Production of Monoclonal Antibodies for Carbaryl from a Hapten Preserving the Carbamate Group

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Monoclonal antibodies (MAbs) for the carbamate insecticide carbaryl (1-naphthyl *N*-methylcarbamate) were obtained by immunizing mice with 6-[[(1-naphthyloxy)carbonyl]amino]hexanoic acid (**3**, CNH) conjugated to bovine serum albumin (BSA). The hapten synthesis strategy was designed to preserve the carbamate group characteristic of *N*-methylcarbamate pesticides. The most promising MAb (LIB/CNH-3.6) was characterized by using an indirect homologous antigen-coated ELISA. With this assay format, LIB/CNH-3.6 showed a high affinity for carbaryl (I_{50} = 3.6 nM) and minimum cross-reactivities for several *N*-methylcarbamates and 1-naphthol, the main carbaryl metabolite. Both affinity and specificity achieved by LIB/CNH-3.6 meant a great improvement with respect to the features displayed by the serum of the mouse from which the MAb was derived. This MAb appears to be a very promising immunoreagent for the future development of a specific and sensitive quantitative ELISA for carbaryl, and the applied hapten synthesis strategy is proposed as a general procedure to obtain immunogenic conjugates for other *N*-methylcarbamate pesticides.

Keywords: *Carbaryl; carbamate; immunoassay; ELISA; hapten synthesis; monoclonal antibody; screening*

INTRODUCTION

Carbaryl, the first *N*-methylcarbamate successfully introduced in the market, is a contact and stomach insecticide, with slight systemic properties, used for horticultural protection from insect pests. It is also extensively used to control ectoparasites in poultry houses (Worthing and Hance, 1983). Although over 20 other carbamates have reached commercial application, carbaryl is still one of the most widely used insecticides (Hodgson and Kuhr, 1990). Carbaryl is an inhibitor of acetylcholinesterase with low cholinergic toxicity in mammals and is highly susceptible to chemical hydrolysis and to biodegradation. The main metabolite resulting from carbaryl hydrolysis is 1-naphthol, which does not accumulate in the body but is excreted in urine, feces, and respiratory gases in a short period of time. Although carbaryl is generally considered a safe insecticide because of its low toxicity in mammals, some adverse effects have been reported, including subchronic neurotoxicity after long-term exposure when used as a household insecticide (Branch and Jacqz, 1986), long-term alterations in chick locomotion with reductions in certain neurotransmitter metabolites in the CNS (Farage-Elawer and Blaker, 1992), and inhibition of IL-2-dependent proliferation of T cells (Casale et al., 1993). Carbaryl is also toxic to honeybees (Westlake et al., 1985). These recently reported adverse effects, the wide use of carbaryl, and its frequency of occurrence in food analysis, mainly in grape- and apple-derived products (Schattenberg and Hsu, 1992; de Kok and Hiemstra, 1992; Yess et al., 1993), have increased the concern over carbaryl residues and have reemphasized the need for methods to detect small quantities of this insecticide.

Methods used for the determination of carbaryl include spectrophotometric (Chiba, 1981), spectrofluorometric (Argauer et al., 1970), infrared spectrometric (Gallignani et al., 1993), and enzymic techniques (Kumaran and Tran-Minh, 1992), gas chromatography (GC) (Seiber, 1972; Kendrick et al., 1991; Färber and Schöler, 1993), and high-performance liquid chromatography (HPLC) (Krause, 1985; Strait et al., 1991; de Kok et al., 1992; Garvey, 1993; Simon et al., 1993). HPLC is generally regarded as the best technique for *N*-methylcarbamate pesticide analysis, since many of these pesticides lack the thermal stability neccesary for GC determination (Barceló, 1993). Although the method works well, it involves laborious extraction and cleanup procedures (de Kok and Hiemstra, 1992), and derivatization as well as concentration steps are often necessary to obtain the desired sensitivity.

Enzyme-linked immunosorbent assay (ELISA) methods have been developed for a significant number of pesticides over the past few years (Van Emon and López-Ávila, 1992; Sherry, 1992). The application of immunoassays for the analysis of trace chemicals constitutes a very promising field that has already proved its suitability in several cases (Feng et al., 1990; Thurman et al., 1990; Abad et al., 1993). In fact, there is a tendency to consider that immunoassays could become alternative or complementary methods for the routine analysis of pesticide residues because they are sensitive, selective, rapid, and inexpensive.

A polyclonal-based immunoassay for carbaryl determination has been recently reported (Marco et al., 1993). The authors used chemically stable mimics of carbaryl for inducing an immune response in rabbits, with excellent results. In the present paper, we describe the first results in the production of monoclonal antibodies (MAbs) for carbaryl using a hapten which preserves the characteristic carbamate group of this insecticide. Several hybridomas secreting MAbs that recognize carbaryl were selected by a simultaneous noncompetitive/competitive ELISA, cloned, and expanded. Regarding its potential use for a carbaryl immunoassay, the characterization of one of these MAbs is presented. A comparison between the MAb characteristics and those of the mice sera is also included.

