Development of an Enzyme-Linked Immunosorbent Assay to Carbaryl. 1. Antibody Production from Several Haptens and Characterization in Different Immunoassay Formats

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With the aim of developing a highly sensitive immunoassay for the insecticide carbaryl, seven compounds were synthesized, conjugated to carrier proteins, and used as immunizing haptens in mice and rabbits. Polyclonal and monoclonal antibodies were subsequently obtained and characterized for their affinity to carbaryl. Three monoclonal antibodies (MAbs) that showed the highest affinity (I_{50} < 3.0 nM) were chosen for immunoassay development and further tested in the coating conjugate and coating antibody enzyme-linked immunosorbert assay (ELISA) formats, against both homologous and heterologous conjugates. Among several immunoassays developed, the combination of LIB-CNH36 MAb with the heterologous conjugate ovalbumin−*N*-(1-naphthoyl)-6-aminohexanoic acid in the coating conjugate ELISA format afforded the highest sensitivity for carbaryl determination (I_{50} = 1.1 nM). This work also provided some insights into the influence of the immunizing hapten structure on the affinity of the antibodies obtained, as well as into the effect of the ELISA format and hapten heterology on the assay sensitivity.

Keywords: *ELISA; immunoassay; carbaryl; monoclonal antibody; N-methylcarbamate; pesticide; hapten design; hapten heterology; ELISA format*

INTRODUCTION

Carbaryl is an *N*-methylcarbamate pesticide extensively used as a broad-spectrum insecticide (D'Amico and Bollinger, 1991; Worthing and Hance, 1991). Carbaryl is an inhibitor of the acetylcholinesterase, and although some adverse effects have been reported (Farage-Elawar and Blaker, 1992; Casale et al., 1993), it is considered a safe insecticide because of its low toxicity in mammals. Carbaryl shows a high susceptibility to chemical hydrolysis and biodegradation, 1-naphthol being its main metabolite. Among the different methods that have been used for carbaryl determination, HPLC with postcolumn fluorescence derivatization is currently the preferred technique (Krause, 1985; Barceló, 1993; McGarvey, 1993). However, laborious extraction, cleanup, and concentration steps are often necessary to obtain the desired sensitivity (De Kok and Hiemstra, 1992).

The concern about the presence of pesticide residues in water, soil, and food has prompted the search for alternative methods able to detect low levels of these compounds in a simple way. In this respect, immunochemical methods are gaining importance as analytical techniques in the agrochemical field, and the number of pesticides for which immunoassays have been developed is constantly increasing (Sherry, 1992; Van Emon and López-Ávila, 1992; Meulenberg et al., 1995).

The initial step in the development of immunoassays for pesticides is the choice and synthesis of the hapten(s) to be used as immunogen(s). The critical role generally attributed to this step depends on the fact that the properties of the antibodies are primarily determined by the hapten structure. This consideration, however, contrasts with the practical difficulty in predicting which chemical structure is required for the production of specific and sensitive antibodies for a particular analyte (Harrison et al., 1991a). Recently, Marco et al. (1993) reported the production of high-affinity rabbit polyclonal antibodies for carbaryl, from a hapten very similar to that synthesized in our laboratory and succesfully used to produce MAbs for this insecticide (Abad and Montoya, 1994). Excellent monoclonal antibodies have also been obtained for triasulfuron (Schlaeppi et al., 1992) and for azinphos-methyl (Mercader et al., 1995) from haptens consisting of only a part of the pesticide structure. Thus, the question arising is when a hapten can be considered similar enough to the analyte to reasonably ensure the production of high-affinity antibodies for a given pesticide.

Provided that a panel of antibodies is available, the next step in the development of immunoassays for pesticides is the selection of the most suitable immunoreagents to be incorporated into a defined ELISA procedure. It is currently well established that ELISA format influences the sensitivity of immunoassays, but there is no general agreement on the configuration that provides the most sensitive assays. Thus, in several studies the coating antibody format was more sensitive than the coating conjugate format (Skerritt et al., 1992; Bekheit et al., 1993; Lucas et al., 1993), whereas in other studies no difference between formats, or even the opposite behavior, was found (Schlaeppi et al., 1991; Böcher et al., 1992; Muldoon et al., 1993; Karu et al., 1994). Likewise, a precoating step has been sometimes successfully included in the coating antibody format to save immunoreagents and to improve sensitivity (Schneider and Hammock, 1992; Giersch, 1993), but the same strategy has proved to be irrelevant in other cases (Bekheit et al., 1993; Schneider et al., 1994). Further-

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more, the influence of ELISA format on assay sensitivity seems to depend not only on the format by itself but also on the hapten used to obtain the coating antigen or the enzyme conjugate, i.e., on hapten heterology.

With the aim of developing a highly sensitive immunoassay for carbaryl, seven haptens were conjugated to proteins and used to immunize mice and rabbits. Polyclonal and monoclonal antibodies were subsequently obtained and characterized for their affinity to carbaryl. Those antibodies showing the highest affinity were chosen for immunoassay development and further tested in the coating conjugate and coating antibody ELISA formats against homologous and heterologous conjugates. This systematic experimental approach allowed us to select the most sensitive combination of antibody, assay hapten conjugate, and ELISA format for carbaryl determination. Moreover, this work provided some interesting insights into the influence of the immunizing hapten structure on the affinity of the antibodies obtained, as well as into the effect of the ELISA format and hapten heterology on the assay sensitivity.

