Development of Monoclonal ELISAs for Azinphos-methyl. 1. Hapten Synthesis and Antibody Production

Josep V. Mercader and Angel Montoya*

Laboratori Integrat de Bioenginyeria, Universitat Politècnica de València, Camí de Vera s/n, E-46022 València, Spain

The development of monoclonal antibody-based enzyme-linked immunosorbent assays for azinphos-methyl is described. A panel of haptens was synthesized for immunoconjugate preparation, and a series of haptens for heterologous, coating or tracer, conjugates was also prepared. Hapten synthesis was based on a strategy in which only a fragment of the whole target molecule was present (fragmentary haptens). From immunized mice, a set of monoclonal antibodies was obtained and ELISA sensitivities were assayed in different formats. Affinities estimated as I_{50} values in the low nanomolar range for azinphos-methyl and phosmet were observed for several monoclonal antibodies in the conjugate-coated format and in the antibody-coated format under nonoptimized assay conditions.

Keywords: *Organodithiophosphates; azinphos-methyl; azinphos-ethyl; phosmet; guthion; imidan; fragmentary haptens; monoclonal antibodies; ELISA*

INTRODUCTION

Azinphos-methyl [*O*,*O*-dimethyl *S*-[(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl)methyl] phosphorodithioate], also called Guthion or Gusathion, is an organodithiophosphorus insecticide and acaricide introduced in 1954. It has found widespread applications in agricultural pest control on some ornamental plants, top-quality fruits, and vegetables. Phosmet [*N*-(mercaptomethyl)phthalimide-*S*-(*O*,*O*-dimethylphosphorodithioate)], also called Imidan, is another organodithiophosphorus insecticide and acaricide introduced in 1956 with similar applications. Their extensive usage constitutes an important risk for nontarget species (Tanner and Knuth, 1995), including humans, and has already caused serious environmental problems (Edge et al., 1996).

Biotechnology-based methods such as immunoassays (IAs) are being increasingly reported for pesticide analysis since their promising application was first envisioned by Ercegovich (1971). Since then, many IAs have been described for pesticides of different families (Kaufman and Clower, 1995; Meulenberg et al., 1995; Szurdoki et al., 1996). The high affinity of antibodies toward their target analytes has led to the development of pesticide analytical techniques for both laboratory and field uses. Furthermore, immunochemical detection methods have been proved to be simple, cost-effective, and rapid (Sherma, 1997; Sherry, 1997). These features convert IAs into very valuable tools for residue analytical laboratories involved in large monitoring programs when high sample throughput and/or on-site screening analyses are required (Ellis, 1996; Hayes et al., 1996). Most IAs have been developed using polyclonal antisera raised in rabbits because the production of monoclonal antibodies (MAbs) has usually been considered as an expensive and laborious technology. However, as immunoassays are becoming more sophisticated, this high initial cost becomes less important considering the subsequent expense of assay validation and the potential of an unlimited supply of uniform immunoreagents.

IA development requires the production of antibodies to the analyte and the optimization and validation of an assay, usually an enzyme-linked immunosorbent assay (ELISA). The successful generation of specific antibodies and sensitive assays against a small molecule is greatly dependent upon a proper design of immunizing and assay haptens. It could be stated that assay specificity depends on the immunogenic hapten, whereas assay sensitivity depends on the competitive hapten (Carlson, 1995). The phosphoric ester of organophosphates seems to be the most adequate moiety to be derivatized for the synthesis of immunizing haptens, although other strategies can be followed (Skerritt and Lee, 1996). Recently, a very interesting strategy has been published to prepare such haptens for any type of organophosphates (Ten Hoeve et al., 1997). Such approaches, however, require too complex synthetic chemistry, especially for organodithiophosphorus pesticides. The design of a simple hapten corresponding to a substructure of the molecule may constitute a valuable approach to compound-specific antibody generation, as exemplified for triasulfuron by Schlaeppi et al. (1992). All of these considerations led us to design what we called *fragmentary haptens*, which are haptens containing only a fragment of the whole target compound. Moreover, hapten and carrier hydrophobicity greatly influence the interactions between them, so that the optimal linker lengths will be different for each case (Fasciglione et al., 1996; Wortberg et al., 1996).

Almost 30 years ago, Centeno et al. (1970) obtained polyclonal antibodies to the dithiophosphorus pesticide malathion. Since then, no reports have appeared describing the production of antibodies to other dithiophosphorus insecticides. Just recently, two papers were published describing the production of MAbs to azin-

* Author to whom correspondence should be addressed (telephone 34-96-3877093; fax 34-96-3877093; e-mail amontoya@upvnet.upv.es).

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phos-methyl (Mercader et al., 1995; Jones et al., 1995). Hapten design has been an important hindrance for antibody production to this family of insecticides, which probably has caused this delay. In this work, a simple strategy to circumvent these difficulties is described. With the aim of obtaining high-affinity MAbs, the ring system of the concerned pesticide was attached to a spacer arm with different lengths. The influence of this heterology on the immunogenicity was studied, together with its effect when different proteins were used for assay conjugates in different ELISA formats.

MATERIALS AND METHODS

Reagents and Instruments. Azinphos-methyl (CAS Registry No. 86-50-0) and phosmet (CAS Registry No. 732-11-6), Pestanal grade, were purchased from Riedel-de-Haën AG (Seelze, Germany). Starting products for hapten synthesis, hapten–protein coupling reagents, and Tween 20 were obtained from Fluka-Sigma-Aldrich Química (Madrid, Spain). Ovalbumin (OVA), *o*-phenylenediamine (OPD), and Freund's adjuvants were from Sigma Química (Madrid, Spain). Bovine serum albumin (BSA) fraction V, horseradish peroxidase (HRP), PEG 1500, and hybridoma fusion and cloning supplement (HFCS) were from Boehringer Mannheim (Barcelona, Spain). Analytical grade solvents, $CDCl_3$, and acetone-d_6 (Spectrosol grade) were from Scharlau (Barcelona, Spain). *N*-Phthaloylglycine and anhydrous *N*,*N*-dimethylformamide (DMF) were obtained from Aldrich Química (Madrid, Spain). Peroxidase-labeled rabbit anti-mouse immunoglobulins (Igs) and affinity-isolated goat anti-mouse Igs were from Dako (Glostrup, Denmark). A Mouse Typer EIA grade isotyping kit from Bio-Rad Laboratories (Richmond, CA) was used. P3-X63-Ag 8.653 mouse plasmacytoma cell line was from American Type Culture Collection (Rockville, MD). Cell culture media, fetal bovine serum, and supplements were purchased from Gibco BRL (Paisley, Scotland). All other chemicals were of reagent grade or better.

