Hapten Synthesis and Production of Monoclonal Antibodies to the *N*-Methylcarbamate Pesticide Methiocarb

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With the aim of developing an immunoassay for the detection of the insecticide methiocarb, three compounds with carboxylic spacer arms of different lengths introduced at the carbamate group were synthesized, conjugated to proteins, and used as immunizing haptens in mice. Monoclonal antibodies (MAbs) were subsequently obtained and characterized for their affinity to methiocarb. In the homologous conjugate-coated assay format, MAbs exhibited I_{50} values in the 1−10 nM range. Thereafter, a collection of methiocarb haptens with modifications in the aromatic ring structure or in the spacer arm were synthesized to be used as heterologous haptens. These haptens and MAbs were incorporated into several ELISA configurations. Some of the new combinations of immunoreagents and assay format provided a significant improvement in assay sensitivity, reaching I_{50} values around 0.1 nM. MAbs seem to be very specific for methiocarb, as evidenced by the negligible cross-reactivity shown by methiocarb metabolites. These immunoreagents are very promising analytical tools for the rapid and sensitive determination of methiocarb in food and in the environment.

Keywords: *ELISA; immunoassay; insecticide; monoclonal antibodies; hapten design; hapten heterology; ELISA format; carbamate*

INTRODUCTION

Methiocarb [4-(methylthio)-3,5-xylyl methylcarbamate], also known as mercaptodimethur or mesurol, is a nonsystemic *N*-methylcarbamate insecticide and acaricide with contact and stomach action that is particularly effective for control of mites, including organophosphate-resistant strains. Likewise, methiocarb is a molluscicide with neurotoxic action that has been employed to control slugs and snails in a wide range of agricultural situations, and it has also been used as a bird repellent on blueberries, cherries, and grapes (Worthing and Hance, 1991; Dolbeer et al., 1994). Methiocarb hydrolyzes quite rapidly in water under natural sunlight to afford 4-(methylthio)-3,5-xylenol (Chiron et al., 1996). In plants, methiocarb is metabolized to its sulfoxide and sulfone derivatives (Miller et al., 1985). Like the parent compound, methiocarb sulfoxide and methiocarb sulfone are cholinesterase inhibitors.

Methiocarb, like most *N*-methylcarbamates, is thermally unstable and decomposes to the phenol under the usual gas chromatography conditions (Liška and Slobodník, 1996). This problem can be overcome by varying the operating conditions (Mueller and Stan, 1990) or by derivatization of the carbamate pesticides (Ballesteros et al., 1993; Färber and Schöler, 1993; Bakowski et al., 1994). Several reports have been recently published dealing with the coupling of mass spectrometry with liquid chromatography for the determination of *N*-methylcarbamates (Chiron et al., 1996; Di Corcia et al., 1996; Slovodnik et al., 1997), but these methods seem to be more appropriate for confirmation purposes than for the highly sensitive routine analysis of carbamate residues. Based on the pioneering work by Moye et al. (1977), further modified for use on food samples by Krause (1985), HPLC using postcolumn derivatization and fluorescence detection (EPA method 531.1) is the preferred technique for the sensitive and selective determination of *N*-methylcarbamate pesticides in environmental waters and food (McGarvey, 1993; Yang et al., 1996). However, the method presents some limitations derived from the fact that laborious cleanup, concentration, and derivatization steps are needed to obtain the desired sensitivity. Thus, the method requires sophisticated equipment that is not available in most analytical laboratories, and it is not very well suited for the analysis of a large number of samples. In fact, most pesticide regulatory programs analyze for *N*-methylcarbamates only a part of the total number of food samples analyzed for other active ingredients, such as organophosphorus pesticides (Gunderson, 1995).

Immunoassays are analytical methods based on the interaction of an analyte with an antibody that recognizes it with high affinity and specificity. They are simple, cost-effective, and field-portable, do not require sophisticated instrumentation, and are able to analyze a large number of samples simultaneously. All of these features, along with the large number of pesticides for which antibodies have been produced over the past few years, have promoted the acceptance of immunochemical techniques among analytical chemists as alternative and/or complementary methods for the analysis of agrochemicals (Hammock and Gee, 1995; Meulenberg et al., 1995; Dankwardt and Hock, 1997). This is particularly true for pesticides that are difficult and/or costly to determine due to their physicochemical char-

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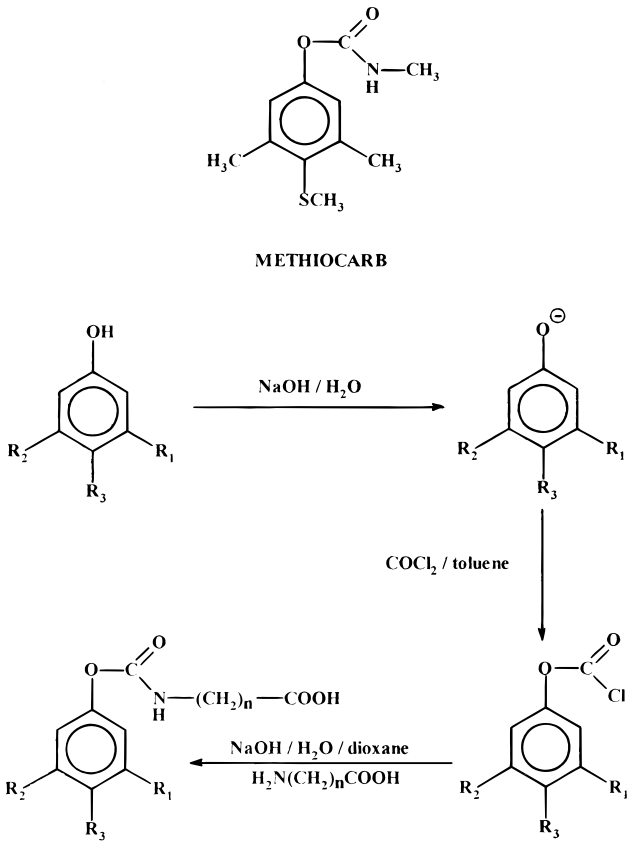


hapten	n	R_1	R_2	R_3
MXNP	2	CH_3	CH_3	SCH_3
MXNB	3	CH_3	CH_3	SCH_3
MXNH	5	CH_3	CH_3	SCH_3
MCNP	2		CH_3	SCH_3
MCNB	3		CH_3	SCH_3
MCNH	5		CH_3	SCH_3
DPNP	2	CH_3	CH_3	
DPNB	3	CH_3	CH_3	
DPNH	5	CH_3	CH_3	
MPNP	2			SCH_3
MPNB	3			SCH_3
MPNH	5			SCH_3
PNP	2			
PNB	3			
PNH	5			

Figure 1. Reaction scheme of the synthesis of hapten series MXN*, MCN*, DPN*, MPN*, and PN*.

acteristics or for large monitoring programs. In these situations the use of immunoassays as a screening technique could provide a great saving of resources, effort, and toxic solvents, since fortunately most samples do not contain residues or they contain them below violative levels. Therefore, although immunoassays are not the panacea, there is nowadays little doubt that they constitute a sensitive, selective, and inexpensive method for the rapid analysis of pesticide residues in a variety of matrixes.