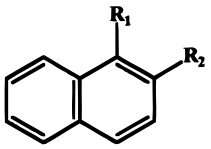
MATERIALS AND METHODS

Chemicals, Immunoreagents, and Instruments. Stock solutions of carbaryl (analytical standard grade, Dr. Ehrenstorfer, Augsburg, Germany) were prepared in DMF and stored at 4 °C. Phosgene (20% solution in toluene) was obtained from Fluka Química (Madrid, Spain). BSA, OVA, Tween 20, OPD, and Freund's complete and incomplete adjuvants were purchased from Sigma-Aldrich Química (Madrid, Spain). HRP was from Boehringer Mannheim (catalog no. 814 407). Peroxidase-labeled rabbit anti-mouse immunoglobulins, peroxidase-labeled goat anti-rabbit immunoglobulins, and affinity-isolated goat anti-mouse immunoglobulins were from Dako (Glostrup, Denmark). 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). Cell culture media (DMEM), fetal calf serum, and supplements were obtained from Gibco BRL (Paisley, Scotland). All other chemicals and organic solvents were of reagent grade or better.

Polystyrene ELISA plates were from Corning, Newport, U.K. (Easy Wash Plates, catalog no. 25805-96) and from Costar, Cambridge, MA (High Binding Plates, catalog no. 3590). 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.). ^{1}H NMR spectra were obtained with a Varian VXR-400S (400 MHz) spectrometer. Chemical shifts are given relative to TMS as an internal reference.

Hapten Synthesis. The structures of the haptens used in this work are depicted in Figure 1. 1NA and 2NA are commercially available compounds ready to be conjugated to proteins. The synthesis of 1NAH, 2NAH, and CPNU was essentially performed as previously described by Marco et al. (1993), and hapten structures were confirmed by NMR. 1NAH, 2NAH, and CPNU correspond to haptens 4, 5, and 8, respectively, in the mentioned paper. The synthesis of CNA was based on that of CNH (Abad and Montoya, 1994) and is briefly described here.

CNA. To a solution of 5.6 g (0.139 mol) of sodium hydroxide in 56 mL of water was added 20 g of 1-naphthol (0.139 mol). The mixture was heated at 85 °C in a water bath for 1 h with magnetic stirring. The solution was cooled at room temperature, and a slight excess of phosgene (100 mL of a 20% phosgene solution in toluene, 0.193 mol) was added slowly. (WARNING: Phosgene is a highly toxic gas. Work in a well-ventilated fume hood and handle carefully.) The reaction was stirred for 1 h at room temperature, and the organic phase was evaporated to dryness at reduced pressure. The resulting brown oil was dissolved in CH_2Cl_2 and distilled at 100 °C (1 mmHg); 3.75 g of the resulting product (1-naphthyl chloroformate, 18.2 mmol) in 6.3 mL of cold 1,4-dioxane was slowly added to a solution of 3.1 g of β-alanine (35.3 mmol) in 4.7 mL of 4 M sodium hydroxide. The reaction mixture was stirred in an ice bath for 1.5 h. After acidification to pH 4.0 with concentrated hydrochloric acid, the carboxylic derivative, which separated as an oil, was extracted with ethyl acetate (three 35 mL portions). The ethyl acetate phase was washed several times with diluted hydrochloric acid and extracted with 1 M bicarbonate solution (three 50 mL portions). After the solution had been cooled in an ice bath, it was acidified again with concentrated hydrochloric acid. The precipitated product was collected, washed with water, and dried to yield 1.3 g of CNA (28%): ^{1}H NMR (acetone-d_6) δ 2.66 (t, 2 H, CH_2), 3.53 (t, 2 H, CH_2), 7.30−8.00 (m, 7 H, aromatic $C_{10}H_7$).



Compound name	R_1	R_2
Carbaryl	$OCONHCH_3$	H
CNH	$OCONH(CH_2)_5COOH$	H
CNA	$OCONH(CH_2)_2COOH$	H
CPNU	$NHCONH(CH_2)_5COOH$	H
1NAH	$CONH(CH_2)_5COOH$	H
2NAH	H	$CONH(CH_2)_5COOH$
1NA	OCH_2COOH	H
2NA	H	OCH_2COOH

Figure 1. Chemical structures of carbaryl and haptens used in this work.

Preparation of Immunizing Conjugates. Haptens were covalently attached to BSA using the modified active ester method (Langone and Van Vunakis, 1982). One hundred 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 3.5 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. The extent of coupling of each hapten to BSA was determined by UV spectrophotometry. By assuming additive absorbance values, hapten to protein molar ratios were evaluated as 32, 20, 23, 28, 38, 41, and 37 for CNH, CNA, 1NA, 2NA, CPNU, 1NAH, and 2NAH, respectively.

Preparation of Coating Conjugates. 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 ovalbumin in 2 mL of 50 mM carbonate buffer, pH 9.6. The coupling reaction was incubated at room temperature for 2 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 3, 4, 4, 4, 5, 6, and 6 for CNH, CNA, 1NA, 2NA, CPNU, 1NAH, and 2NAH, 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 1.8 mL of DMF was added, 100 μL of this diluted solution of activated hapten was incubated for 4 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. The extent of coupling of each hapten to HRP was determined by UV spectrophotometry. By assuming additive absorbance values, hapten to protein molar ratios were evaluated as 2.2, 2.0, 3.0, 2.0, 2.0, 1.7, and 0.4 for CNH, CNA, CPNU, 1NAH, 2NAH, 1NA, and 2NA, respectively.