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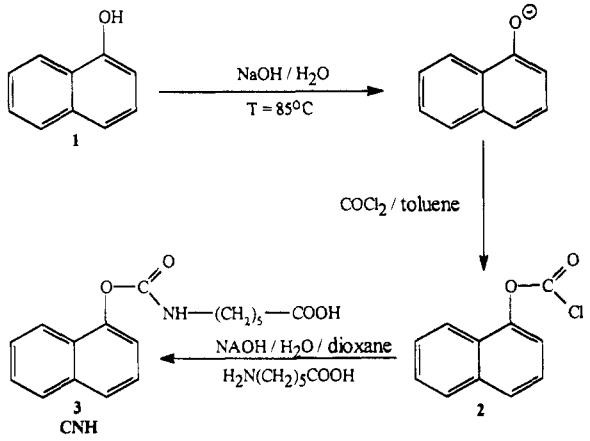


Figure 1. Reaction scheme of the synthesis of 6-[[(1-naphthyloxy)carbonyl]amino]hexanoic acid (CNH).

MATERIALS AND METHODS

Chemicals and Instruments. Analytical standards of carbaryl, 1-naphthol, carbofuran, aldicarb, methomyl, methiocarb, and propoxur were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock solutions of each pesticide were prepared in *N,N'*-dimethylformamide (DMF) and stored a 4 °C. Phosgene (20% solution in toluene) was obtained from Fluka Química (Madrid, Spain). Bovine serum albumin (BSA), ovalbumin (OVA), Tween 20, *o*-phenylenediamine (OPD), and Freund's complete and incomplete adjuvants were purchased from Sigma–Aldrich Química (Madrid, Spain). Peroxidase-labeled rabbit 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 (Dulbecco's modified Eagle's medium, 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 (Nunc MaxiSorp, Roskilde, Denmark) were washed with a Ultrawash II microplate washer, and absorbances were read in dual-wavelength mode (490–630 nm) with a MR 700 microplate reader, both from Dynatech (Sussex, U.K.). ^{1}H nuclear magnetic resonance (NMR) spectra were obtained with a Varian VXR-400S (400 MHz) spectrometer. Chemical shifts are given relative to TMS (tetramethylsilane) as an internal reference. Electron impact mass spectra (EIMS) were recorded on a 5988A Hewlett-Packard apparatus at 70 eV, and data are reported as m/z (relative intensity). A Hewlett-Packard 5890 gas chromatograph equipped with a 20 m × 0.20 mm (i.d.) HP-5 column was interfaced to this mass spectrometer for GC–MS analyses. Infrared spectra (IR) were obtained on a Perkin-Elmer 781 instrument, and ultraviolet (UV) spectra were recorded on a UV-160A Shimadzu apparatus.

Hapten Synthesis. The synthesis strategy (Figure 1) was based on previously reported procedures for similar compounds (Wolf and Seligman, 1951; Carpenter and Gish, 1952; Lambrech, 1961).

1-Naphthyl Chloroformate (**2**). 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 (**1**, 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. Diphosgene and triphosgene could be used as alternative reagents since they are less toxic and less volatile than phosgene.) 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) to give 16.5 g (58%) of the chloroformate as a light yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.26–8.00 (m, aromatic $C_{10}H_7$); EIMS, m/z (relative intensity) 208 (M + 2, 10), 206 (M, 31), 164 (18), 162 (5), 144 (5), 143 (39), 127 (25), 115 (100), 89 (14), 63 (12).

6-[[(1-Naphthyloxy)carbonyl]amino]hexanoic Acid (CNH, **3**). Aminohexanoic acid (7.37 g, 56.1 mmol) was dissolved in 7.5 mL of 4 M sodium hydroxide, and the solution was cooled at 4 °C. Naphthyl chloroformate (6.25 g, 30.3 mmol) was dissolved in 11 mL of 1,4-dioxane and cooled at 4 °C. The dioxane solution, along with 7.5 mL of cold (4 °C) 4 M sodium hydroxide, was added to the aminohexanoic solution in five equal portions, with at least 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, 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 with concentrated hydrochloric acid. The precipitated product was collected, washed with water, and dried to yield 5.0 g of CNH (68%): ^{1}H NMR (acetone-d_6) δ 1.50 (m, 2 H, CH_2), 1.69 (m, 4 H, 2 CH_2), 2.35 (t, 2 H, CH_2COOH), 3.29 (q, 2 H, CH_2NH), 7.14–8.03 (m, 7 H, aromatic $C_{10}H_7$); EIMS, m/z (relative intensity) 301 (M^+, 1), 236 (1), 144 (100), 116 (27), 115 (57), 55 (9). The IR spectrum was also in agreement with the expected structure.

