibody and unsuitable to develop competitive immunoassays. In the authors' experience, suitable heterologous haptens are those prepared by introducing slight changes in the hapten structure (change or elimination of a substituent), by changing the type and/or length of the spacer arm, or by changing the attachment site to a position proximal to that used in the immunogen (Manclús and Montoya, 1996; Manclús *et al.*, 1996; Abad *et al.*, 1997).

The competitive behavior of the MAbs in the direct and indirect antibody-coated formats was also evaluated. MAbs directly immobilized to the plate at 1 μg/mL were not able to recognize any of the HRP−hapten conjugates, so no competitive assay could be performed with this assay configuration (the maximum concentration of enzyme conjugate tested was 10 μg/mL). On the contrary, MAbs immobilized to the plate through a capture immunoglobulin (indirect antibody-coated format) recognized their corresponding homologous enzyme conjugates, while the heterologous ones remained unrecognized. Therefore, competitive assays were performed with the HRP−**1** conjugate for the LIB**1**-11 MAb and with the HRP-**5** conjugate for the LIB**5**-* MAbs. For all of the MAbs, the I_{50} value obtained in the indirect antibody-coated format was higher than in the conjugate-coated format, so less sensitive immunoassays were obtained (Table 2).

Specificity. The specificity of the MAbs was evaluated by performing competitive assays with several compounds of the DDT family as competitors, and the obtained I_{50} values were used to calculate cross-reactivities. Except for LIB**1**-11 MAb, antibody cross-reactivities were investigated in both the conjugate-coated format (Table 3) and the antibody-coated format (Table 4). Comparison of the data in Tables 3 and 4 suggests that specificity is mainly determined by the MAb more than by the assay format used. The only noticeable change in the MAb cross-reactivity pattern is a lower recognition of DDD isomers in the indirect antibody-coated format with respect to the CR values found in the conjugate-coated format.

According to the recognition pattern exhibited, MAbs could be classified into three different groups. First, monoclonal antibodies LIB**5**-21, -25, -28, and -212 exhibited high cross-reactivity values for all of the compounds of the DDT family, particularly for the *p,p′*-isomers, which are the most relevant from an analytical point of view. Therefore, these MAbs can be considered

as unspecific antibodies or, more appropriately, *class-specific* antibodies. Second, monoclonal antibodies LIB**5**-51, -52, and -53 were *DDT-specific* antibodies, since they recognized almost equally *p,p′*- and *o,p′*-DDT isomers, while displaying low CR values for the rest of compounds (<11%). Third, LIB**1**-11 MAb displayed a different behavior characterized by CRs of 56.2% to *p,p′*-DDD and 5.6−13.9% for the rest of compounds. Not surprisingly, this MAb was the only one that recognized dicofol to some extent (CR = 8.2%), which is in accordance with the fact that this MAb derives from hapten **1,** and both dicofol and hapten **1** have a hydroxyl group joined to the central carbon atom. Nevertheless, the ideal antibody for analytical purposes should be completely specific for a determined analyte or completely specific of a certain class of compounds, showing very similar cross-reactivity values for all of them. Therefore, the analytical uselfulness of an antibody displaying an intermediate situation, like LIB**1**-11 MAb, is uncertain. This fact, together with the lower affinity to DDT-related compounds shown by LIB**1**-11 MAb, led us to focus our attention on LIB**5**-* MAbs.

Among class-specific antibodies, LIB**5**-25 MAb showed the highest and most homogeneous cross-reactivity values, so this antibody seems to be the most adequate to develop an immunoassay for DDT isomers and metabolites. With respect to the development of an immunoassay specific for DDT, LIB**5**-52 and LIB**5**-53 MAbs displayed the lowest cross-reactivity values for DDT metabolites along with a similar affinity to DDT isomers. The choice of LIB**5**-52 MAb for this purpose was based on the fact that it can be used at a concentration 10 times lower than LIB**5**-53 MAb. Competitive curves obtained with LIB**5**-25 and LIB**5**-52 MAbs in the conjugate-coated format to DDT isomers and metabolites are presented in Figure 6.