The aim of this work was to obtain high-affinity monoclonal antibodies (MAbs) to methiocarb and to develop enzyme immunoassays as an alternative to liquid chromatographic techniques for the screening of large numbers of agricultural and environmental samples. The achievement of this objective strongly relies on a proper hapten design since the structure of the immunizing hapten is primarily responsible for the antibody recognition properties. Moreover, assay sensitivity is often influenced by the structure of the assay hapten (Harrison et al., 1991a; Goodrow et al., 1995; Szurdoki et al., 1995). Accordingly, a high number of haptens with different spacer arms and with modifications in the aromatic moiety of the methiocarb structure were synthesized and conjugated to carrier proteins and to horseradish peroxidase (HRP). 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.

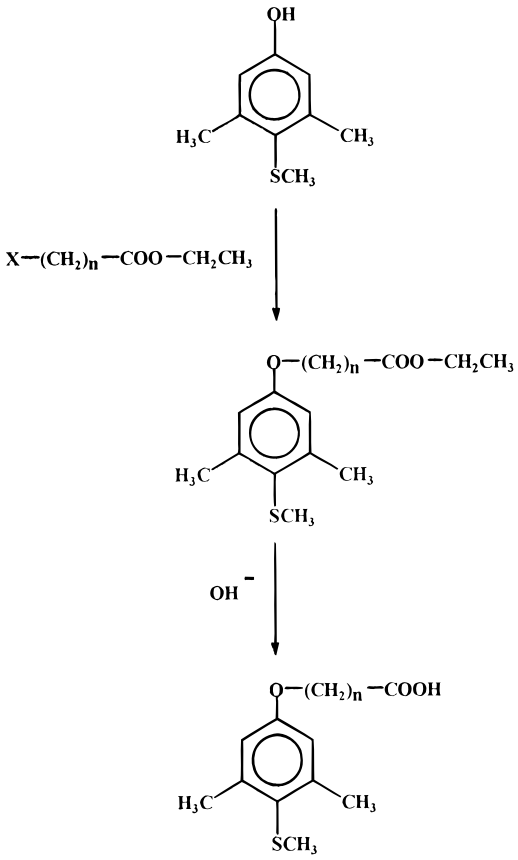
MATERIALS AND METHODS

Chemicals, Immunoreagents, and Instruments. Standards of methiocarb, methiocarb sulfoxide, and methiocarb sulfone were from Riedel-de Haën (Seelze, Germany). Stock solutions were prepared in dry *N*,*N*-dimethylformamide (DMF) and stored at 4 °C. Starting products for the synthesis of haptens and hapten−protein coupling reagents were obtained from Fluka-Aldrich Química (Madrid, Spain).

Ovalbumin (OVA), Freund's adjuvants, and *o*-phenylenediamine (OPD) were obtained from Sigma Química (Madrid, Spain). Bovine serum albumin fraction V (BSA), enzyme immunoassay grade HRP, and poly(ethylene glycol) (PEG) 1500 were purchased from Boehringer Mannheim (Barcelona, Spain). Peroxidase-labeled rabbit anti-mouse immunoglobulins and affinity-isolated goat anti-mouse immunoglobulins were obtained from Dako (Glostrup, Denmark). Culture media (Dulbecco's Modified Eagle's Medium, DMEM), fetal calf serum (Myoclone Super Plus), and supplements were from GibcoBRL (Paisley, Scotland). Culture plasticware was from Bibby Sterilin Ltd. (Stone, U.K.). P3-X63-Ag8.653 mouse plasmacytoma line was from American Tissue 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 (490−650 nm) with a Emax microplate reader from Molecular Devices (Sunnyvale, CA). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a Varian Gemini 300 spectrometer (Sunnyvale, CA), operating at 300 MHz for ^1H and at 75 MHz for ^{13}C. Chemical shifts are reported relative to tetramethylsilane. Liquid secondary ion (LSI) (3-nitrobenzyl alcohol as matrix) mass spectra (MS) were obtained on a VG Autospec mass spectrometer (Kyoto, Japan). Ultraviolet−visible (UV−vis) spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan).

Hapten Synthesis. Methiocarb haptens used in this work were prepared by introduction of alkyl chain spacers, ending in a carboxylic acid, at the hydroxyl group of the phenolic precursor, either after being reacted with phosgene to form the carbamate group (Figure 1), or directly by O-alkylation (Figure 2).



hapten	n	X
MXOA	1	Cl
MXOB	3	Br
MXOH	5	Br

Figure 2. Reaction scheme of the synthesis of hapten series MXO*.

6-[[1-(4-(Methylthio)-3,5-xylyloxy)carbonyl]amino]hexanoic Acid (MXNH, Figure 1). To a solution of 14.31 g (85.2 mmol) of 4-(methylthio)-3,5-xylenol in 100 mL of 2.5 M sodium hydroxide in water was added 50 mL of a 20% phosgene solution in toluene (96.5 mmol). (WARNING: phosgene is a highly toxic gas. Work in a well ventilated fume hood and handle carefully.) The mixture was stirred for 4 h at room temperature, and the organic phase was evaporated to dryness at reduced pressure to afford 18.21 g of a yellow oil, 15.48 g of which was identified as the chloroformate by GC (78.8%). This crude product was used without further purification in the following step: 2.96 g of aminohexanoic acid (22.6 mmol) was dissolved in 4.0 mL of 4 M sodium hydroxide, and the solution was cooled at 4 °C. 1-[4-(Methylthio)-3,5-xylyl] chloroformate (3.0 g, 11.3 mmol) was dissolved in 4 mL of 1,4-dioxane and cooled at 4 °C. The dioxane solution, along with 3.0 mL of cold (4 °C) 4 M sodium hydroxide, was added to the aminohexanoic solution in five equal portions, with ~5 min being allowed between additions. The reaction mixture was stirred in an ice bath for 1.5 h. After acidification to pH 4 with concentrated hydrochloric acid, the carboxylic derivative was extracted with ethyl acetate (three 50 mL portions). The ethyl acetate phase was washed several times with diluted hydrochloric acid and extracted with 1 M bicarbonate solution (two 50 mL portions). The aqueous phase was acidified again with concentrated hydrochloric acid, extracted with ethyl acetate, and dried over anhydrous sodium sulfate, and the solvent was evaporated at reduced pressure to afford 1.19 g of the crude product as an oil. The product was crystallized from hexane/ethyl acetate (70:30) to obtain 840 mg of the pure hapten (22.9%): ^{1}H NMR (acetone-d_6) δ 1.38−1.70 (m, 6 H, CH_2−CH_2−CH_2), 2.20 (s, 3 H, S−CH_3), 2.32 (t, 2 H, CH_2−COOH), 2.52 (s, 6 H, 2 CH_3), 3.21 (q, 2 H, CH_2−NH), 6.90 (s, 2 H, aromatic); ^{13}C NMR (acetone-d_6) δ 18.32, 21.84, 25.30, 26.95, 34.07, 41.52, 121.97, 131.80, 144.55, 152.05, 155.05, 174.63; LSI-MS, *m/z* (relative intensity) 325 (90, M), 168 (100).