Thin-layer chromatography (TLC) was performed on 0.25 mm precoated silica gel 60 on aluminum sheets Alugram Sil G/UV$_{254}$ from Macherey-Nagel (Düren, Germany). Column chromatography was carried out on silica gel 60 (0.06–0.20 mm particle size, 70–230 mesh ASTM) from Scharlau (Barcelona, Spain). Sephadex G-50 used for protein–hapten conjugate purification was from Sigma Química (Madrid, Spain). DEAE-Sepharose CL-6B from Pharmacia Biotech (Uppsala, Sweden) was employed for MAb purification. Culture plasticware was from Bibby Sterilin Ltd. (Stone, U.K.). Pyrex borosilicate glass disposable culture tubes and polystyrene easy wash ELISA plates were from Corning Inc. (Corning, NY). ELISA plates were washed with an Ultrawash II microplate washer, and absorbances were read in dual-wavelength mode (490–630 nm) with an MR-700 microplate reader, both from Dynatech (Sussex, U.K.). Ultraviolet–visible spectra were recorded in a UV-160A Shimadzu apparatus (Kyoto, Japan). ^{1}H NMR and ^{13}C NMR spectra were obtained with a Varian Gemini 300 (operating at 300 and 75 MHz, respectively). Chemical shifts are given relative to tetramethylsilane as an internal reference. GC/EI-mass spectra were recorded with a Varian Saturn II apparatus equipped with a 25 m column with 5% phenylmethyl silicone and a flame ionization detector. Data are reported as relative intensity (*m*/*z*). Fast atom bombardment mass spectra (FAB-MS) were obtained with a VG-Autospec apparatus from Fisons Instruments, using Cs^+ for ionization and 3-nitrobenzyl alcohol as matrix. Data are also reported as relative intensity (*m*/*z*).

Hapten Synthesis. Two types of haptens were synthesized depending on the aromatic moiety to which the spacer arm was attached (Figure 1). Type I haptens contained the 1,2,3-benzotriazine ring system characteristic of azinphos-methyl (AM), whereas type II haptens contained the phthalimido group of phosmet (P). Most of the compounds used in this work present minor or usual safety concerns. Nevertheless, it is recommended to operate in a well-ventilated fume hood.

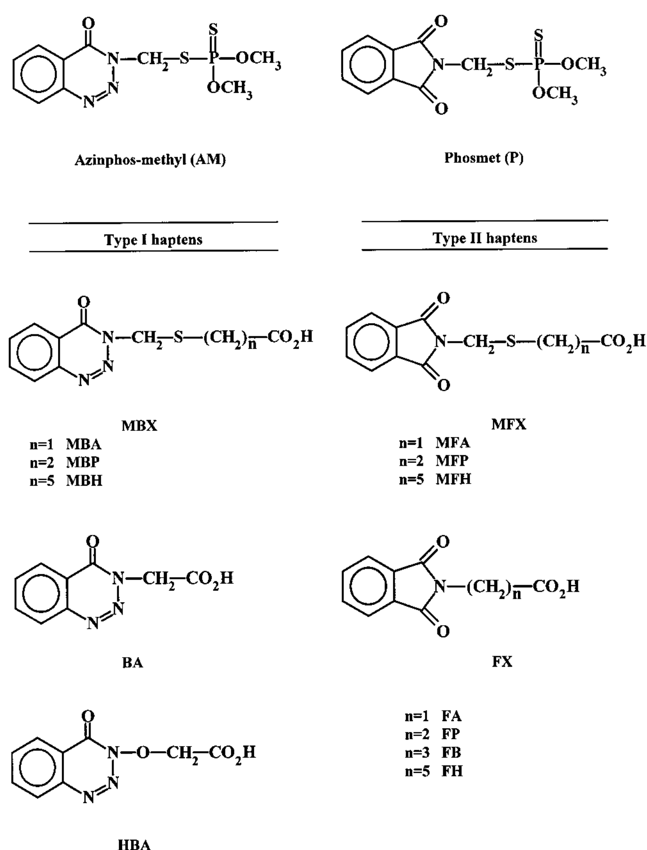

Figure 1. Chemical structures of azinphos-methyl, phosmet, and synthesized haptens.

Type I Haptens. This class of haptens can be divided into two subgroups. MBX-type haptens (MBA, MBP, and MBH) contained the CH_2S moiety of organodithiophosphorus pesticides and were prepared from *N*-(chloromethyl)benzazimide and the appropriate mercaptoacid. Haptens BA and HBA, which did not contain the CH_2S moiety, were prepared from benzazimide and *N*-hydroxybenzazimide, respectively.

2-[(4-Oxo-1,2,3-benzotriazin-3-yl)methylthio]acetic Acid (MBA, Figure 1). 3-Chloromethyl-1,2,3-benzotriazin-4(3*H*)-one [*N*-(chloromethyl)benzazimide, CMB] was prepared as previously described (Mercader et al., 1995). A solution of 2.5 mmol of CMB (489 mg) in 30 mL of absolute ethanol was slowly added to a solution of 5 mmol of mercaptoacetic acid (348 μL) and 10 mmol of potassium hydroxide (560 mg) in 20 mL of absolute ethanol, previously heated until complete dissolution. After 30 min of stirring, the solvent was removed under reduced pressure. A 5% solution of $NaHCO_3$ was then added and the new solution extracted with dichloromethane. The aqueous phase was acidified with 1 N HCl, extracted with ethyl acetate, dried over Na_2SO_4, and concentrated. The pure product was crystallized from hexane and ethyl acetate (1:5), providing 378 mg (60% yield). When analyzed by TLC, only one spot could be seen (R_f 0.3 using hexane/ethyl acetate 1:1 with 1% acetic acid as the mobile phase): ^{1}H NMR (acetone-d_6) δ 8.32–7.95 (m, 4H, ArH_4), 5.66 (s, 2H, NCH_2S), 3.66 (s, 2H, SCH_2CO); ^{13}C NMR (acetone-d_6) δ 171.12, 155.58, 145.03, 136.08, 133.59, 129.19, 125.57, 120.72, 51.58, 34.02; FAB-MS, *m*/*z* 252 (M + H^+, 100), 160 (33), 154 (41), 136 (39), 132 (27).

3-[(4-Oxo-1,2,3-benzotriazin-3-yl)methylthio]propanoic Acid (MBP, Figure 1). The synthesis and structural characterization of this hapten have previously been described (Mercader et al., 1995). The same scheme as for MBA was followed, but using 3-mercaptopropanoic acid instead of mercaptoacetic acid.

6-[(4-Oxo-1,2,3-benzotriazin-3-yl)methylthio]hexanoic Acid (MBH, Figure 1). 6-Mercaptohexanoic acid was prepared according to the method reported by Gee et al. (1988). MBH was synthesized as MBA. Briefly, 2.5 mmol of CMB (489 mg) in 30 mL of absolute ethanol was slowly added to a solution

of 5 mmol of 6-mercaptohexanoic acid (740 mg) and 7.5 mmol of potassium hydroxide (420 mg) in 20 mL of absolute ethanol, previously heated until complete dissolution. Fifteen minutes later, the solvent was removed under reduced pressure and the residue was dissolved in 5% $NaHCO_3$. After acidification with 1 N HCl, the precipitate was extracted with ethyl acetate and the organic phase dried over Na_2SO_4. Only one spot could be seen on TLC (R_f 0.48, hexane/ethyl acetate 1:1 with 1% acetic acid). The product was crystallized twice with hexane/ethyl acetate (2:1), allowing 216 mg of pure product (28% yield): ^{1}H NMR (acetone-d_6) δ 8.32−7.95 (m, 4H, ArH_4), 5.53 (s, 2H, NCH_2S), 2.81 (t, 2H, SCH_2C), 2.28 (t, 2H, CH_2CO_2), 1.71−1.40 (m, 6H, $3CH_2$); ^{13}C NMR (acetone-d_6) δ 174.49, 155.00, 145.03, 136.11, 133.62, 129.19, 125.57, 121.00, 51.24, 33.96, 32.41, 30.56, 28.83, 25.13; FAB-MS, m/z 308 (M + H$^+$, 100), 160 (76), 132 (42).