HRP Labeling of LIB-CNH36 Anti-carbaryl MAb. HRP was coupled to LIB-CNH36 MAb according to the periodate method (Wilson and Nakane, 1978). Two hundred microliters of freshly prepared 0.1 M $NaIO_4$ in distilled water was added to 1.0 mL of 4 mg/mL HRP in distilled water, and the reaction was incubated at room temperature for 20 min with stirring. To remove the periodate excess and to exchange the buffer, the oxidized HRP was chromatographied on Sephadex G-50 using 1 mM acetate buffer, pH 4.4, as eluant. The protein volume recovered (12 mL) was concentrated to 2.0 mL and the pH adjusted to 9.0 with 200 mM carbonate buffer, pH 9.6. This activated HRP solution was immediately added to 1 mL of 7.9 mg/mL LIB-CNH36 MAb in 10 mM carbonate buffer, pH 9.5. The mixture was incubated for 2 h at room temperature with gentle stirring. The Schiff bases formed were stabilized by reduction with 100 μL of freshly prepared 0.1 M $NaBH_4$ in distilled water. After 2 h at 4 °C, labeled MAb was purified by gel filtration on Sephacryl S-300. Those chromatographic fractions giving a constant ratio of the absorbance at 403 nm (HRP) to the absorbance at 280 nm (total protein) were pooled, since they represented a quite homogeneous HRP-labeled MAb preparation. The HRP to MAb molar ratio, as determined from the molar adsorptivities of both proteins at 280 and 403 nm by assuming additive absorbance values, was 1.2.

Mice Immunization. At least four BALB/c female mice (8−10 weeks old) were immunized with each BSA−hapten conjugate. Mice were injected intraperitoneally with 200 μL of a 1:1 (v/v) mixture of Freund's complete adjuvant and PBS (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 100 μg of immunogen. Three subsequent injections were given at 2−3 week intervals using incomplete Freund's adjuvant. Three weeks after the fourth injection, mice were boosted intraperitoneally with 200 μg of immunogen in 200 μL of PBS. Blood was collected from mice immediately before cell fusions, and the sera obtained were precipitated with ammonium sulfate up to 50% saturation. Subsequently, sera were characterized by determining their titer and affinity to carbaryl. The titer was defined as the serum dilution that gave an absorbance of 1.0 in noncompetitive conditions. Serum affinity was estimated as the concentration of carbaryl that reduced the maximum signal in a competitive ELISA to 50% (I_{50}).

Rabbit Immunization. Two New Zealand rabbits of around 2 kg weight received a subcutaneous injection into two different sites at the shoulder with 1 mL of a 1:1 (v/v) mixture of Freund's complete adjuvant and PBS containing 300 μg of immunogen (BSA−CNH or BSA−CPNU conjugates). Four subsequent injections were given at 3 week intervals using incomplete Freund's adjuvant. Ten days after the last injection, blood was collected from the marginal ear vein, and the sera were characterized as described for mice.

Production of Monoclonal Antibodies to Carbaryl. The procedure for cell fusion and hybridoma production was based on a modification of the original Köhler and Milstein method (Nowinski et al., 1979). Details were previously published (Abad and Montoya, 1994). Briefly, mouse spleen cells were fused with the murine myeloma cell line P3-X63-Ag8.653 using polyethyleneglycol 1500 and dispensed into 96-well microtiter culture plates at a cellular density of (4−5) × 10^5 cells/well. Hybridomas were selected in HAT medium, and 12 days after fusion cell culture supernatants were tested for the presence of anti-carbaryl antibodies by a simultaneous noncompetitive/competitive ELISA. Wells giving a strong positive response in the noncompetitive ELISA (absorbance ≥ 1.0) and showing a very significant carbaryl recognition (usually a signal reduction of 80% or more in the competitive format with 1 μM carbaryl) were cloned by limiting dilution to ensure monoclonality. Clones were expanded and stored in liquid nitrogen, and MAbs were purified from cell culture supernatants and/or ascites fluid by chromatography on DEAE-Sepharose. Purified MAbs were stored at 4 °C as ammonium sulfate precipitates.

Immunoassays. *General Conditions.* All incubations were carried out at room temperature. After each step, plates were washed four times with 0.15 M NaCl containing 0.05% Tween 20. Plates were coated by adding 100 μL/well of a 1 μg/mL (ovalbumin conjugates and anti-carbaryl MAbs) or 2 μg/mL (goat anti-mouse Igs) solution in 50 mM carbonate buffer, pH 9.6, followed by overnight incubation. HRP activity bound to the wells was determined by adding 100 μL/well of 2 mg/mL OPD in reaction buffer (25 mM citrate, 62 mM sodium phosphate, pH 5.4, contaning 0.012% H_2O_2). The enzymatic reaction was stopped after 10 min by adding 100 μL/well of 2.5 M sulfuric acid, and the absorbance at 490 nm was read.

Prior to inhibition studies, the concentrations of the reagents (antisera or MAbs for format I, enzyme tracers for formats II and III, and HRP-labeled MAb for format IV) to be added to the plate together with carbaryl standards were determined under noncompetitive conditions to obtain a maximum absorbance around 1.0 in the absence of carbaryl.

The following four competitive ELISA formats were used.

Coating Conjugate Indirect ELISA (Format I). Fifty microliters per well of different carbaryl concentrations in PBS and 50 μL/well of a predetermined antiserum dilution or MAb concentration in PBST (PBS with 0.05% Tween 20) were added to plates previously coated with OVA−hapten conjugates. After 2 h of incubation, plates were washed and incubated for 1 h with 100 μL/well of a 1/2000 dilution of HRP-labeled rabbit anti-mouse Igs in PBST. Color was developed as described under General Conditions.

Coating Antibody Direct ELISA (Format II). Fifty microliters per well of different carbaryl concentrations in PBS and 50 μL/well of a predetermined concentration of enzyme conjugate in PBST were added to plates previously coated with anti-carbaryl MAbs and incubated for 2 h. Color was developed as described under General Conditions.

Precoating Capture Antibody Direct ELISA (Format III). One hundred microliters per well of anti-carbaryl antibodies in PBST (1 μg/mL) was added to plates previously coated with goat anti-mouse immunoglobulins. After 2 h of incubation, plates were washed and the assay performed as described for format II.