From appropriate amino acids and phenolic precursors, the following compounds were also synthesized with similar yields by using the same procedure.

3-[[1-(4-(Methylthio)-3,5-xylyloxy)carbonyl]amino]propanoic Acid (MXNP): ^{1}H NMR (acetone-d_6) δ 2.20 (s, 3 H, S−CH_3), 2.52 (s, 6 H, 2 CH_3), 2.60 (t, 2 H, CH_2−COOH), 3.46 (q, 2 H, CH_2−NH), 6.90 (s, 2 H, aromatic); ^{13}C NMR (acetone-d_6) δ 18.32, 21.84, 34.45, 37.73, 121.94, 131.86, 144.60, 152.05, 155.05, 173.100; LSI-MS, *m/z* (relative intensity) 283 (100, M), 168 (95).

4-[[1-(4-(Methylthio)-3,5-xylyloxy)carbonyl]amino]butanoic Acid (MXNB): ^{1}H NMR (acetone-d_6) δ 1.86 (m, 2 H, CH_2−CH_2−CH_2), 2.20 (s, 3 H, S−CH_3), 2.40 (t, 2 H, CH_2−COOH), 2.52 (s, 6 H, 2 CH_3), 3.26 (q, 2 H, CH_2−NH), 6.91 (s, 2 H, aromatic); ^{13}C NMR (acetone-d_6) δ 18.33, 21.85, 25.84, 31.37, 41.10, 122.00, 131.86, 144.58, 152.15, 155.20, 174.40; LSI-MS, *m/z* (relative intensity) 297 (100, M), 168 (80).

3-[[1-(3,5-xylyloxy)carbonyl]amino]propanoic Acid (DPNP): ^{1}H NMR (acetone-d_6) δ 2.25 (s, 6 H, 2 CH_3), 2.60 (t, 2 H, CH_2−COOH), 3.45 (q, 2 H, CH_2−NH), 6.71−6−81 (m, 3 H, aromatic); ^{13}C NMR (acetone-d_6) δ 21.09, 34.44, 37.66, 120.07, 127.12, 139.46, 152.33, 155.33, 173.12.

4-[[1-(3,5-xylyloxy)carbonyl]amino]butanoic Acid (DPNB): ^{1}H NMR (acetone-d_6) δ 1.84 (m, 2 H, CH_2-CH_2−CH_2), 2.25 (s, 6 H, 2 CH_3), 2.40 (t, 2 H, CH_2−COOH), 3.25 (q, 2 H, CH_2−NH), 6.71−6−81 (m, 3 H, aromatic); ^{13}C NMR (acetone-d_6) δ 21.14, 25.86, 31.37, 41.03, 120.13, 127.10, 139.45, 152.44, 155.52, 174.43.

6-[[1-(3,5-xylyloxy)carbonyl]amino]hexanoic Acid (DPNH): ^{1}H NMR (acetone-d_6) δ 1.35−1.70 (m, 6 H, CH_2−CH_2−CH_2), 2.25 (s, 6 H, 2 CH_3), 2.30 (t, 2 H, CH_2−COOH), 3.19 (q, 2 H, CH_2−NH), 6.70−6.80 (m, 3 H, aromatic); ^{13}C NMR (acetone-d_6) δ 21.14, 25.28, 26.94, 34.07, 41.49, 120.13, 127.04, 139.43, 152.43, 155.55, 174.66; LSI-MS, *m/z* (relative intensity) 279 (100, M), 123 (50).

3-[[1-(4-(Methylthio)-3-cresyloxy)carbonyl]amino]propanoic Acid (MCNP): ^{1}H NMR (acetone-d_6) δ 2.27 (s, 3 H, CH_3), 2.44 (s, 3 H, S−CH_3), 2.60 (t, 2 H, CH_2−COOH), 3.45 (q, 2 H, CH_2−NH), 6.94 (m, 2 H, aromatic), 7.17 (m, 1 H, aromatic); ^{13}C NMR (acetone-d_6) δ 15.45, 19.93, 34.42, 37.69, 120.62, 123.87, 126.74, 134.58, 137.39, 149.81, 155.28, 173.10.

4-[[1-(4-(Methylthio)-3-cresyloxy)carbonyl]amino]butanoic Acid (MCNB): ^{1}H NMR (acetone-d_6) δ 1.86 (m, 2 H, CH_2−CH_2−CH_2), 2.29 (s, 3 H, CH_3), 2.41 (t, 2 H, CH_2−COOH), 2.46 (s, 3 H, S−CH_3), 3.26 (q, 2 H, CH_2−NH), 6.96 (m, 2 H, aromatic), 7.20 (m, 1 H, aromatic); ^{13}C NMR (acetone-d_6) δ 21.14, 25.86, 31.37, 41.03, 120.13, 127.10, 139.45, 152.44, 155.52, 174.43.

6-[[(1-(4-(Methylthio)-3-cresyloxy)carbonyl]amino]hexanoic Acid (MCNH): ^{1}H NMR (acetone-d_6) δ 1.35−1.70 (m, 6 H, CH_2−CH_2−CH_2), 2.29 (s, 3 H, CH_3), 2.32 (t, 2 H, CH_2−COOH), 2.45 (s, 3 H, S−CH_3), 3.20 (q, 2 H, CH_2−NH), 6.95 (m, 2 H, aromatic), 7.19 (m, 1 H, aromatic); ^{13}C NMR (acetone-d_6) δ 15.59, 19.93, 25.24, 26.91, 34.00, 41.36, 120.68, 123.90, 126.78, 134.40, 137.39, 149.96, 155.28, 174.56; LSI-MS, *m/z* (relative intensity) 311 (100, M).