2-(4-Oxo-1,2,3-benzotriazin-3-yl)acetic Acid (BA, Figure 1). The synthesis of this hapten was carried out in two steps. First, the benzyl ester of BA was obtained as follows. Ten millimoles of benzyl bromoacetate (1.6 mL) was added to a solution of 6.8 mmol of 1,2,3-benzotriazin-4(3*H*)-one (1 g, benzazimide, CAS 90-16-4) in 30 mL of tetrahydrofuran containing 10 mmol of triethylamine (1.4 mL). The mixture was refluxed overnight and then filtered and concentrated. The solution was then diluted with dichloromethane and washed twice with 1 N HCl. The product in the organic layer was dried over Na_2SO_4 and chromatographed on silica gel column using dichloromethane as the mobile phase, the benzyl ester of BA being the main product observed by TLC (R_f 0.4, same solvent, 61.7% yield). The pure solid product was analyzed by GC/EI-MS: m/z 296 (M + 1, 5), 160 (4), 132 (83), 133 (14), 117 (6), 107 (7), 106 (10), 105 (100), 104 (95), 103 (5), 91 (39), 77 (29), 76 (11), 65 (15); ^{1}H NMR ($CDCl_3$) δ 8.37−7.83 (m, 4H, ArH_4), 7.36 (s, 5H, ArH_5), 5.25 (s, 4H, NCH_2CO+OCH_2Ar); ^{13}C NMR ($CDCl_3$) δ 167.00, 156.00, 144.00, 135.13, 132.65, 128.56, 125.16, 120.00, 67.68, 50.78. Next, the cleavage of the benzyl ester of BA was performed to obtain BA as follows. 1.36 mmol of the ester (400 mg) was dissolved in 1 mL of dichloromethane, and 2 mL of hydrogen bromide was added. Reaction was kept at room temperature for 3 h and the excess of acid was displaced with a flow of nitrogen. The residue was then dissolved in dichloromethane and extracted with 5% $NaHCO_3$. Thereafter, the aqueous solution was acidified, forming a precipitate, and extracted with ethyl acetate. Finally, the organic phase was dried over Na_2SO_4. BA was purified using a silica column in hexane/ethyl acetate 1:1 with 1% acetic acid. The pure product appeared as a single spot on TLC (R_f 0.6, same solvent, 24% yield): ^{1}H NMR (acetone-d_6) δ 8.32−7.99 (m, 4H, ArH_4), 5.18 (s, 2H, NCH_2CO); ^{13}C NMR (acetone-d_6) δ 168.99, 155.90, 145.14, 136.15, 133.62, 129.20, 125.56, 120.54, 51.24; FAB-MS, m/z 206 (M + H$^+$, 100), 149 (36), 132 (24).

2-[(4-Oxo-1,2,3-benzotriazin-3-yl)oxy]acetic Acid (HBA, Figure 1). This hapten was synthesized in a similar manner as BA. First, the benzyl ester of HBA was prepared by overnight incubation under reflux of 5 mmol of 3-hydroxy-1,2,3-benzotriazin-4(3*H*)-one (814 mg, hydroxybenzazimide, CAS 28230-32-2) in 30 mL of dichloromethane containing 7.5 mmol of benzyl bromoacetate (1.2 mL) and 5.5 mmol of triethylamine (765 μL). Afterward, the solution was diluted with dichloromethane, washed once with 5% $NaHCO_3$, twice with 1 N HCl, and again with 5% $NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated. The residue was subjected to silica gel chromatography using dichloromethane with 1% ethyl acetate as the mobile phase. The combination of those fractions showing only one spot on TLC (R_f 0.4, same solvent) provided the benzyl ester of HBA (70% efficiency): ^{1}H NMR ($CDCl_3$) δ 8.37−7.83 (m, 4H, ArH_4), 7.35 (s, 5H, ArH_5), 5.25 (s, 2H, OCH_2CO), 5.09 (s, 2H, OCH_2Ar); ^{13}C NMR (acetone-d_6) δ 167.16, 151.00, 145.03, 136.56, 136.11, 133.41, 129.43, 129.28, 129.14, 129.07, 125.91, 123.42, 73.98, 67.38; FAB-MS, m/z 312 (M + H$^+$, 100). Next, the acid was deprotected as for BA. The benzyl ester of HBA (0.64 mmol; 200 mg) in 2 mL of dichloromethane containing 2 mL of hydrogen bromide was stirred for 1.5 h at room temperature. Excess of acid was removed under a flow of nitrogen and the residue was dissolved in dichloromethane. Then, the product was extracted with 5% $NaHCO_3$, the solution was acidified, forming a precipitate that was dissolved in ethyl acetate, the organic layer was dried over Na_2SO_4, and the solvent was evaporated, leaving a solid residue (130 mg, 92% yield). Only one spot could be seen on TLC (R_f 0.1, hexane/ethyl acetate 1:1 with 1% acetic acid): ^{1}H NMR (acetone-d_6) δ 8.35−7.98 (m, 4H, ArH_4), 5.05 (s, 2H, OCH_2CO); ^{13}C NMR (acetone-d_6) δ 168.01, 136.23, 133.51, 129.48, 125.92, 123.25, 74.07; FAB-MS, m/z 222 (M + H$^+$, 100).

Type II Haptens. This class of haptens can also be divided into two subgroups depending on whether they contained the CH_2S moiety or not. MFX-type haptens (MFA, MFP, and MFH) were prepared from *N*-(bromomethyl)phthalimide (CAS 5332-26-3) and contained the CH_2S group of organodithiophosphorus pesticides. FX-type haptens (FA, FP, FB, and FH) did not contain any heteroatom between the rings and the spacer arm and were prepared from the corresponding *n*-amino acid.

2-(N-Phthalimidoylmethylthio)acetic Acid (MFA, Figure 1). *N*-(Bromomethyl)phthalimide (2.5 mmol; 600 mg) dissolved in 40 mL of absolute ethanol (heating was needed to completely dissolve the compound) was slowly added to a solution of 5 mmol of mercaptoacetic acid (348 μL) and 10 mmol of potassium hydroxide (560 mg) in 20 mL of absolute ethanol. After 45 min of stirring, the mixture was filtered and the solvent removed. The residue was dissolved with 5% $NaHCO_3$, the solution washed with dichloromethane, the product precipitated with acid and extracted with ethyl acetate, and the organic layer dried over Na_2SO_4. The crude product was chromatographed on silica gel using hexane/ethyl acetate 1:1 with 1% acetic acid. Those fractions showing only one spot on TLC were collected (R_f 0.4, same solvent) and crystallized with hexane/ethyl acetate (1:1.5, 16% yield): ^{1}H NMR (acetone-d_6) δ 7.88 (br s, 4H, ArH_4), 4.90 (s, 2H, NCH_2S), 3.60 (s, 2H, SCH_2CO); ^{13}C NMR (acetone-d_6) δ 171.18, 168.07, 135.21, 133.04, 123.97, 40.07, 34.40; FAB-MS, m/z 252 (M + H$^+$, 91), 160 (100).