Coating Conjugate Direct ELISA (Labeled-Antibody, Format IV). Fifty microliters per well of different carbaryl concentrations in PBS and 50 μL/well of a predetermined concentration of HRP-labeled MAb in PBST were added to plates previously coated with OVA−hapten conjugates. After 1 h of incubation, plates were washed and the color was developed as described under General Conditions.

Competitive ELISA standard curves were obtained in quadruplicate. When required, curves were normalized by expressing experimental absorbance values (B) as $(B/B_0) \times 100$, where B_0 is the absorbance at zero carbaryl concentration. Absolute or normalized signals were fitted to a four-parameter logistic equation using a commercial package software (Sigmaplot, Jandel Scientific).

RESULTS AND DISCUSSION

Hapten Design. The development of a highly sensitive ELISA for pesticides depends to a great extent on the synthesis of a panel of haptens that should preserve as far as possible the analyte structure. To evaluate the influence of the hapten structure on the affinity of the antibodies eventually obtained, seven compounds sharing the aromatic moiety of carbaryl and with a carboxylic group were used as immunizing haptens (Figure 1). 1NA and 2NA are commercially available

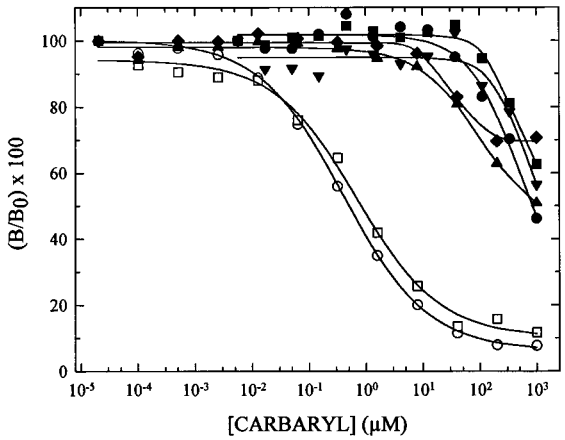


Figure 2. Recognition of carbaryl by mouse antisera as tested by homologous indirect competitive ELISA. Normalized inhibition curves correspond to the best antisera from each immunizing hapten: CNH (○), CNA (□), CPNU (●), 1NAH (■), 2NAH (▼), 1NA (▲), and 2NA (◆).

molecules ready for protein conjugation, whereas the rest where synthesized from commercial precursors in one (CPNU, 1NAH, and 2NAH) or two steps (CNH and CNA).

CNH and CNA could theoretically be considered the best haptens for the obtention of antibodies to carbaryl, because in both molecules the spacer arm is attached to the methyl group of the analyte, thus preserving the *N*-methylcarbamate characteristic of this insecticide. In CPNU and 1NAH haptens, the spacer arm is located at the same position, but the carbamate group is not present. 2NAH differs from 1NAH only in the aromatic ring position used to introduce the spacer arm, and 1NA and 2NA are the simplest and presumably less adequate haptens, differing between them also in the ring position to which the functional group is attached.

Characterization of Polyclonal Antisera. A total of 33 mouse antisera from 7 BSA−hapten conjugates were collected immediately before cell fusions. These sera were tested by noncompetitive ELISA against 7 ovalbumin coating conjugates with similar hapten densities. All mouse antisera exhibited good antibody titer when tested against their respective homologous conjugates (>1/40000). However, antisera from different haptens showed a very different recognition pattern of heterologous conjugates. As an example, whereas antisera obtained from CNH and CNA conjugates highly recognized coating conjugates derived from CPNU and 1NAH haptens, the opposite was not true.

The ability of all mouse antisera to recognize carbaryl was subsequently tested by homologous competitive ELISA in the coating conjugate format. For each immunizing hapten, the carbaryl inhibition curve obtained from the best antiserum is depicted in Figure 2. Consistent with their greater structural similarity to the analyte, CNH and CNA were the most adequate haptens for the obtention of mouse polyclonal antibodies to carbaryl, as evidenced by the complete signal inhibition shown by their respective antisera in the competitive ELISA. On the contrary, little or no inhibition was observed for the antisera derived from the rest of the haptens.

When competitive ELISAs are performed, it is well established that different hapten conjugates can modify the apparent affinity of antibodies for the target molecule (Wie and Hammock, 1984; Harrison et al., 1991b). Therefore, the 25 mouse antisera obtained from CNH, CNA, CPNU, and 1NAH haptens were further tested by competitive ELISA using heterologous conjugates.