3-[[1-(4-(Methylthio)phenyloxy)carbonyl]amino]propanoic Acid (MPNP): ^{1}H NMR (acetone-d_6) δ 2.47 (s, 3 H, S−CH_3), 2.60 (t, 2 H, CH_2−COOH), 3.45 (q, 2 H, CH_2−NH), 7.08 (m, 2 H, aromatic), 7.25 (m, 2 H, aromatic); ^{13}C NMR (acetone-d_6) δ 16.65, 34.91, 38.20, 123.60, 128.77, 135.95, 150.63, 155.70, 173.60.

4-[[1-(4-(Methylthio)phenyloxy)carbonyl]amino]butanoic Acid (MPNB): ^{1}H NMR (acetone-d_6) δ 1.84 (m, 2 H, CH_2−CH_2−CH_2), 2.40 (t, 2 H, CH_2−COOH), 2.46 (s, 3 H, S−CH_3), 3.25 (q, 2 H, CH_2−NH), 7.08 (m, 2 H, aromatic), 7.25 (m, 2 H, aromatic); ^{13}C NMR (acetone-d_6) δ 16.69, 26.29, 31.83, 41.56, 123.64, 128.79, 135.85, 150.72, 155.83, 174.91.

6-[[1-(4-(Methylthio)phenyloxy)carbonyl]amino]hexanoic Acid (MPNH): ^{1}H NMR (acetone-d_6) δ 1.35−1.70 (m, 6 H, CH_2−CH_2−CH_2), 2.30 (t, 2 H, CH_2−COOH), 2.46 (s, 3 H, S−CH_3), 3.19 (q, 2 H, CH_2−NH), 7.08 (m, 2 H, aromatic), 7.25 (m, 2 H, aromatic); ^{13}C NMR (acetone-d_6) δ 16.70, 25.76, 27.40, 30.71, 34.53, 42.00, 123.61, 128.80, 135.77, 150.76, 155.73, 175.14.

3-[[1-(Phenyloxy)carbonyl]amino]propanoic Acid (PNP): ^{1}H NMR (acetone-d_6) δ 2.61 (t, 2 H, CH_2−COOH), 3.46 (q, 2 H, CH_2−NH), 7.09−7.37 (m, 5 H, aromatic); ^{13}C NMR (acetone-d_6) δ 34.69, 37.69, 122.46, 125.62, 129.86, 152.45, 155.21, 173.10.

4-[[1-(Phenyloxy)carbonyl]amino]butanoic Acid (PNB): ^{1}H NMR (acetone-d_6) δ 1.88 (m, 2 H, CH_2−CH_2−CH_2), 2.40 (t, 2 H, CH_2−COOH), 3.26 (q, 2 H, CH_2−NH), 7.09−7.37 (m, 5 H, aromatic); ^{13}C NMR (acetone-d_6) δ 25.80, 31.34, 41.04, 122.50, 125.54, 129.85, 152.52, 155.37, 174.42.

6-[[1-(Phenyloxy)carbonyl]amino]hexanoic Acid (PNH): ^{1}H NMR (acetone-d_6) δ 1.36−1.68 (m, 6 H, CH_2−CH_2−CH_2), 2.30 (t, 2 H, CH_2−COOH), 3.20 (q, 2 H, CH_2−NH), 7.09−7.37 (m, 5 H, aromatic); ^{13}C NMR (acetone-d_6) δ 25.42, 26.91, 30.20, 34.01, 41.34, 41.48, 122.49, 125.48, 129.83, 152.57, 155.30, 174.62.

6-[1-(4-(Methylthio)-3,5-xylyloxy)]hexanoic Acid (MXOH, Figure 2). To 50 mL of dry acetone were added stoichiometric amounts (20 mmol) of 4-(methylthio)-3,5-xylenol, potassium carbonate, and ethyl 6-bromohexanoate. After reflux for 12 h, the mixture was filtered and the solvent was removed under reduced pressure. The residue was dissolved in 50 mL of ethyl acetate, washed with water (2 × 50 mL), 1 M NaOH (2 × 50 mL), and 4 M NaCl (2 × 50 mL), and finally dried over Na_2SO_4. GC analysis confirmed that 43% of the crude product was ethyl 6-[(1-(4-(methylthio)-3,5-xylyloxy)]hexanoate. After evaporation of the solvent, 50 mL of 1 M NaOH was added to the residue (4.91 g), and the solution was stirred while heated under reflux for 1.5 h. The solution was then acidified with concentrated hydrochloric acid, extracted with ethyl acetate, and dried over Na_2SO_4. The oily product obtained after solvent evaporation (1.55 g, 27%) was crystallized from hexane to obtain 640 mg of the pure hapten: ^{1}H NMR (acetone-d_6) δ 1.40−1.80 (m, 6 H, CH_2−CH_2−CH_2), 2.15 (s, 3 H, S−CH_3), 2.33 (t, 2 H, CH_2−COOH), 2.49 (s, 6 H, 2 CH_3), 3.40 (t, 2 H, O-CH_2), 6.72 (s, 2 H, aromatic); ^{13}C NMR (acetone-d_6) δ 18.60, 21.97, 25.34, 26.29, 34.06, 68.12, 114.89, 126.59, 144.82, 159.76, 174.61.

From appropriate spacers, the following compounds were synthesized also by O-alkylation of 4-(methylthio)-3,5-xylenol.

2-[1-(4-(Methylthio)-3,5-xylyloxy)]acetic Acid (MXOA): ^{1}H NMR (acetone-d_6) δ 2.16 (s, 3 H, S−CH_3), 2.50 (s, 6 H, 2 CH_3), 4.70 (s, 2 H, CH_2−COOH), 6.75 (s, 2 H, aromatic); ^{13}C NMR (acetone-d_6) δ 18.51, 21.98, 65.04, 114.93, 127.62, 144.94, 158.66, 170.07.

4-[1-(4-(Methylthio)-3,5-xylyloxy)]butanoic Acid (MXOB): ^{1}H NMR (acetone-d_6) δ 2.04 (m, 2 H, CH_2−CH_2−CH_2), 2.15 (s, 3 H, S−CH_3), 2.49 (t, 2 H, CH_2−COOH), 2.49 (s, 6 H, 2 CH_3), 4.02 (t, 2 H, O-CH_2), 6.74 (s, 2 H, aromatic); ^{13}C NMR (acetone-d_6) δ 18.58, 21.97, 25.34, 67.30, 114.90, 126.79, 144.86, 159.58, 174.32.