3-(N-Phthalimidoylmethylthio)propanoic Acid (MFP, Figure 1). This hapten was obtained in a similar manner as MFA, but 5 mmol (435 μL) of 3-mercaptopropanoic acid was used instead of mercaptoacetic acid. The reaction was kept under stirring for 20 min, and the solvent was then removed under reduced pressure. After precipitation with acid and extraction with ethyl acetate, the crude extract was crystallized with ethyl acetate. Two hundred and seventeen milligrams of pure product was obtained (14% yield), and only one spot could be seen on TLC (R_f 0.38, hexane/ethyl acetate 1:1 with 1% acetic acid): ^{1}H NMR (acetone-d_6) δ 7.89 (br s, 4H, ArH_4), 4.79 (s, 2H, NCH_2S), 2.94 (t, 2H, SCH_2C), 2.64 (t, 2H, CCH_2CO_2); ^{13}C NMR (acetone-d_6) δ 172.82, 168.04, 135.29, 132.98, 123.98, 39.44, 34.73, 27.75; FAB-MS, m/z 266 (M + H$^+$, 76), 160 (100).

6-(N-Phthalimidoylmethylthio)hexanoic Acid (MFH, Figure 1). The synthesis of MFH was performed according to the strategy followed for MBH preparation, but *N*-(bromomethyl)-phthalimide was used instead of CMB. The reaction was stirred for 30 min at room temperature and then filtered. Afterward, the solvent was removed under reduced pressure and the residue dissolved in 5% $NaHCO_3$. The solution was then washed with dichloromethane and precipitated with acid. The pellet formed was dissolved with ethyl acetate and the organic layer dried over Na_2SO_4. The concentrated product was chromatographed on silica gel using hexane/ethyl acetate 1:1 with 1% acetic acid as the mobile phase. Fractions showing only one spot on TLC (R_f 0.57, same solvent) were collected and the residue was crystallized twice with a mixture of hexane/ethyl ether (1:1), rendering 80 mg of pure product (10% yield): ^{1}H NMR (acetone-d_6) δ 7.89 (br s, 4H, ArH_4), 4.76 (s, 2H, NCH_2S), 2.73 (t, 2H, SCH_2C), 2.29 (t, 2H, CH_2CO_2), 1.69−1.43 (m, 6H, $3CH_2$); ^{13}C NMR (acetone-d_6) δ 175.04, 168.52, 135.77, 133.46, 124.46, 39.90, 39.57, 34.47, 33.00, 25.67, 25.64; FAB-MS, m/z 308 (M + H$^+$, 100), 307 (M, 92), 294 (34), 290 (46), 289 (42), 176 (23).

N-Phthaloylglycine (FA, Figure 1). This hapten is a commercial product and was purchased from Aldrich Química (Madrid, Spain) (CAS 4702-13-0).

N-Phthaloyl-β-alanine (FP, Figure 1). This class of haptens was prepared according to the method of Nefkens et al. (1960) in which phthaloylation of amino acids is described. Briefly, 20 mmol of β-alanine (1.78 g) and 20 mmol of sodium carbonate (2.12 g) was dissolved in 30 mL of water, to which 4.5 g of *N*-(carbethoxy)phthalimide (CAS 22509-74-6) was added. The mixture was stirred until practically all of the *N*-(carbethoxy)-phthalimide had gone into solution (~1 h). The solution was then filtered and acidified with acid, and the precipitated product was extracted with ethyl acetate, dried over Na_2SO_4, and concentrated. Pure product (1.76 g; 40% yield) was rendered after crystallization with hexane/ethyl acetate (1:2). R_f on TLC was 0.43 using hexane/ethyl acetate 1:1 with 1% acetic acid as the mobile phase: ^{1}H NMR (acetone-d_6) δ 7.85 (br s, 4H, ArH_4), 3.94 (t, 2H, NCH_2), 2.76 (t, 2H, CH_2CO); ^{13}C NMR (acetone-d_6) δ 172.71, 168.94, 135.45, 133.58, 124.19, 34.90, 33.24; FAB-MS, *m*/*z* 220 (M + H$^+$, 100), 202 (48), 160 (34).

4-(N-Phthalimidoyl)butanoic Acid (FB, Figure 1). The same procedure as for FP was followed but 20 mmol of 4-aminobutanoic acid (2.10 g) was used. Crystallization was performed with hexane/ethyl acetate (1:1), and 2.21 g of pure product was produced (48% yield). R_f was 0.49 when hexane/ethyl acetate 1:1 with 1% acetic acid was used for TLC analysis: ^{1}H NMR (acetone-d_6) δ 7.85 (br s, 4H, ArH_4), 3.74 (t, 2H, NCH_2), 2.41 (t, 2H, CH_2CO_2), 1.97 (m, 2H, CCH_2C); ^{13}C NMR (acetone-d_6) δ 173.97, 168.83, 134.86, 133.13, 123.63, 37.80, 31.39, 24.50; FAB-MS, *m*/*z* 234 (M + H$^+$, 100), 216 (48), 160 (4).

6-(N-Phthalimidoyl)hexanoic Acid (FH, Figure 1). This hapten was obtained as the preceding FP and FB haptens, but only 30 min was needed for complete reaction using 6-aminohexanoic acid. The product was crystallized with methanol at 4 °C, rendering 1.23 g of pure product (24% yield). Measured R_f after TLC was 0.57 for hexane/ethyl acetate 1:1 with 1% acetic acid: ^{1}H NMR (acetone-d_6) δ 7.85 (br s, 4H, ArH_4), 3.66 (t, 2H, NCH_2), 2.31 (t, 2H, CH_2CO_2), 1.68 (m, 4H, $2CH_2$), 1.40 (m, 2H, CCH_2C); ^{13}C NMR (acetone-d_6) δ 174.44, 168.75, 134.88, 133.10, 123.62, 38.21, 33.87, 28.91, 26.95, 25.13; FAB-MS, *m*/*z* 262 (M + H$^+$, 100), 244 (59).

Preparation of Hapten−Protein Conjugates. All haptens synthesized contained a carboxylic group at the end of the spacer arm, suitable to react with free amine groups of proteins. Immunizing conjugates were prepared according to the mixed anhydride method described by Rajkowski et al. (1977), whereas coating and tracer conjugates were prepared by using the active ester method described by Langone and Van Vunakis (1975).

Immunizing Conjugates. Haptens MBA, MBP, MBH, MFA, MFP, and MFH were coupled to BSA with an initial hapten to protein molar ratio (MR) of 50:1. Conjugates were purified by gel filtration chromatography on Sephadex G-50, using 10 mM phosphate, 137 mM NaCl, and 2.7 mM KCl, pH 7.4 (PBS) as eluant. Conjugation of haptens to protein was measured spectrophotometrically. If conjugation occurred, the UV−vis spectrum of the conjugate was slightly different from that of the free protein, mainly in the region over 300 nm. Therefore, the hapten to protein MRs were calculated from the absorbance values at 280 and 310 nm by assuming that the molar absorptions of haptens and proteins were the same for the free and conjugated forms. As an exception, haptens MBA and MFA were conjugated to BSA according to the active ester method. All purified conjugates were kept frozen at −20 °C.

Coating Conjugates. All synthesized haptens were coupled to OVA for coating conjugates with an initial hapten to protein MR of 30:1. Conjugates were purified by gel filtration chromatography, and the extent of conjugation was measured spectrophotometrically at 280 and 310 nm as before. Hapten FP was conjugated to OVA using the mixed anhydride method. All purified conjugates were stored at −20 °C.