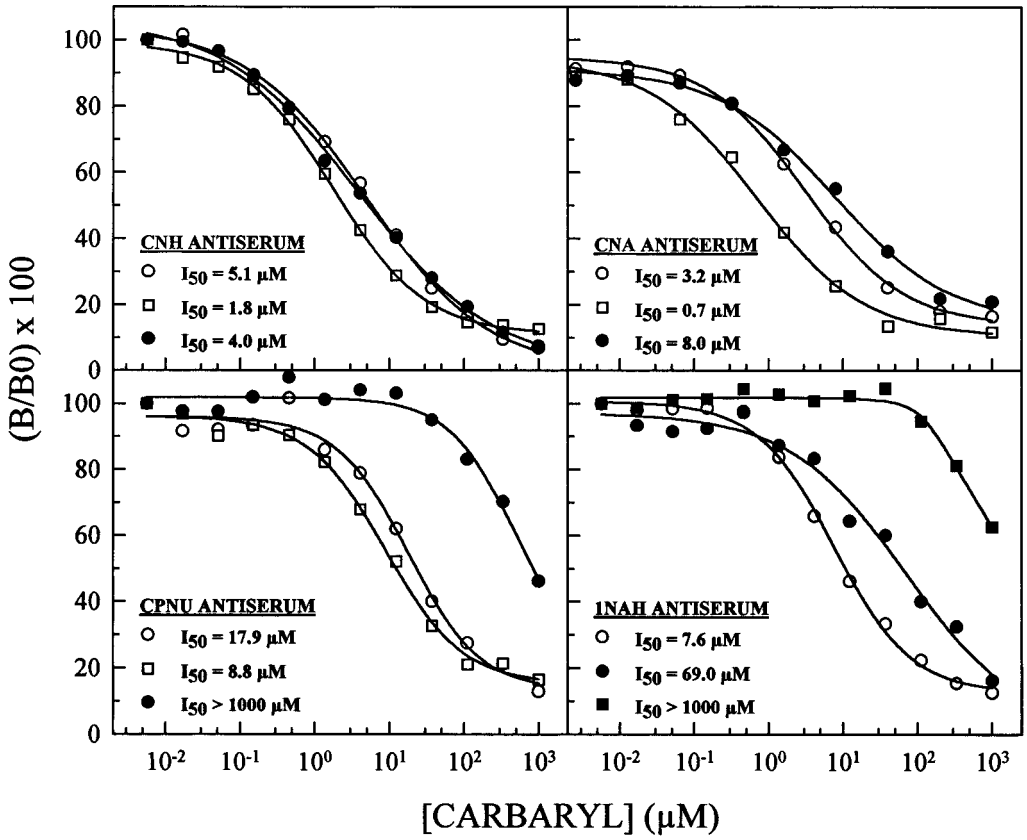


Figure 3. Recognition of carbaryl by mouse antisera as tested by homologous and heterologous indirect ELISA. Besides the homologous assay, only the best combinations of each antiserum with heterologous coating conjugates are depicted in each graph. Coating conjugates were as follows: OVA−CNH (○), OVA−CNA (□), OVA−CPNU (●), and OVA−1NAH (■).

Figure 3 shows a series of graphs, each one representing the inhibition curves for the best antiserum from each immunizing hapten in combination with several coating conjugates. With only one exception, for all the antisera it was possible to find at least one heterologous conjugate giving an I_{50} lower than that obtained with the homologous conjugate. Thus, I_{50} of CNH antisera decreased 3−15 fold, and improvements of more than 2 orders of magnitude were obtained for CPNU- and 1NAH-derived antisera. Nevertheless, the lowest I_{50} continued corresponding to CNH and CNA antisera, which confirmed the previous conclusion about the suitability of CNH and CNA haptens to produce the mouse antibodies with the highest affinity for carbaryl.

As mentioned, excellent polyclonal antibodies to carbaryl were obtained in rabbits using CPNU as the immunizing hapten (Marco et al., 1993). With the aim of evaluating if CNH is a more suitable hapten than CPNU to induce anti-carbaryl antibodies also in rabbits, BSA conjugates of these two haptens were injected into rabbits and the antisera obtained subsequently compared. Antisera from CNH showed a lower I_{50} than antisera from CPNU, in both the homologous and heterologous formats (data not shown). These results confirmed that, regardless of the animal species, CNH is the most adequate hapten to produce antibodies for carbaryl.

Production and Characterization of Monoclonal Antibodies. Cell fusions were performed with all of the immunized mice, irrespective of their serum affinities. Consistent with the behavior of mouse antisera, monoclonal antibodies that significantly recognized carbaryl were obtained only from mice immunized with CNH and CNA conjugates (13 and 2 hybridomas, respectively). When assayed in the homologous coating conjugate format, 6 of the 15 antibodies showed an I_{50} to carbaryl lower than 15 nM. Such high affinity meant a great improvement with respect to mouse and rabbit antisera, which confirmed the potential of the hybridoma technology to select MAbs with better analyte recognition properties than polyclonal antisera.

These six MAbs were further tested against heterologous conjugates. All of them recognized several immobilized conjugates, and conversely nearly all coating conjugates were recognized by most antibodies. Carbaryl inhibition curves were then obtained for those MAb/coating conjugate combinations that provided a sufficient signal under noncompetitive conditions. The I_{50} values for these combinations are summarized in Table 1. All MAbs increased their apparent affinity to carbaryl by using heterologous conjugates. Nevertheless, the best coating conjugate, as well as the improvement in sensitivity achieved, strongly depended on each antibody. I_{50} values around 3 times lower than those obtained with the homologous coating conjugate were found for LIB-CNH36 MAb with OVA−1NAH and for LIB-CNA38 MAb with OVA−2NAH.

From the data obtained in this format, three MAbs (LIB-CNH36, LIB-CNH45, and LIB-CNA38) displaying an I_{50} to carbaryl lower than 3.0 nM with at least one of the coating conjugates were selected to be tested in the immobilized antibody ELISA formats.

Recognition of Enzyme Conjugates by Immobilized MAbs. *Antibodies Directly Immobilized to the Plate.* Before inhibition experiments were performed, the three selected MAbs were evaluated for their ability to recognize different HRP−hapten conjugates (Figure 4b). LIB-CNH36 MAb recognized very well only two enzyme conjugates (HRP−CNH and HRP−CPNU); LIB-CNH45 MAb bound two additional enzyme conjugates (HRP−1NAH and HRP−2NAH), and LIB-CNA38 MAb performed the worst, since it weakly recognized only one enzyme conjugate (HRP−CPNU). Thus, most enzyme conjugates were poorly recognized by the three MAbs, in contrast with the fact that OVA−hapten conjugates were properly recognized in the coating conjugate