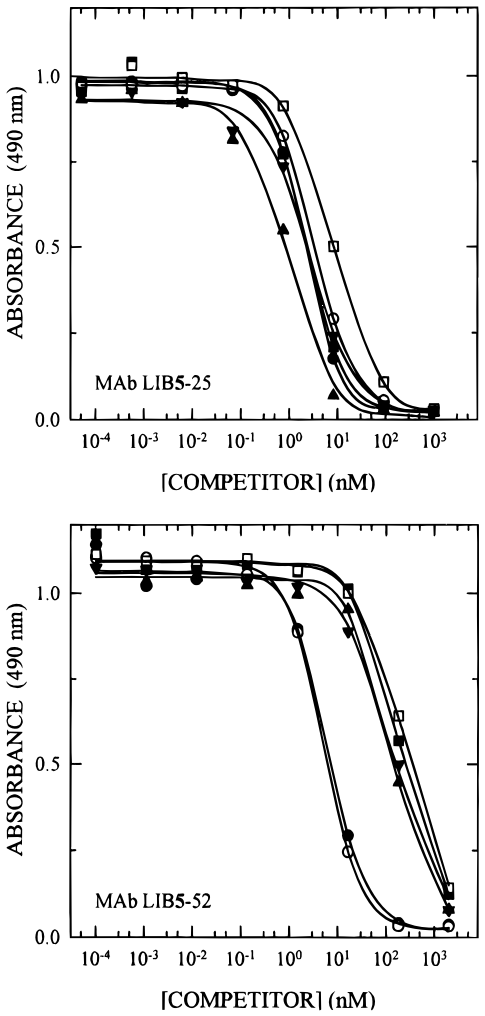


Figure 6. Competitive curves in the homologous conjugate-coated ELISA format obtained with the MAbs LIB**5**-25 (top) and LIB**5**-52 (bottom). The coating conjugate OVA-**5** was used at 1 μg/mL, and the MAbs were used at 0.1 and 0.2 μg/mL (LIB**5**-25 and LIB**5**-52, respectively). Standards in the range $10^{-4}-2 \times 10^3$ nM were prepared by serial dilutions in PBS from the stock solutions in DMF. Points represent the average of absorbance values from triplicate wells, and they were fitted to a four-parameter logistic equation (lines). Competitors: *p,p′*-DDT (●); *o,p′*-DDT (○); *p,p′*-DDE (■); *o,p′*-DDE (□); *p,p′*-DDD (▲); *o,p′*-DDD (▼).

CONCLUSIONS

Following general guidelines about hapten design, several haptens were synthesized and examined for their ability to produce monoclonal antibodies to the DDT family of pesticides. Two types of immunizing haptens, characterized by the presentation of different parts of the DDT molecular structure, were prepared. Although only the polyclonal response from hapten **5** showed the presence of anti-DDT antibodies, high-affinity MAbs were obtained from mice immunized with both hapten **1** and hapten **5** immunogens (I_{50} values in the low nanomolar range). In fact, the affinity to *p,p′*-DDT of these MAbs, particularly that of the antibodies derived from hapten **5**, is higher by far than that exhibited by previously reported antibodies (Bürgisser *et al.*, 1990) Taking into account the affinity and the number of MAbs obtained, hapten **5** seems to mimic better the DDT molecular structure than hapten **1** does. To our knowledge, this is the first time that a hapten derivatized at the aromatic ring has been used to produce antibodies to DDT. All previous attempts to obtain anti-DDT antibodies used the central carbon atom as the derivatization site to prepare the hapten.

In the evaluation of the analytical properties of immunoassays performed by combining several assay haptens and formats, no improvement was found. Competitive assays with DDT isomers and metabolites evidenced that anti-DDT MAbs may be classified into two main groups according to their cross-reactivity pattern: class-specific antibodies and DDT-specific antibodies. Interestingly, both classes of antibodies were elicited from the same hapten (**5**) simply by applying a different selection criterion in the screening of fusion supernatants. This fact points out the importance of a well-designed fusion screening for selecting MAbs with the desired characteristics.

Two of the MAbs produced were selected for further studies on the basis of their high affinity and different specificity to DDT-related compounds. The most sensitive assays with these antibodies, named LIB**5**-25 and LIB**5**-52, were based on the homologous conjugate OVA−**5** in the conjugate-coated ELISA format (I_{50} values of 2.1 and 6.4 nM, respectively). Work is in progress to assess the influence of physicochemical factors on the performance of both immunoassays and to study their feasibility for the analysis of DDT residues in biological and environmental samples.

ABBREVIATIONS USED

BSA, bovine serum albumin; CR, cross-reactivity; DMEM, Dulbecco's Modified Eagle's Medium; DDA, bis-(*p*-chlorophenyl)acetic acid; DDD, dichlorodiphenyldichloroethane; DDE, dichlorodiphenyldichloroethene; DDT, dichlorodiphenyltrichloroethane; DMF, *N,N*-di-

methylformamide; ELISA, enzyme-linked immunosorbent assay; HFCS, Hybridoma Fusion and Cloning Supplement; HRP, horseradish peroxidase; I_{50}, concentration giving 50% inhibition of maximum response; Ig, immunoglobulin; MAb, monoclonal antibody; NMR, nuclear magnetic resonance; OPD, *o*-phenylenediamine; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.05% Tween 20; PEG, poly(ethylene glycol); TLC, thin-layer chromatography; UV−vis, ultraviolet−visible.

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