Enzyme Conjugates. Each of the prepared haptens was also conjugated to HRP for direct and capture antibody-coated assays. The active ester method was employed for this purpose with an initial hapten to protein MR of 20:1. The reaction mixture was purified by gel chromatography as described, and final hapten to protein MRs were determined spectrophotometrically at 280 and 402.5 nm with the same assumptions as before. None of the free haptens absorbed at 402.5 nm, whereas HRP does. Conjugates (enzyme tracers) were diluted with an equal volume of a saturated solution of ammonium sulfate, bubbled with pure argon for 15 min, and kept sealed at 4 °C.

Production of Monoclonal Antibodies. *Immunization.* BALB/c female mice (8−10 weeks old) were immunized with BSA−MBA, −MBP, −MBH, −MFA, −MFP, and −MFH conjugates by intraperitoneal injections. Doses consisted of 250 μL of a solution of 100 μg of protein conjugate, estimated as protein concentration, in PBS and emulsified with Freund's adjuvants. First dose contained complete Freund's adjuvant, and subsequent doses were given at weeks 2, 4, and 6 using incomplete Freund's adjuvant. After a resting period of at least 2 weeks from the last injection with adjuvant, mice received a booster intraperitoneal injection of 250 μg of protein conjugate in 250 μL of PBS, 4 days before cell fusion.

Cell Fusion and Culture. Mouse spleen lymphocytes were fused with myeloma cells according to established protocols (Nowinsky et al., 1979). Just before spleen extraction, mouse blood was collected by heart puncture and serum diluted with 4 volumes of PBS, then precipitated with 1 volume of a saturated ammonium sulfate solution, and stored at 4 °C. P3-X63/Ag 8.653 murine myeloma cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX-I (catalog no. 31966-021) supplemented with 1 mM MEM nonessential amino acids, 25 μg/mL gentamicin, and 10% Myoclone Super Plus fetal bovine serum (referred to as S-DMEM). For more details, see Abad et al. (1997a).

Hybridoma Selection and Cloning. A screening of fusion culture supernatants was performed 10−12 days after cell fusion. The screening consisted of a simultaneous noncompetitive and competitive homologous conjugate-coated ELISA to test the presence of antibodies with the ability to bind the OVA conjugate and to recognize free analytes (Abad and Montoya, 1994). AM or P was used at 1 or 10 μM as competitor. Before the screening of each fusion was performed, the optimum concentration of coating OVA conjugate was selected by bidimensional checkerboard titration in a noncompetitive assay using serial dilutions of the homologous coating species and of the fusion serum of that mouse. In this way, the lowest coating concentration that provided the highest signal for a given antibody concentration was chosen as the optimum one for that fusion screening. Those wells showing positive (clearly over the background) and competitive signals (inhibition ≥50% of the positive control) were searched for growing hybridomas. Afterward, cells in the selected wells were cloned by limiting dilution until monoclonality was ensured. Cells were cultured on HT medium (DMEM medium with 100 μM hypoxanthine and 16 μM thymidine) containing 2% HFCS (v/v) or on a feeder-layer of BALB/c thymocytes (~10^6 cells/well) and peritoneal macrophages (~5000 cells/well). Culture supernatants containing MAbs were used for affinity analysis by conjugate-coated noncompetitive and competitive assays.

MAb Purification. MAbs were purified from late stationary phase culture supernatants. First, Igs were precipitated with 1 volume of saturated ammonium sulfate and stored at 4 °C. When needed, precipitated antibodies were centrifuged, redissolved in 5 mM phosphate and 40 mM NaCl buffer, pH 7.0, extensively dialyzed against the same buffer, and purified by ion-exchange chromatography on DEAE-Sepharose using a NaCl gradient in 5 mM phosphate buffer, pH 7.0. Purified MAbs were stored at 4 °C as ammonium sulfate precipitates. The isotype of purified MAbs was determined using a commercial kit.

ELISAs. *Noncompetitive Conjugate-Coated ELISA Format.* Bidimensional checkerboard titrations of mice sera, culture supernatants, or purified MAbs versus coating conjugate were performed according to this format. Ninety-six well polystyrene ELISA plates were coated with 100 μL/well of serial OVA−conjugate dilutions in 50 mM carbonate−bicarbonate buffer, pH 9.6 (coating buffer), by overnight incubation at 4 °C. The coated plates were then washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20) and

received 100 μL/well of Ig solution (serum, culture supernatant, or MAb) serially diluted in PBS containing 0.05% Tween 20 (PBST). Immunological reaction took place for 1 h at 37 °C, and plates were washed again as described. Next, 100 μL/well of a 1/2000 dilution of peroxidase-labeled rabbit anti-mouse Igs in PBST was added to the wells, and plates were incubated for 30 min at 37 °C. After washing, retained peroxidase activity was determined by adding 100 μL/well of freshly prepared 2 mg/mL OPD and 0.012% H_2O_2 in 25 mM citrate and 62 mM sodium phosphate buffer, pH 5.4 (substrate solution). The enzymatic reaction was stopped after 10 min at room temperature by adding 100 μL/well of 2.5 M sulfuric acid. The absorbance was immediately read at 490 nm with a reference wavelength at 630 nm. The titer was defined as the serum or supernatant dilution that provided an absorbance value around 1.0 when the lowest coating concentration affording saturating signal was used.

Competitive Conjugate-Coated ELISA Format. Microtiter plates were coated as described with the appropriate concentration of OVA conjugate. The washed plates then received 50 μL/well of standard solution in PBST plus 50 μL/well of Ig solution in PBST at twice the selected assay concentrations. In this way, a competition was established between the immobilized hapten and the free analyte for the antibody binding sites. Plates were incubated at 37 °C for 1 h and washed four times. Then was added 100 μL/well of a 1/2000 dilution of peroxidase-labeled rabbit anti-mouse Igs in PBST. After 30 min at 37 °C, the enzymatic activity bound to the wells was determined as described for the noncompetitive ELISA. Serum and MAb affinity was estimated as the concentration of analyte that reduced 50% the maximum signal reached at the zero dose of analyte (I_{50}).

Direct Antibody-Coated ELISA Format. Before competitive assays were performed, enzyme tracers were titrated by noncompetitive assays. Those reagent concentrations affording an absorbance around 1.0 were chosen. Plates were coated with 1 μg/mL (100 μL/well) of antibody in coating buffer by overnight incubation at 4 °C. After washing, 100 μL/well of a solution of enzyme tracer in PBST for noncompetitive assays, or 50 μL/well of standards in PBST plus 50 μL/well of enzyme tracer in PBST at twice the desired concentrations for competitive assay, was used. Immune reaction was allowed to occur for 1 h at 37 °C and, subsequently, after washing, HRP activity bound to the well was measured as previously described for the conjugate-coated ELISA format.

Capture Antibody-Coated ELISA Format. This format added one step to the previous format. Briefly, plates were coated with goat anti-mouse Igs at 2 μg/mL in coating buffer (100 μL/well) by overnight incubation at 4 °C. The washed plates then received 100 μL/well of a solution of specific MAb at 1 μg/mL in PBST and were incubated for 1.5 h at 37 °C. From now on, this assay format was performed as the direct antibody-coated format.

Stocks and Standard Curves. Analytes were prepared as concentrated solutions in DMF and kept at 4 °C in amber glass vials. For standard curves, a 1:100 dilution was prepared in PBST from the stock in DMF, and it was then serially diluted in PBST, always using borosilicate glass tubes. Triplicates or quadruplicates of each standard were run in each ELISA plate. Competitive curves were obtained by plotting mean absorbance values versus the logarithm of analyte concentration. Sigmoidal curves were mathematically fitted to a four-parameter logistic equation using a SigmaPlot software package from Jandel Scientific (Erkrath, Germany).