Table 1. Carbaryl I_{50} (nM) Values of the Best MAbs Obtained, Determined by Competitive Indirect ELISA with Different Coating Conjugates

	coating conjugate[a]						
MAb	CNH	CNA	CPNU	1NAH	2NAH	1NA	2NA
LIB-CNH32	**14.2**[b]	13.7	13.0	7.1	13.3	10.8	nd[c]
LIB-CNH33	**5.6**	5.7	4.7	3.9	3.7	4.5	nd
LIB-CNH36	**3.9**	4.4	3.0	1.1	nd	3.4	nd
LIB-CNH37	**10.4**	9.3	7.1	4.4	nd	5.4	nd
LIB-CNH45	**3.6**	2.3	4.8	2.9	2.4	2.8	nd
LIB-CNA38	3.9	**3.1**	25.3	2.2	1.4	12.6	nd

[a] Haptens in the table were coupled to OVA to obtain the corresponding coating conjugates. ELISA plates were coated using the same concentration (1 μg/mL) for all coating conjugates. The MAb concentration used in the competitive assays was previously determined for each MAb/coating conjugate combination to obtain an absorbance around 1.0 in the absence of carbaryl. [b] Homologous combinations are indicated in boldface print. [c] nd, not determined, because this MAb/coating conjugate combination did not provide a sufficient maximum absorbance with the maximum MAb concentration tested (1 μg/mL).

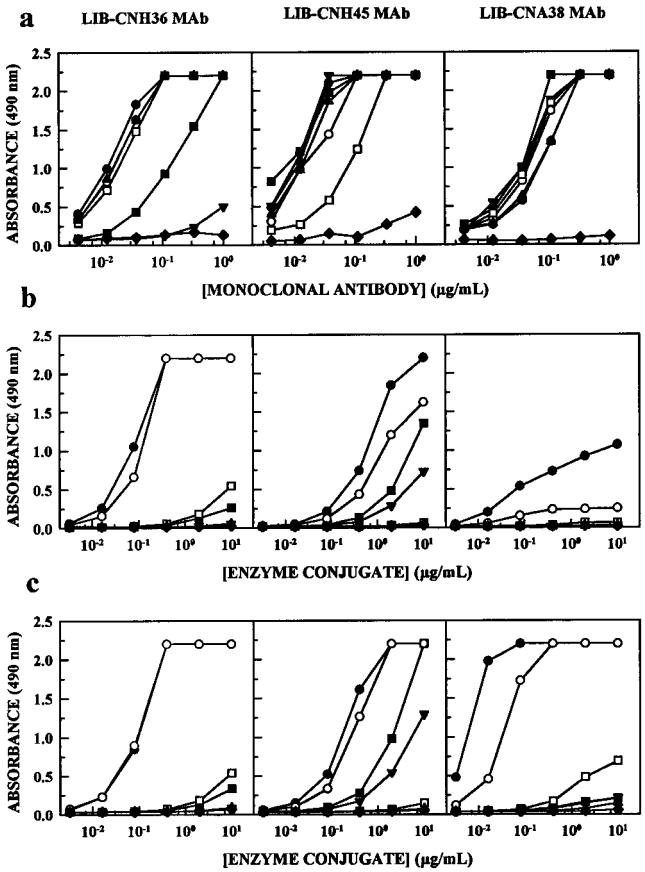


Figure 4. Recognition of OVA−hapten conjugates (a) or HRP−hapten conjugates (b and c) by LIB-CNH36 MAb (left), LIB-CNH45 MAb (center), and LIB-CNA38 MAb (right) in the coating conjugate indirect ELISA format (a), in the coating antibody direct ELISA format (b), and in the precoating capture antibody direct ELISA format (c). Wells were coated with 100 μL of a 1 μg/mL solution in carbonate buffer of coating conjugate (a) or monoclonal antibody (b and c). Conjugated haptens were CNH (○), CNA (□), CPNU (●), 1NAH (■), 2NAH (▼), 1NA (▲), and 2NA (◆).

Table 2. Study of the Influence of ELISA Format and Hapten Heterology on the Sensitivities of Three MAbs for Carbaryl

	I_{50} (nM)									
	LIB-CNH36				LIB-CNH45			LIB-CNA38		
hapten	format I	format II	format III	format IV	format I	format II	format III	format I	format II	format III
CNH	**3.9**[a]	**3.0**	**3.2**	**33.6**	**3.6**	**3.2**	**3.7**	3.9	nd[b]	3.7
CNA	4.4	nd	nd	28.3	2.3	nd	nd	**3.1**	**nd**	**16.5**
CPNU	3.0	2.0	2.1	34.9	4.8	2.9	2.9	25.3	207.6	2.3
1NAH	1.1	nd	nd	38.8	2.9	3.7	3.0	2.2	nd	nd
2NAH	nd	nd	nd	34.5	2.4	5.8	4.1	1.4	nd	nd
1NA	3.4	nd	nd	14.0	2.8	nd	nd	12.6	nd	nd

[a] Homologous combinations are indicated in boldface print. [b] nd, not determined, because this particular combination did not provide a sufficient maximum absorbance in the absence of the analyte.

format (Figure 4a). CNA conjugates constitute the most representative example of this behavior: while the OVA−CNA coating conjugate was highly recognized by the MAbs, the HRP−CNA conjugate was unrecognized even by the CNA-derived LIB-CNA38 MAb, when it is well-known that antibodies usually recognize their homologous conjugates with high affinity (Goodrow et al., 1990). A low activity of the enzyme conjugates was tentatively thought as the cause of the observed behavior. However, when new enzyme conjugates with higher and lower hapten to enzyme molar ratios were synthesized, the results were essentially the same. Therefore, the number of and the extent at which enzyme conjugates were recognized should be attributed to the binding properties of the immobilized MAb rather than to the conjugate preparation.

Antibodies Immobilized through a Capture Immunoglobulin. The effect of a precoating step with goat anti-mouse Igs on the ability of immobilized antibodies to recognize enzyme conjugates was also evaluated. As shown in Figure 4c, very different results were obtained for each MAb. On the one hand, LIB-CNH36 MAb showed exactly the same recognition pattern of enzyme conjugates, regardless of whether the antibody was bound directly to the plate or through a trapping immunoglobulin. On the other hand, a slight improvement in tracer recognition was noticed with LIB-CNH45 MAb. This allowed us to reduce the immunoreagent concentrations, a fact already reported for other antibodies immobilized through capture Igs (Schneider and Hammock, 1992; Giersch, 1993).