Preparation of Immunizing Conjugates. Haptens MXNP, MXNB, and MXNH 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 the carrier protein 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 27, 16, and 18 for haptens MXNP, MXNB, and MXNH, 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 spectrophotometry. By assuming additive absorbance values, hapten to protein molar ratios were evaluated as 5, 5, 6, 9, 8, 8, 2, 4, 4, 2, 2, 4, 9, 6, 2, 2, 4, and 6 for haptens MXNP, MXNB, MXNH, DPNP, DPNB, DPNH, MCNP, MCNB, MCNH, MPNP, MPNB, MPNH, PNP, PNB, PNH, MXOA, MXOB, and MXOH, respectively.

Preparation of Enzyme Conjugates. The mixed-anhydride method was also used for covalent coupling of haptens to HRP. Typically, 2.9 μL of tributylamine 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 the addition of 1.8 mL of DMF, 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 molar ratios were 1.7, 3.0, 4.6, 11.5, 11.3, 11.5, 2.3, 1.4, 3.2, 1.7, 3.4, and 1.5 for haptens MXNP, MXNB, MXNH, DPNP, DPNB, DPNH, MCNP, MCNB, MCNH, MXOA, MXOB, and MXOH, respectively.

Production of Monoclonal Antibodies to Methiocarb. *Immunization.* BALB/c female mice (8−10 weeks old) were immunized with BSA−MXNP, −MXNB, and −MXNH conjugates. First dose consisted of 30 μg of conjugate intraperitoneally injected as an emulsion of PBS and complete Freund's adjuvant. Two subsequent injections were given at 3-week intervals emulsified in incomplete Freund's adjuvant. One week after the last injection, mice were tail-bled and sera tested for anti-hapten antibody titer by indirect ELISA and for analyte recognition properties by competitive indirect ELISA. After a resting period of at least 3 weeks from the last injection in adjuvant, mice selected to be spleen donors for hybridoma production received a final soluble intraperitoneal injection of 100 μg of conjugate in PBS, 4 days prior to cell fusion.

Cell Fusion. P3-X63/Ag 8.653 murine myeloma cells (ATCC, Rockville, MD) were cultured in high-glucose DMEM supplemented with 2 mM L-glutamine, 1 mM nonessential amino acids, 25 μg/mL gentamicin, and 15% fetal bovine serum (referred to as s-DMEM). Cell fusion procedures were carried out essentially as described by Nowinski et al. (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 (4−5) × 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 day 4 postfusion and by HT medium (HAT medium without aminopterine) on day 8 postfusion.

Hybridoma Selection and Cloning. Eight to 11 days after cell fusion, culture supernatants were screened for the presence of antibodies that recognized methiocarb. 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 methiocarb, 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 ($\sim 10^6$ cells/well) and peritoneal macrophages ($\sim$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 ammonium sulfate 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.

Enzyme-Linked Immunosorbent Assays (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 was used for the study of antibody sensitivity and specificity to methiocarb. 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 (hapten−HRP conjugates).

For competition assays, optimum concentrations of antibodies, hapten conjugates, or enzyme tracers were previously determined. 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. Different antibody concentrations in PBS containing 0.1% BSA (PBSB) were then added and incubated for 2 h. Next, plates were incubated for 1 h with peroxidase-labeled rabbit anti-mouse immunoglobulins diluted 1/2000 in PBST (PBS containing 0.05% Tween 20). Finally, peroxidase activity bound to the wells was determined by adding the substrate solution (2 mg/mL OPD and 0.012% H_2O_2 in 25 mM citrate, 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 methiocarb standards 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 methiocarb standards and the selected concentrations of enzyme tracers. Peroxidase activity was measured as above.

Indirect Antibody-Coated Format. The difference from 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. Methiocarb, like most pesticides, is a small and simple organic molecule non-immunogenic by itself and lacking a functional group for coupling to proteins. Therefore, the synthesis of haptens resembling as much as possible the structure and electronic distribution of methiocarb is a necessary and critical step in the production of high-affinity antibodies. Accordingly, we synthesized three immunizing haptens, namely MXNP, MXNB, and MXNH, by the introduction of spacer arms of different lengths through the carbamate group characteristic of this pesticide (Figure 1). In this way, that is, by elongation of an aliphatic chain already present in the analyte, both the structure and the electronic distribution of the methiocarb molecule are hardly modified, as demostrated by the high-affinity MAbs previously obtained following this chemical approach for carbaryl (Abad and Montoya, 1994), carbofuran (Abad et al., 1997c), and bendiocarb (unpublished results).

Apart from the immunizing haptens required for antibody production, the development of an ELISA for pesticides relies on the synthesis of proper assay haptens. These haptens can be the same used to derive the antibodies (homologous haptens) or different ones (heterologous haptens). Although excellent immunoassays may be developed using homologous haptens, especially with MAbs, the synthesis of heterologous haptens is usually a very valuable approach to improve the sensitivity of immunoassays for pesticides. In this work this possibility has been tested by synthesizing a panel of 15 haptens, grouped into 5 series, that differ from the immunizing haptens in the number of ring substituents (MCN*, DPN*, MPN*, and PN* series; Figure 1) or in the type of spacer arm (MXO* series; Figure 2). Haptens in the same series differ from one another in the spacer arm length. Thus, *P haptens have a spacer arm derived from amino**p**ropanoic acid, *B haptens have a spacer arm derived from amino**b**utyric acid, and *H haptens have a spacer arm derived from amino**h**exanoic acid.

Production of Antibodies to Methiocarb. *Mouse Polyclonal Response.* To evaluate the suitability of the synthesized haptens to raise anti-methiocarb antibodies, three mice were immunized with each of the BSA conjugates of the MXN* hapten series. After three injections, the titer (serum dilution giving an absorbance of 1.0 in the established conditions) of antibodies recognizing conjugated haptens was estimated by indirect ELISA using the OVA−MXN* conjugates as coating antigens. The three immunogens showed anti-conjugate reactivity with titers around 1×10^5 for the three coating conjugates, so no influence of the spacer arm length was observed at this point. Sera were subsequently tested for their ability to recognize methiocarb by indirect competitive ELISA. All sera bound competitively to methiocarb with a concentration producing 50% inhibition of antibody binding (I_{50}) in the high nanomolar range (Table 1). No noticeable influence of the hapten spacer arm length was observed. Thus, the three haptens seem to be equally suitable for the production of MAbs to methiocarb. Accordingly, cell fusions were performed with the nine immunized mice, and the obtained results are summarized in Table 1. All of the fusions rendered wells with antibodies recognizing the corresponding homologous conjugated haptens, but usually only a few of these wells recognized 0.1 μM methiocarb in competitive assays. From competitive wells, 12 hybridomas showing the highest affinity to methiocarb were finally cloned and stabilized: 2 hybridomas came from the hapten with the shortest spacer arm (MXNP), 2 came from the hapten