RESULTS AND DISCUSSION

Hapten Design. In a previous work, MAbs that recognized AM and highly cross-reacted with P were obtained (Mercader et al., 1995). In the present work, the immunizing haptens MBX- and MFX-types (Figure 1) contained the specific moieties of AM and P, respectively, including the CH_2S group characteristic of organodithiophosphates. To these haptens was covalently bonded through the sulfur atom a spacer arm with different lengths (n = 1, 2, 5 not including the CH_2S moiety), which terminated in a carboxyl group for carrier linking. Although these *fragmentary haptens* do not mimic the complete molecule of the target compounds, the planarity of the ring system (Rohrbaugh et al., 1976), its rotational flexibility, and the specific and determinant moieties of the analytes are preserved. Essentially, the strategy was to react an *n*-mercaptoacid with *N*-(chloromethyl)benzazimide or *N*-(bromomethyl)phthalimide. Thus, the CH_2S moiety can be considered as a *neodeterminant* (Fasciglione et al., 1996) introduced by the coupling arm, but at the same time it corresponds to the structure of the target compounds, making it difficult to know what the apparent length of the spacer arm will be. Therefore, three different linker lengths were tested for the immunogens.

For coating haptens and enzyme tracers, the similarity requirement is less rigorous because, to obtain a sensitive assay, these reagents should be less recognized than the target analyte. Moreover, hapten heterology usually improves assay sensitivity, variation in handle length being enough in many cases for this purpose (Eremin, 1995; Manclús and Montoya, 1996). Also, Wortberg et al. (1996) observed that the optimal spacer length for HRP tracers was different from those of OVA conjugates. Nevertheless, other heterologies such as changes in composition affecting steric and electronic properties may also be very valuable. Therefore, both heterologies were taken into account in this work (Figure 1). Structurally heterologous haptens attached to a linker with several different lengths were prepared and coupled to OVA and HRP for different assay formats. Besides, BA and HBA haptens were similar to MBA except that the former did not contain the CH_2S moiety, and furthermore, HBA introduced an oxygen atom between the rings and the spacer arm, which probably modified more drastically the electronic properties. Also, heterologous P haptens were prepared with the phthalimido rings directly attached to a linear handle of 1, 2, 3, or 5 carbon atoms, with no CH_2S moiety.

Hapten−Protein Conjugates. MRs estimated for BSA conjugates were very similar, all of them being between 22 and 25, except for MBP conjugate. Initial MR used for MBP conjugation to BSA was lower (25:1) than usual, affording a lower final MR of 17. Surprisingly, MBA and MFA (n = 1) could not be coupled to BSA using the mixed anhydride method, and the active ester method had to be used instead. Probably, the anhydride formed is spatially too close to the sulfur atom or to the rings, so this method may not be suitable for such short handles. Determined MRs for OVA conjugates were between 8 and 16 except for HBA, which was 4. MRs for HRP conjugates were 1 or 2 except for haptens MBP, MFP, MFH, and FB, with longer spacer arms, which were between 5 and 7. Also, MR for HRP−FH conjugate was 19, a too high value, probably indicating important modifications of the protein structure that altered the molar absorptivity of HRP. Besides, hapten FP could not be coupled to OVA using the active ester method, and the mixed anhydride method had to be employed.

Characterization of Polyclonal Mouse Sera. It is known that preliminary studies of mice antisera may provide some information about the immunogenicity of the synthetic antigens and about further work perfor-

Table 1. Summary of Coating Conjugate Concentrations and Sera Titers Used in Homologous Competitive Assays

immunizing hapten	fusion no.	titer[a] ($\times 10^4$)	coating conjugate[b] (ng/mL)	I_{50} (μM) azinphos-methyl	phosmet
MBA	1	3	100	4.7	13.6
	2	1	60	0.5	37.7
	3	3	60	0.5	1.0
MBP	4	nd[c]	nd	nd	nd
	5	2	1000[d]	40.3	nd
	6	5	1000[d]	>50	nd
MBH	7	5	60	48.4	156.8
	8	3	60	13.3	154.4
	9	2	60	7.0	69.7
MFA	10	2	60	ni[e]	48.4
	11	4	30	ni	39.5
	12	3	30	ni	55.9
MFP	13	5	60	ni	169.0
	14	3	30	ni	ni
	15	5	30	ni	ni
MFH	16	3	30	ni	109.1
	17	3	300	ni	ni
	18	3	30	ni	109.5

[a] The titer was defined as the serum or supernatant dilution that provided an absorbance value around 1.0 when the lowest coating concentration affording saturating signal was used. [b] Coating conjugate concentration used in competitive assays. [c] Not determined. [d] Nonoptimized coating conjugate concentration. [e] No inhibition was detected up to 100 μM.

mance such as the screening of fusion cultures and the use of heterologous haptens. At least three mice were immunized with each BSA−hapten conjugate. Next, fusion sera (sera collected immediately before cell fusion) were titrated by bidimensional noncompetitive homologous conjugate-coated assays, and optimum coating conjugate concentrations and sera dilutions for competitive assays were determined as described under Materials and Methods. As shown in Table 1, optimum coating conjugate concentrations were different for each fusion sera but, for most, they were around 30 or 60 ng/mL. As an exception, competitive assays for MBP fusion sera were performed with a nonoptimized coating conjugate concentration (Mercader et al., 1995). Regarding specificity, competitive assays showed that MBX-type fusion sera recognized both AM and P pesticides, whereas MFX-type fusion sera were more specific to P. In both cases, antisera raised from haptens with shorter spacer arms (MBA and MFA) resulted in lower I_{50} values.

Production and Characterization of MAbs. Fusions were performed from all of the mice despite the results of some fusion sera, since it has been reported that high-affinity MAbs can be produced from mice with low-affinity antisera (Manclús et al., 1996). Screenings of fusion cultures were conducted with standard (1 μg/mL) or optimum coating concentrations using the homologous conjugate-coated ELISA format in simultaneous noncompetitive and competitive assays as described. The standard coating concentration was enough to identify many positive wells. However, the use of optimum coating concentration for each culture supernatant was revealed as essential to find the highest number of competitive wells. From 18 cell fusions, a total of 29 hybridomas were successfully expanded in culture medium and their culture supernatants assayed in the homologous conjugate-coated ELISA format to select those MAbs with lower I_{50} and better specificity. In all cases, a fixed coating conjugate concentration of 50 ng/mL was used, estimated as the mean optimum coating concentration obtained from fusion sera analysis. First, supernatants were titered to select that dilution affording a maximum absorbance around 1.0, and next, competitive assays were performed using AM and P as competitors. Those MAbs having an I_{50} lower than 100 nM for AM and/or P, or being specific for either of them, were selected. Finally, 2, 2, 1, 1, and 5 MAbs were obtained from MBA, MBP, MBH, MFP, and MFH haptens, respectively. All of them were IgG_1 (κ) isotype except LIB-MFP12, which was IgG_{2a} (κ) isotype. Figure 2 depicts the inhibition curves obtained with the culture supernatants of the best or specific MAbs for AM and P. Only LIB-MFP12 and LIB-MFH111 were specific of P, with I_{50} values of 1.7 and 2.7 μM, respectively. In contrast, the rest of the MAbs recognized both AM and P, with I_{50} values for AM of 28.3, 10.0, 2.0, and 2.0 nM for LIB-MBP34, LIB-MFH13, LIB-MFH14, and LIB-MFH110, respectively, and I_{50} values for P of 3.0, 1.0, and 2.0 nM for LIB-MFH13, LIB-MFH14, and LIB-MFH110, respectively.