The behavior of LIB-CNA38 MAb deserves special attention. After precoating with anti-mouse Igs, this MAb bound the HRP−CPNU and HRP−CNH conjugates even better than the two other antibodies did, even though these tracers were nearly unrecognized when the antibody was directly immobilized to the plate. Furthermore, the previously unrecognized HRP−CNA conjugate provided a sufficient signal to perform a competitive assay in this format. Therefore, the use of the precoating step is essential for this antibody, since it constitutes not only a way of drastically decreasing the concentration of the enzyme conjugate (more than 1000 times for HRP−CPNU and HRP−CNH) but also the only possibility of performing an immunoassay based on tracers otherwise unsuitable (HRP−CNH and HRP−CNA). These results suggest that, when directly put in contact with the plastic surface of the ELISA plate, LIB-CNA38 antibody undergoes an important conformational change that affects its HRP−hapten recognition properties.

Irrespective of whether a precoating step was used or not, the number of enzyme conjugates bound by immobilized MAbs was lower than the number of coating conjugates recognized by the same MAbs in solution. Similar results have been previously reported concerning poor or even null recognition of haptens when coupled to HRP, while they efficiently interacted with the antibodies when coupled to carrier proteins (Bekheit et al., 1993). Particularly, the behavior of CNA agrees with the results found by other authors indicating that haptens with short handles are less suitable for enzyme conjugates, probably due to steric hindrances (Skerritt et al., 1992; Bekheit et al., 1993; Schneider et al., 1994).

Competitive Experiments with Immobilized MAbs. Carbaryl inhibition curves were performed for all those tracer/MAb combinations providing a sufficient signal. With LIB-CNH36 and LIB-CNH45 MAbs directly immobilized to the plate, I_{50} values in the 2.0−6.0 nM range were obtained (Table 2, format II), which were very similar to those found in the coating conjugate format (Table 2, format I). On the contrary, LIB-CNA38 MAb displayed a I_{50} value clearly higher than that obtained in the coating conjugate format for the only recognized tracer.

No improvement in sensitivity was achieved for the antibodies LIB-CNH36 and LIB-CNH45 when the precoating with goat anti-mouse Igs was included (Table 2, format III). However, the behavior of the LIB-CNA38 MAb clearly improved with the precoating, as compared with the direct immobilization procedure. In the only case in which the comparison was possible (using the HRP−CPNU conjugate), an improvement in the I_{50} of around 100 times was obtained. In fact, for this particular hapten/antibody combination, format III was around 10 times more sensitive than format I. Nevertheless, if an absolute comparison is established independently of the hapten used, for the three MAbs tested, it was always possible to find a more sensitive assay for carbaryl in format I than in formats II and III.

HRP-Labeled MAb ELISA Format. The MAb that provided the most sensitive immunoassay in the coating conjugate format (LIB-CNH36) was labeled with HRP to develop a coating conjugate direct ELISA (format IV). This format, although it has not found a wide application for pesticide analysis, provided optimal sensitivities in the few cases in which it was used for this purpose (Skerritt et al., 1992; Böcher et al., 1992; Giersch et al., 1993). When the HRP-labeled MAb was tested against different coating conjugates, most of them were recognized, but the recognition pattern (Figure 5) was very different from that previously found with the unlabeled MAb (Figure 4a). Thus, whereas the OVA−2NAH conjugate was nearly unrecognized by the unlabeled antibody, it was the best bound coating conjugate by the labeled MAb. The OVA−1NAH conjugate was also better recognized in format IV than in format I, while

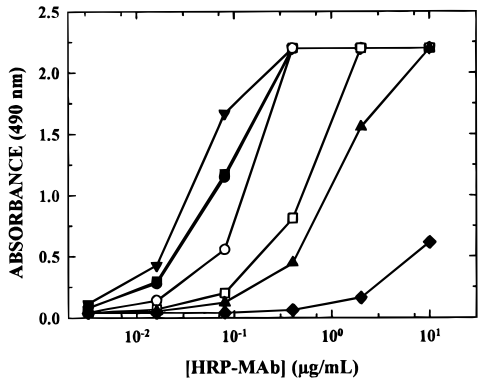


Figure 5. Recognition of immobilized ovalbumin−hapten conjugates by LIB-CNH36 MAb labeled with HRP. For all coating conjugates, wells were coated with 100 μL of a 1 μg/mL solution in carbonate buffer. Coating conjugates were OVA−CNH (○), OVA−CNA (□), OVA−CPNU (●), OVA−1NAH (■), OVA−2NAH (▼), OVA−1NA (▲), and OVA−2NA (◆).

OVA−CNA and OVA−1NA conjugates were less recognized in format IV. On the other hand, when competitive curves were performed in format IV, carbaryl I_{50} values were higher than those obtained in other formats for any hapten−protein conjugate (Table 2). All of these results suggest that the antibody binding site was appreciably altered as a consequence of the covalent coupling of HRP.

CONCLUSIONS

Within the general objective of developing a MAb-based immunoassay for carbaryl, a systematic study was undertaken dealing with the influence of the immunizing hapten structure on the affinity of the resulting antibodies. This work confirmes the relevance of proper hapten design in the production of antibodies for low molecular weight compounds. From both mouse and rabbit antisera, we have proved that even minor differences in the hapten structure, while affecting an important antigenic determinant, have dramatic consequences on the antibody binding properties. Thus, the two haptens preserving the characteristic carbamate group of carbaryl (CNH and CNA) provided the best antisera by far, regardless of whether homologous or heterologous coating antigens were used to establish the comparison.