Table 1. Summary of Cell Fusion and Hybridoma Selection Results

immunizing hapten	fusion no.	serum[a] I_{50} (nM)	no. of wells seeded	no. of wells positive[b] (conjugate)	no. of wells competitive[c] (analyte)	no. of cloned hybridomas[d]
MXNP	1	329	288	22	2	2[e]
	2	121	288	46	46	2[e]
	3	428	288	150	0	0
MXNB	1	618	288	14	1	1
	2	156	288	4	0	0
	3	1128	384	177	16	2
MXNH	1	273	384	150	12	4
	2	713	288	141	20	4
	3	694	288	4	0	0

[a] ELISA plates were coated with 1 μg/mL of the homologous conjugate, and the antiserum dilution used was previously determined to obtain a maximum absorbance around 1.0. [b] Wells with antibodies that recognized the OVA−hapten conjugates (homologous assays) by indirect ELISA (absorbance > 0.5). [c] Wells with antibodies that recognized 0.1 μM methiocarb in solution (inhibition > 50%). [d] Only hybridomas secreting antibodies with the lowest I_{50} for methiocarb were stabilized and cloned. [e] Indicates that these antibodies were rejected during the cloning proccess because better antibodies were obtained in further fusions.

Table 2. Recognition of Haptens Coupled to Ovalbumin by Anti-Methiocarb MAbs in the Conjugate-Coated Format[a]

conjugated hapten	MAb LIB-MXNP22	LIB-MXNB31	LIB-MXNB33	LIB-MXNH14	LIB-MXNH15	LIB-MXNH22
MXNP	+	+	+	+	+	+
MXNB	+	+	+	+	+	+
MXNH	+	+	+	+	+	+
MCNP		+	±	±	±	
MCNB		+	+	+	+	±
MCNH		+	+	+	+	±
DPNP				±		
DPNB		+	±	+	±	
DPNH		+		+	+	
MPNP						
MPNB		+				
MPNH		+		±		
PNP						
PNB						
PNH						
MXOA		+		±		
MXOB		+	+			
MXOH		+				

[a] ELISA plates were coated with 1 μg/mL of OVA conjugates of haptens displayed in the table. Symbols indicate the antibody concentration that produces absorbances of 1.0 in the established conditions: <0.02 μg/mL (+); between 0.02 and 0.3 μg/mL (±); >0.3 μg/mL (no entry).

with the medium-sized spacer arm (MXNB), and 8 came from the hapten with the largest spacer arm (MXNH). The fact that the highest number of hybridomas was derived from the MXNH hapten should not be necessarily attributed to the spacer arm length but rather to the high intrinsic variability of the cell fusion process.

MAbs obtained were small-scale-purified from culture supernatant, and their ability to recognize methiocarb was roughly estimated from inhibition curves in the conjugate-coated ELISA format using homologous haptens. Under these conditions, I_{50} values in the 1−10 nM range were obtained, which means an improvement of 2 orders of magnitude with respect to the affinity displayed by mouse antisera evaluated in the same conditions. According to these data, a group of six MAbs displaying the lowest I_{50} values were selected for further studies using the complete panel of synthesized haptens coupled either to OVA (conjugate-coated format) or to HRP (antibody-coated format and indirect antibody-coated format).

Characterization of the MAbs in the Conjugate-Coated Format. *Recognition of Conjugated Haptens.* Before inhibition experiments were performed, the six selected MAbs were evaluated for their ability to recognize conjugates of ovalbumin with haptens displaying modifications in the aromatic moiety and/or in the spacer arm, with respect to the compounds used to obtain the MAbs. The obtained results are summarized in Table 2, and they essentially show a very different recognition pattern of OVA−hapten conjugates by each MAb. Thus, whereas LIB-MXNP22 MAb recognized only 3 immobilized conjugates, LIB-MXNB31 MAb recognized 12 of 18 conjugates. As would be expected, the best recognized conjugates by all MAbs were those derived from haptens belonging to the MXN* series, since they are homologous or differ only in the spacer arm length. On the opposite side, conjugates derived from haptens of the PN* series, which lack all ring substituents, were not recognized by any of the MAbs, and conjugates from haptens with only one ring substituent (MPN* series) were well recognized only by LIB-MXNB31 MAb, the antibody less sensitive to changes in the assay hapten structure. An intermediate situation was found for immobilized conjugates from haptens with two ring substituents (MCN* and DPN* series), because they were recognized with a high titer by most antibodies, especially those haptens with spacer arms of 4−6 carbon atoms. On the other hand, haptens of the MXO* series, with no modifications in the aromatic structure but with the spacer arm directly attached to the oxygen atom of the phenolic precursor, were recognized only by LIB-MXNB31 MAb. This finding calls into question the general usefulness of this promising chemical approach to obtain heterologous

Table 3. Study of the Influence of ELISA Format and Hapten Heterology with Anti-Methiocarb MAbs[a]

conjugated hapten		LIB-MXNB31 format			LIB-MXNB33 format			LIB-MXNH14 format			LIB-MXNH15 format		
		CC	AC	IAC	CC	AC	IAC	CC	AC	IAC	CC	AC	IAC
MXNB	I_{50}	**1.65**		**0.53**	**0.92**	**0.62**	**0.80**	0.85		0.47	0.46		
	A_{max}	**0.86**		**1.44**	**1.13**	**0.87**	**1.10**	1.22		0.84	1.34		
MXNH	I_{50}	1.13	0.39	0.92	0.93	0.89	1.43	**0.91**		**0.63**	**0.69**	**0.61**	**0.81**
	A_{max}	1.04	0.48	1.90	1.26	1.02	1.19	**1.32**		**0.95**	**1.39**	**0.68**	**0.87**
MCNH	I_{50}	0.21	0.11	0.60	0.22	0.39	0.69	0.14	0.20	0.47	0.14	0.30	0.53
	A_{max}	1.41	0.76	2.26	0.83	0.85	1.14	1.44	0.76	1.21	1.15	1.03	1.14
DPNH	I_{50}	0.12		0.33			0.53	0.22	0.19	0.32	0.14	0.23	0.31
	A_{max}	1.13		0.93			0.84	1.22	0.90	1.19	0.97	0.81	1.01

[a] I_{50} values are expressed in nanomolar, A_{max} was measured at 490 nm, and homologous combinations are indicated in boldface print. No entry indicates that this particular combination was not tested because it did not provide a sufficient maximum absorbance in the absence of the analyte. [b] CC, conjugate-coated format; AC, antibody-coated format; IAC, indirect antibody-coated format.

haptens for this class of pesticides and emphasizes the importance of maintaining the carbamate group in the structure of the assay hapten.