Preparation of Hapten–Protein Conjugates. The CNH hapten was covalently attached to bovine serum albumin (BSA) and ovalbumin (OVA) using the modified active ester method (Langone and Van Vunakis, 1982) and the mixed-anhydride method (Rajkowski et al., 1977), respectively. BSA–CNH conjugate was used as the immunogen and prepared as follows: A mixture of 30.2 mg of CNH (0.1 mmol), 13.9 mg of *N*-hydroxysuccinimide (0.12 mmol), and 24.3 mg of dicyclohexylcarbodiimide (0.118 mmol) in 1 mL of DMF was stirred at room temperature for 4 h. 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. OVA–CNH was used as coating conjugate and prepared as follows: To 3.68 mg of CNH (12.2 μmol) in 197 μL of DMF was added 2.9 μL of tri-*n*-butylamine (12.2 μmol) followed by 1.6 μL of isobutyl chloroformate (12.3 μmol). The mixture was stirred for 30 min at room temperature, and 100 μL 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 6.5 h with stirring, and the conjugate obtained was purified as described for the BSA–CNH immunogen.

Although both CNH and proteins show absorbance peaks at almost the same wavelength (280 nm), the CNH spectrum displays little characteristic shoulders in this spectral zone, so when conjugation occurred, the modification of the protein spectra was very evident. Then, the extent of coupling of CNH to proteins was determined by UV absorbance espectrophotometry at 280 nm by assuming additive absorbance values. The estimated hapten to protein molar ratios were about 32 and 3 for the BSA–CNH and OVA–CNH conjugates, respectively.

Immunoassays. *Noncompetitive Indirect ELISA.* The titer of mice sera and the antisera dilution or MAb concentration to be used in the competitive assays were determined according to this format. Ninety-six-well microtiter plates were coated with the OVA–CNH conjugate (1 μg/mL, 100 μL/well) in coating buffer (50 mM carbonate–bicarbonate buffer, pH 9.6) by overnight incubation at 4 °C. Plates were washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20), and 100 μL/well of serial dilutions of antiserum or MAb in phosphate-buffered saline (PBS; 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) was added. After incubation at 37 °C for 2 h, the plates were washed as before, and 100 μL of a 1/2000 dilution of peroxidase-labeled rabbit

anti-mouse immunoglobulins in PBST (PBS containing 0.05% Tween 20) was added to the wells. Plates were incubated at 37 °C for 1 h, and after washing 100 μL of 2 mg/mL OPD in reaction buffer (0.012% H_2O_2 in 25 mM citrate, 62 mM sodium phosphate, pH 5.4) was added. After 10 min at room temperature, the enzymic reaction was stopped by adding 100 μL of 2.5 M sulfuric acid and the absorbance at 490 nm was read.

Competitive Indirect ELISA. To microtiter plates coated with the OVA–CNH conjugate as described above, 50 μL/well of serial dilutions of the analyte in PBS was added, followed by 50 μL/well of a previously determined antiserum dilution or MAb concentration. In this way, a competition was established between immobilized hapten and free analyte for the antibody binding sites. Plates were incubated at 37 °C for 2 h and washed four times, and a 1/2000 dilution of peroxidase-labeled rabbit anti-mouse immunoglobulins in PBST was added. After 1 h at 37 °C, the enzymic activity bound to the wells was determined as described for the noncompetitive ELISA.

Production of Monoclonal Anti-Carbaryl Antibodies. *Immunization.* BALB/c female mice (8–10 weeks old) were injected intraperitoneally with 200 μL of a 1:1 (v/v) mixture of Freund's complete adjuvant and 100 μg of BSA–CNH conjugate dissolved in PBS. Three subsequent injections were given at 2–3-week intervals using incomplete Freund's adjuvant. One week after the third injection, mice were tail-bled and 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 the described noncompetitive assay conditions. Serum affinity was estimated as the concentration of carbaryl that reduced the maximum signal in a competitive ELISA to 50% (I_{50}). Three weeks after the fourth injection, mice were boosted intraperitoneally with 200 μg of immunogen in 200 μL of PBS.