Assay Selection. It is known that the assay format may strongly influence the sensitivity of the ELISAs. On the basis of the results obtained from culture supernatant characterization, purified MAbs displaying the lowest I_{50} or which were specific for one of the analyzed pesticides were assayed in different ELISA formats.

Conjugate-Coated ELISA Format. LIB-MBP34, LIB-MFP12, LIB-MFH13, LIB-MFH14, LIB-MFH110, and LIB-MFH111 MAbs were assayed in this format against all of the OVA conjugates prepared. To ensure that all of the conjugates would be recognized by the MAbs, assays were performed with a fixed coating conjugate concentration of 1 μg/mL of all of the homologous and heterologous conjugates. Optimum MAb concentrations were titered to reach a final maximum absorbance around 1.0 in the absence of analyte. It could be observed that homologous assays were much less sensitive if reagent concentration was not previously optimized. LIB-MFP12 and LIB-MFH111 MAbs did not recognize the heterologous haptens or no inhibition was detected under these conditions, not even with the homologous conjugate (data not shown). For the remaining MAbs (Table 2), assay sensitivities, defined as the I_{50} value of the standard curve, were either improved or not depending on the heterologies used. The lower I_{50} values for AM and P were obtained in all cases when BA, HBA, FA, or FP ($n = 1$ or 2) conjugates were used. These are the haptens that showed greater heterologies with respect to the immunizing hapten. Interestingly, with LIB-MFH-type MAbs, conjugates with ring heterologies (MBX type) produced a reduction of sensitivity, even with the MBH conjugate, which has a homologous spacer arm length ($n = 5$). When only the spacer arm length heterology was introduced, lower I_{50} values were obtained with those conjugates of the shortest spacer length, $n = 1$ (MBA and MFA for LIB-MBP34 and LIB-MFH-type MAbs, respectively), in accordance with other authors' results (Harrison et al., 1991). The introduced heterologies did not involve changes on assay specificity. The best assays were those of LIB-MFH14 MAb, with I_{50} values of 1.1 and 0.8 nM for AM and P, respectively, when OVA−HBA was used as coating conjugate.

Direct Antibody-Coated ELISA Format. The same six MAbs were tested in this format with all, homologous and heterologous, enzyme tracers prepared. In this format, a standard coating antibody concentration (1 μg/mL) was employed. Tracers were first titered to find a

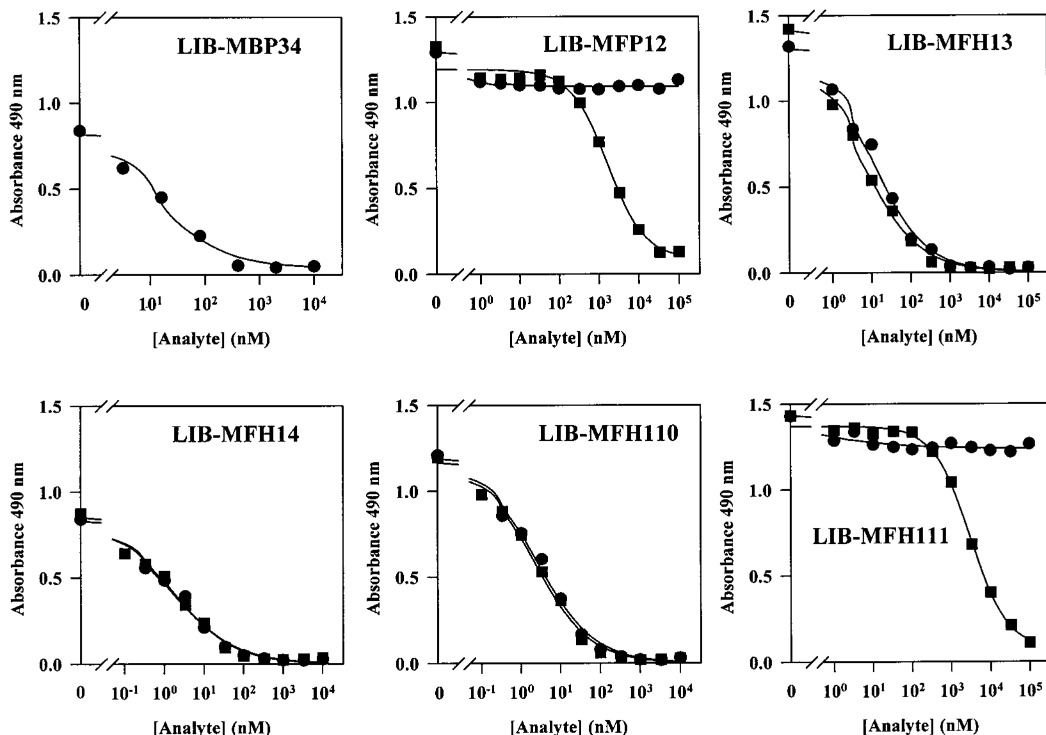

Figure 2. Competitive homologous conjugate-coated ELISA for azinphos-methyl (●) and phosmet (■) with six selected culture supernatants. No data for phosmet with LIB-MBP34 culture supernatant were available. Coating conjugate concentration was fixed at 50 ng/mL, and optimum supernatant titers were first determined.

Table 2. I_{50} Values for Azinphos-methyl and Phosmet in the Conjugate-Coated ELISA Format[a]

monoclonal antibody	coating hapten											
	MBA	MBP	MBH	MFA	MFP	MFH	BA	HBA	FA	FP	FB	FH
					I_{50} (nM) for Azinphos-methyl							
LIB-MBP34	1200	**2000**[b]	3700	140	500	780	13	3.7	5.0	10	38	200
LIB-MFH13	950	930	1100	86	180	**190**	6.3	1.4	3.1	6.4	56	200
LIB-MFH14	340	580	490	24	64	**62**	8.8	1.1	2.8	7.4	30	140
LIB-MFH110	980	1200	1100	68	230	**230**	9.2	1.3	4.5	9.5	46	190
					I_{50} (nM) for Phosmet							
LIB-MBP34	860	**1300**	3300	96	430	630	12	3.5	4.3	8.0	50	210
LIB-MFH13	820	870	1100	71	170	**170**	6.0	0.9	2.4	4.9	45	150
LIB-MFH14	190	340	300	20	45	**35**	5.4	0.8	1.9	4.8	16	73
LIB-MFH110	430	600	590	33	130	**100**	5.4	0.9	2.3	5.6	27	110

[a] Assays were performed at a fixed coating conjugate concentration of 1 μg/mL. [b] Results with the homologous conjugates are indicated in boldface print.