As a conclusion, although the most potentially suitable assay haptens were those involving slight modifications of the immunizing hapten structure, the relationship between the hapten structure and its uselfulness as an assay hapten is strongly dependent on the antibody. Therefore, the availability of a wide panel of antibodies is essential to study such a basic question in immunoassay development.

Affinity. Heterologous assays are well-known procedures to improve the sensitivity of immunoassays, because the structure of the assay hapten modulates the equilibrium conditions of the competition (Harrison et al., 1991b; Marco et al., 1995). However, it should be also taken into account that it is nearly impossible to predict the structure of the hapten that will provide a more sensitive assay, as well as the extent of the improvement achievable (Abad et al., 1997a). Therefore, after the number of conjugates and the extent at which they were recognized by each MAb were determined, inhibition curves using methiocarb as competitor were performed for all coating conjugates that were recognized by each antibody.

Most MAbs increased their apparent affinity to methiocarb by using heterologous conjugates, with improvements in sensitivity (I_{50} values) of up to 1 order of magnitude (Table 3, CC format). Thus, the I_{50} value of the MAb LIB-MXNB31 changed from 1.65 nM with the OVA−MXNB conjugate (homologous hapten) to 0.12 nM with the OVA−DPNH conjugate (Figure 3). Sensitivity improvements of 4−6 times were also observed with antibodies LIB-MXNB33, -MXNH14, and -MXNH15 (Table 3). However, no noticeable improvement was found with LIB-MXNP22 and -MXNH22 MAbs (data not shown). A general finding was that assay haptens of the same series, that is, haptens differing only in the spacer arm length, provide immunoassays with very similar I_{50} values. Therefore, although haptens with spacer arm length heterology are normally easily prepared, great sensitivity improvements should not be expected by following this strategy.

Specificity. The specificity of the MAbs was evaluated in the conjugate-coated format using their respective homologous hapten, that is, OVA−MXNP for LIB-MXNP22 MAb, OVA−MXNB for LIB-MXNB31 and -MXNB33 MAbs, and OVA−MXNH for LIB-MXNH14, -MXNH15, and -MXNH22 MAbs. Competitive assays with the methiocarb metabolites 4-(methylthio)-3,5-xylenol, methiocarb sulfoxide, and methiocarb sulfone as competitors were performed, and the obtained I_{50} values were used to calculate cross-reactivities. 4-(Methylthio)-3,5-xylenol lacks the methylcarbamate group characteristic of methiocarb, and methiocarb sulfoxide and sulfone differ from the parent insecticide only in the oxidation state of the sulfur atom (Figure 4). Despite the great structural similarity between methiocarb and its metabolites, all of the MAbs were extraordinarily specific to methiocarb. Hence, cross-reactivity values for methiocarb sulfone, the best recognized metabolite, were 0.03% with the antibodies LIB-MXNB31 and LIB-MXNB33 and 0.4% with the rest of the MAbs.

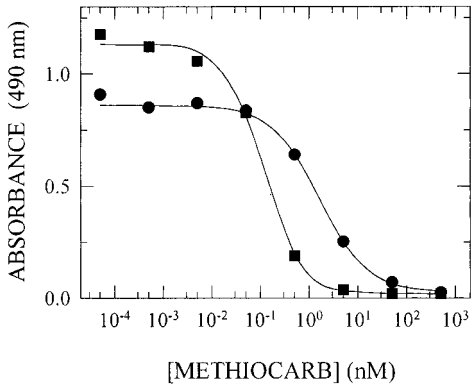


Figure 3. Standard curves for methiocarb obtained with the MAb LIB-MXNB31 (0.015 μg/mL) in the conjugate-coated format, using the assay haptens MXNB (●) and DPNH (■) coupled to ovalbumin at 1 μg/mL. Experimental points, obtained in quadruplicate, were fitted to a four-parameter logistic equation. Enzymatic reactions were stopped after 10 min.

Evaluation of the Antibody-Coated Formats. As in the conjugate-coated format, noncompetitive experiments were carried out prior to inhibition assays in the antibody-coated formats. Concentrations of HRP−hapten conjugates (enzyme tracers) from 1 ng/mL to 1 μg/mL were tested in plates previously coated with the MAbs at a fixed concentration of 1 μg/mL, either directly or through a capture immunoglobulin. Most of the HRP−hapten conjugates were not recognized by the MAbs directly immobilized to the plate. Thus, only enzyme conjugates derived from the haptens MXNB, MXNH, MCNH, and DPNH were recognized, and not even by all of the MAbs. In fact, LIB-MXNP22 and -MXNH22 MAbs did not bind any of the 12 HRP−hapten conjugates assayed, LIB-MXNB31 and -MXNH14 MAbs recognized 2 conjugates, and LIB-MXNH15 and -MXNB33 MAbs recognized 3 conjugates. It should be noticed that the MAb that recognized more coating conjugates (LIB-MXNB31) is not the antibody

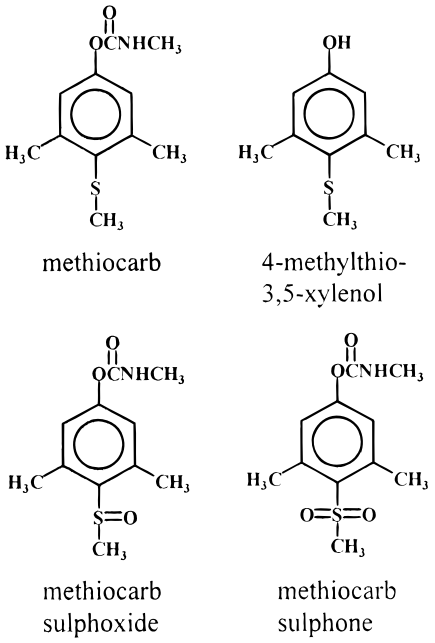


Figure 4. Chemical structures of methiocarb and methiocarb metabolites.

that bound more enzyme tracers and that LIB-MXNH14 and -MXNB31 MAbs recognized only heterologous enzyme conjugates. These results agree with previous authors' experience in this field, which indicates that the number of haptens coupled to HRP recognized by immobilized antibodies is always lower than the number of haptens recognized when coupled to OVA in the conjugate-coated format by the same antibodies (Manclús and Montoya, 1996; Manclús et al., 1996; Abad et al., 1997a,b). Moreover, it is very infrequent that an immobilized MAb recognizes a hapten conjugated to HRP if this hapten, coupled to OVA, is not recognized by the same antibody in the conjugate-coated format. The fact that none of the haptens with spacer arms derived from aminopropanoic acid (*P haptens), and only one of those containing aminobutyric acid (MXNB hapten), was recognized by the immobilized antibodies agree with previous results reported by other authors indicating that haptens with short linking groups are less suitable for enzyme conjugates, probably due to steric hindrances (Skerrit et al., 1992; Bekheit et al., 1993; Schneider et al., 1994).