The pursued goal of obtaining a wide panel of high-affinity MAbs to carbaryl was finally achieved from CNH and CNA conjugates. Most of these antibodies drastically improved the affinity to the analyte exhibited by antisera from mice and rabbits. A high number of competitive curves with very different sensitivities for carbaryl were obtained by combining three monoclonal antibodies, six haptens coupled to both ovalbumin and horseradish peroxidase, and four assay configurations (Figure 6).

With respect to the influence of hapten heterology and ELISA format on the sensitivity of the assay, several conclusions can be established. First, hapten heterology is confirmed as a valuable approach to improve the sensitivity of immunoassays for pesticides. However, it is nearly impossible to predict which hapten will provide the most sensitive assay, as well as the extent of the improvement achievable. Second, very similar sensitivities were obtained with the coating conjugate and the enzyme conjugate formats. Nevertheless, more haptens were recognized when coupled to ovalbumin

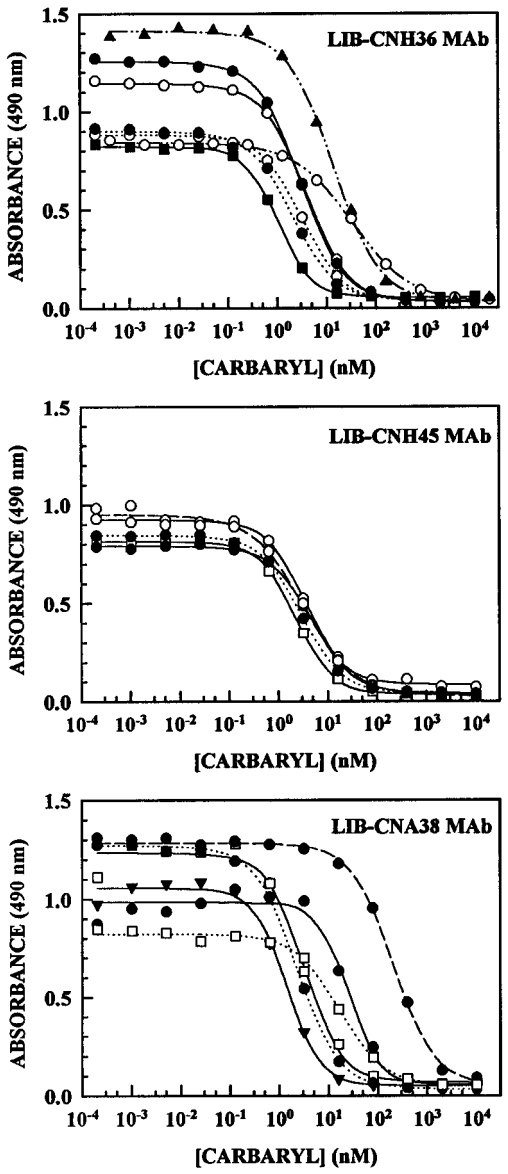


Figure 6. Standard curves for carbaryl obtained with the three selected MAbs assayed in different ELISA formats and against different haptens coupled to ovalbumin or horseradish peroxidase. For visual clarity, only some inhibition curves for each antibody are shown. Enzymatic reactions were stopped in all cases after 10 min. All curves were obtained in quadruplicate. Points represent the average of the four absorbance values, and they were fitted to a four-parameter logistic equation (lines). Each line type represents an ELISA format, and each symbol a hapten, regardless if it was coupled to ovalbumin or HRP. ELISA configurations: format I (—), format II (- - -), format III (· · ·), and format IV (— · · —). Haptens: CNH (○), CNA (□), CPNU (●), 1NAH (■), 2NAH (▼), 1NA (▲), and 2NA (◆).

than when coupled to HRP, so the use of heterologous conjugates seems to be a strategy more widely applicable to format I than to formats II and III. Third, some antibodies are very sensitive to the direct immobilization to the plate. In these cases, the use of a precoating step may be a good strategy to recover antibody activity. Finally, the best hapten for an antibody in a given ELISA format is not necessarily also the best in a different format. Similarly, antibodies performing well in one format may not perform adequately in another one.

Among all of the competitive immunoassays herein developed for carbaryl, the most sensitive one is based on the heterologous ovalbumin conjugate of 1NAH

together with the LIB-CNH36 MAb in the indirect coating conjugate ELISA format (I_{50} = 1.1 nM). This assay will be optimized in the following paper with respect to physicochemical parameters such as pH, ionic strength, reagent concentrations, incubation times, and presence of Tween 20. The immunoassay will be also characterized with respect to organic solvent tolerance and analyte specificity.

ABBREVIATIONS USED

BSA, bovine serum albumin; DEAE, diethylaminoethyl; DMEM, Dulbecco's modified Eagle's medium; DMF, *N*,*N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; HAT, hypoxanthine−aminopterine−thymidine; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; Igs, immunoglobulins; I_{50}, analyte concentration required for 50% inhibition; MAb, monoclonal antibody; NMR, nuclear magnetic resonance; OPD, *o*-phenylenediamine; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.05% Tween 20; TMS, tetramethylsilane; UV, ultraviolet.

Haptens: 1NA, 1-naphthoxyacetic acid; 2NA, 2-naphthoxyacetic acid; 1NAH, *N*-(1-naphthoyl)-6-aminohexanoic acid; 2NAH, *N*-(2-naphthoyl)-6-aminohexanoic acid; CNA, 3-[[(1-naphthyloxy)carbonyl]amino]propanoic acid; CNH, 6-[[1-naphthyloxy)carbonyl]amino]hexanoic acid; CPNU, 1-(5-carboxypentyl)-3-(1-naphthyl)urea.

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