Table 3. I_{50} Values for Azinphos-Methyl and Phosmet in the Antibody-Coated ELISA Format[a]

monoclonal antibody	tracer hapten											
	MBA	MBP	MBH	MFA	MFP	MFH	BA	HBA	FA	FP	FB	FH
					I_{50} (nM) for Azinphos-methyl							
LIB-MBP34	nr[b]	**10.5**[c]	13	nr	nr	7.8	nr	nr	nr	nr	13	nr
LIB-MFH13	nr	nr	2.0	nr	nr	**1.7**	nr	nr	nr	nr	2.2	nr
LIB-MFH14	nr	3.2	4.8	nr	2.3	**4.9**	nr	nr	nr	nr	5.5	nr
LIB-MFH110	nr	nr	1.7	nr	nr	**2.0**	nr	nr	nr	nr	2.0	nr
					I_{50} (nM) for Phosmet							
LIB-MBP34	nr	**11.3**	10	nr	nr	7.5	nr	nr	nr	nr	12	nr
LIB-MFH13	nr	nr	1.4	nr	nr	**1.4**	nr	nr	nr	nr	1.5	nr
LIB-MFH14	nr	2.8	3.5	nr	1.8	**3.1**	nr	nr	nr	nr	3.1	nr
LIB-MFH110	nr	nr	1.0	nr	nr	**1.3**	nr	nr	nr	nr	1.3	nr

[a] Assays were performed at a fixed coating antibody concentration of 1 μg/mL. [b] A maximum absorbance value of 1.0 could not be reached with up to 10 μg/mL enzyme tracer in noncompetitive assays. [c] Results with the homologous conjugates are indicated in boldface print.

concentration that gave a maximum absorbance around 1.0 in the absence of analyte. Again, LIB-MFP12 and LIB-MFH111 MAbs did not show any competition under these conditions (data not shown). For the remaining MAbs, all homologous enzyme tracers were recognized by their corresponding MAbs (Table 3), with I_{50} values much lower than those obtained with the conjugate-coated assays under standardized conditions. Nevertheless, in agreement with the results found by other authors (Schneider and Hammock, 1992), most of the tracers were not recognized or did not provide enough signal. In the case of LIB-MFH-type MAbs, sensitivity

was maintained when MBH enzyme tracer was used (ring system heterology), in contrast with the results observed in the conjugate-coated format where sensitivity was lowered. When the arm length was reduced (MBA enzyme tracer for LIB-MBP34 MAb and MFA and MFP enzyme tracers for LIB-MFH-type MAbs), a general loss of recognition was found. An exception was the case of MFP ($n = 2$), which afforded enough signal with LIB-MFH14 MAb when a concentration of 1.5 μg/mL of enzyme tracer was used (a concentration 500 times higher than that required of MBH enzyme tracer, with homologous linker length). These results suggest that it is important to maintain the spacer arm length of these haptens over a certain value ($n > 2$). Also, FA and FP tracers ($n = 1$ and 2, respectively) were not yet recognized by LIB-MFH-type MAbs, although their ring system was homologous, whereas FB ($n = 3$) enzyme tracer was, with an I_{50} value similar to that of the homologous one (which has a spacer length of $n = 5$). FB enzyme tracer was also recognized by LIB-MBP34. Surprisingly, FH tracer was not recognized by any of the MAbs, whereas OVA−FH conjugate was well recognized in the conjugate-coated format. The reason of this lack of recognition may be related to an anomalous preparation of the enzyme conjugate, for which an extremely high MR was estimated. The best assay was accomplished with LIB-MFH110 MAb and HRP−MBH as enzyme tracer, displaying I_{50} values of 1.7 and 1.0 nM for AM and P, respectively.

Capture Antibody-Coated ELISA Format. Occasionally, recognition of enzyme tracers is enhanced when MAbs are not directly immobilized to the solid support but through a capture antibody (Abad et al., 1997b). Therefore, capture antibody-coated assays were performed under the conditions described under Materials and Methods. No improvement was accomplished for any of the tested MAbs with any of the tracers (data not shown).

CONCLUSIONS

The main goal of this work was to obtain monoclonal ELISAs for azinphos-methyl. Previous work showed that MAbs obtained from some of the designed haptens always cross-reacted with phosmet. Therefore, several new haptens containing any of the specific structures of both pesticides were designed. As mentioned, sera properties depend on the characteristics of the protein and the hapten, which constitute the immunogen, the linker length being an important factor. With this idea in mind, a set of protein−hapten conjugates with different spacer arm lengths was prepared, and the immunological response of the mice to each of the conjugates was studied. In general, titers revealed low immunogenicity, but affinity to the homologous hapten conjugate was high, because low coating concentrations were requiered for optimum signals. Also, short arm conjugates gave rise to antisera with the lowest I_{50} values. Even though the affinity of polyclonal sera to AM and P was very low or nonexistant, high-affinity MAbs were obtained from these mice. Different specificity patterns were observed for MBX-type and MFX-type conjugates. Protein−hapten conjugates containing the characteristic moieties of AM (MBX type) seemed to produce nonspecific antisera, whereas MFX-type conjugates resulted in sera with higher specificity for the phthalimido rings.

Results of the cell fusions were variable, and the greatest number of MAbs came from a mouse immunized with the BSA−MFH conjugate. The screening of culture supernatants revealed that previous optimization of coating conjugate concentration was a crucial step when hybridomas producing competitive MAbs are searched in a simultaneous noncompetitive and competitive assay. Competitive assays performed with culture supernatants helped to focus our attention on those MAbs and coating or tracer conjugates that gave the most sensitive assays. Using purified MAbs, hapten heterology rendered improved assay sensitivities, but to a different extent depending on the ELISA format. Regarding the conjugate-coated format, spacer arm heterologies improved homologous assay sensitivities if shorter spacer linkers were used. Also, ring heterologies alone improved assay sensitivities for LIB-MBP34 MAb, but surprisingly, it worsened assay sensitivities of LIB-MFH-type MAbs. Because all heterologous assays reached sufficient absorbance, more drastic heterologies could be introduced in competitive assays in this format, which provided assays with lower I_{50} values. In the antibody-coated format, hapten heterology is a more restricted strategy to improve assay sensitivity, because only conjugates with long spacer arms provided enough signal. Haptens containing heterologous rings and long spacer arms, which reduced sensitivity in the conjugate-coated format, now provided assays with I_{50} values similar to those of the homologous assay. It was observed that the optimum handle length of these HRP conjugates is clearly longer than those of the OVA conjugates. MAbs assayed could be used in any of the studied ELISA formats with high sensitivities.

High-affinity MAbs have been obtained from haptens that contain only a fragment of the whole target analyte. Therefore, it seems that the degree of mimicking of the analyte required by the immunogen is limited to maintaining the physicochemical properties of the unique functional and immunodeterminant chemical groups. Regarding specificity, none of the MAbs available could, at the same time, discriminate between AM and P and provide assays with enough sensitivity. When MAbs were raised from AM fragments, it seemed difficult to generate specific MAbs that did not cross-react with P. On the contrary, specific MAbs for P were obtained from P fragments. The combination of antibodies with different specificities could be a promising route to the development of IAs for the simultaneous detection of AM and P. In an accompanying paper, optimization of physicochemical parameters of ELISA and its application to water analysis is described.

ABBREVIATIONS USED

AM, azinphos-methyl; BSA, bovine serum albumin; CMB, *N*-(chloromethyl)benzazimide; DMF, *N*,*N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; FX, haptens containing the phthalimido ring system without the CH_2S moiety; HRP, horseradish peroxidase; I_{50}, concentration of analyte giving 50% inhibition of the maximum absorbance; IA, immunoassay; Ig, immunoglobulin; MAb, monoclonal antibody; MBX, haptens containing the 1,2,3-benzotriazine ring system and a CH_2S moiety; MFX, haptens containing the phthalimido ring system with the CH_2S moiety; MR, molar ratio; OVA, ovalbumin; P, phosmet; PBS, 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4.

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