Methiocarb inhibition curves were performed for all enzyme conjugate/MAb combinations that provided a sufficient signal. I_{50} values in the 0.1−0.9 nM range were obtained (Table 3, AC format), which were very similar to those found in the conjugate-coated format (Table 3, CC format). Concerning hapten heterology, assay sensitivity in the antibody-coated format also improved by the use of heterologous enzyme conjugates with respect to that achieved with homologous enzyme conjugates, although at a lower extent than in the conjugate-coated format. Thus, a 3-fold I_{50} decrease was observed with LIB-MXNH15 MAb (Figure 5).

The effect of a precoating step with goat anti-mouse Igs (indirect antibody-coated format) was also evaluated as a well-known procedure to improve the ability of immobilized antibodies to recognize enzyme conjugates (Schneider and Hammock, 1992; Giersch, 1993; Manclús et al., 1996; Abad et al., 1997a,b). This approach proved to be especially successfull for the MAbs LIB-MXNB31 and -MXNH14, which not only recognized in this way

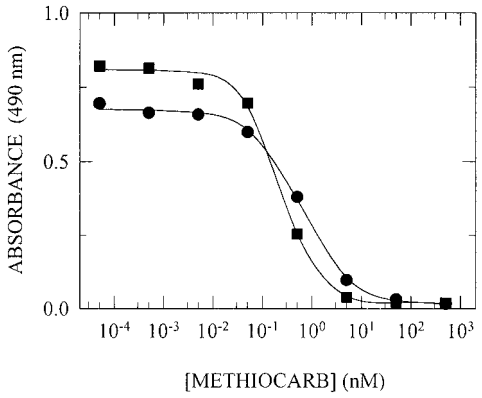


Figure 5. Standard curves for methiocarb using enzyme conjugates of the haptens MXNH (●, 0.3 μg/mL) and DPNH (■, 0.5 μg/mL) obtained with the MAb LIB-MXNH15 (1 μg/mL) in the antibody-coated format. Experimental points, obtained in quadruplicate, were fitted to a four-parameter logistic equation. Enzymatic reactions were stopped after 10 min.

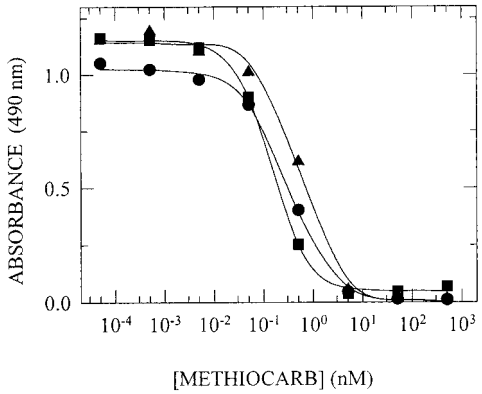


Figure 6. Methiocarb competitive curves obtained for LIB-MXNH15 MAb in combination with the heterologous assay hapten MCNH in the three formats tested: conjugate-coated format (●), antibody-coated format (■), and indirect antibody-coated format (▲). Experimental points, obtained in quadruplicate, were fitted to a four-parameter logistic equation. Enzymatic reactions were stopped after 10 min.

four enzyme conjugates but also improved their affinity to the enzyme tracers previously recognized when directly immobilized to the plate. With respect to assay sensitivity, I_{50} values were in most cases very similar to those found in the other two assay formats (Table 3). In this respect, it is interesting to point out that only slight differences among assay configurations were observed, as perfectly exemplified by the MAb LIB-MXNH15 in combination with the assay hapten MCNH (Figure 6).

CONCLUSIONS

The goal of obtaining a wide panel of high-affinity MAbs to the *N*-methylcarbamate pesticide methiocarb was achieved from mice immunized with BSA conjugates of three haptens derivatized at the carbamate group and differing in the spacer arm length. In the conjugate-coated format with homologous coating conjugates, MAbs exhibited I_{50} values in the 1−10 nM range. This result, along with previous experience with other carbamates, confirms the excellence of this hapten synthesis strategy as a general and simple approach for the obtention of antibodies to this family of pesticides. Competitive assays with methiocarb metabolites evi-

denced the great specificity to methiocarb of most of the MAbs obtained. Thus, the absence of the methylcarbamate group or the oxidation of the sulfur atom severely decreases the affinity of the antibodies to these compounds.

With the idea in mind of a further improvement in assay sensitivity, 15 heterologous haptens with modifications in the aromatic moiety or in the spacer arm were synthesized and evaluated, and the influence of the ELISA format on the assay sensitivity was studied. Our results completely confirm that hapten heterology is a valuable approach to improve the sensitivity of immunoassays for pesticides, as evidenced by decreases in I_{50} values of up to 1 order of magnitude. The best heterologous haptens were those with minor modifications in the ring structure and with long spacer arms of the same type of the immunizing hapten, particularly in the antibody-coated formats. Nevertheless, haptens with major modifications were perfectly adequate for certain antibodies. Very similar sensitivities were obtained in the different assay formats. The main difference among ELISA formats is the number of conjugated haptens suitable for the development of immunoassays, since more haptens were recognized by the antibodies when coupled to OVA and used in the conjugate-coated format than when coupled to HRP and used in the antibody-coated formats.

To our knowledge, the MAbs for methiocarb herein presented are the first reported for this pesticide. The high affinity to methiocarb displayed by some combinations of antibody, assay hapten, and ELISA format, with I_{50} values in the 0.1−0.3 nM range, is one of the highest ever reported for an antipesticide antibody, making them very promising immunoreagents for the development of a sensitive and specific immunoassay for this agrochemical contaminant. In fact, with regard to the sensitivity reached by these immunoreagents, it seems highly feasible to develop an immunoassay able to directly determine residues of methiocarb as low as 5 ppb in fruit and vegetable juices, without sample concentration and/or cleanup and with the only need of a 100-fold sample dilution to enter into the working range of the ELISA standard curve. Therefore, work is in progress to select the most sensitive of the immunoreagent combinations for each assay format and to assess the influence of several physicochemical factors on their performance, as well as to study their suitability for the analysis of methiocarb residues in agricultural and environmental samples.

ABBREVIATIONS USED

BSA, bovine serum albumin; CR, cross-reactivity; DMEM, Dulbecco's Modified Eagle's Medium; DMF, *N,N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; 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; PBSB, phosphate-buffered saline containing 0.1% BSA; PBST, phosphate-buffered saline containing 0.05% Tween 20; PEG, poly(ethylene glycol); UV−vis, ultraviolet−visible.

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