Cell Fusion. Three to four days after the booster injection, mice were sacrificed and spleen cells were fused with the murine plasmacitoma cell line P3-X63-Ag8.653 (Kearney et al., 1979) using PEG-1500 according to a modification of the original Köhler and Milstein method (Nowinski et al., 1979). After fusions, cells were resuspended in DMEM supplemented with 20% fetal calf serum, L-glutamine (4 mM), nonessential amino acids (1 mM), and 2-mercaptoethanol (0.05 mM) (referred to as complete DMEM) and dispensed into sterile 96-well microtiter culture plates at a cellular density of 4×10^5 cells/well. Plates were incubated at 37 °C in an atmosphere of 5% CO_2 in air. Twenty-four hours after fusion, 150 μL of HAT medium (complete DMEM supplemented with 0.1 mM hypoxanthine, 40 μM aminopterin, and 16 μM thymidine) was added to the wells to obtain the selective growth of the hybrid cells. Cell culture plates were resupplied with HAT medium at 3-day intervals by replacing 100 μL/well of supernatant with fresh medium.

Culture Supernatant Screening. Twelve days after fusion, cell culture supernatants were tested for the presence of anti-carbaryl antibodies by a simultaneous noncompetitive/competitive ELISA. The procedure was as follows: PBS (50 μL/well, odd rows) or 2 μM carbaryl in PBS (50 μL/well, even rows) was added to ELISA plates coated with the OVA–CNH conjugate as described above. Then, 50 μL of each culture well supernatant was added to adjacent wells with a multichannel pipet, so the immunoreaction was noncompetitive in odd rows and competitive in even rows. After 2 h at 37 °C, plates were washed four times and 100 μL/well of a 1/2000 dilution of peroxidase-labeled rabbit anti-mouse immunoglobulins in PBST was added. Plates were incubated for 30 min at 37 °C and washed four times, and the enzymic activity was determined as described above. 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.

Cell Cloning. Only wells giving a strong positive response in the noncompetitive ELISA (absorbance ≥1.0) and showing a signal reduction of 80% or more in the competitive format were considered for further work. These most promising cell lines were cloned by limiting dilution to ensure monoclonality.

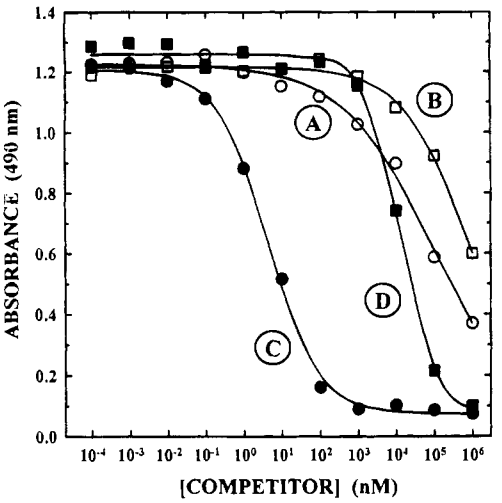


Figure 2. Inhibition curves of LIB/CNH-3.6 MAb and CNH-3 fusion serum by carbaryl and 1-naphthol, using an indirect homologous antigen-coated ELISA: (A) inhibition of fusion serum by carbaryl; (B) inhibition of fusion serum by 1-naphthol; (C) inhibition of MAb by carbaryl; (D) inhibition of MAb by 1-naphthol. All curves were performed with OVO–CNH (1 μg/mL) as coating conjugate. For curves A and B, mouse serum was diluted 1/200000. For curves C and D, MAb was used at 0.03 μg/mL. Each point represents the average of three wells in the same ELISA plate.

Once unique producing clones were identified, they were expanded and finally stored in liquid nitrogen.

Monoclonal Antibody Purification. MAbs were purified from cell culture supernantants and/or ascites fluid. Immunoglobulins were precipitated with ammonium sulfate up to 50% saturation, redisolved in PBS, dialyzed, and chromatographied on DEAE-Sepharose. Most culture supernatants were able to provide enough MAb (3–10 mg/100 mL) for characterization studies and further work, making it unnecessary to grow hybridomas as ascites tumors in mice. Purified MAbs were stored a 4 °C as ammonium sulfate precipitates. In these conditions immunoglobulins are stable for several years (Montoya and Castell, 1987). Working dilutions of MAbs were prepared directly from the ammonium sulfate suspensions.

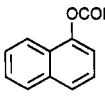
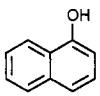
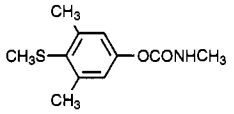
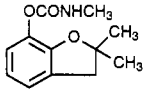
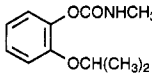
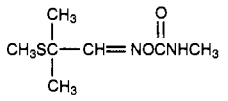
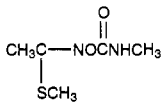
Determination of Cross-Reactivities. A number of compounds were tested for cross-reactivity by performing competitive assays and determining their respective I_{50} values. For this purpose, the inhibition curves were analyzed by mathematically fitting experimental points to a four-parameter logistic equation using a commercial software package (Sigmaplot, Jandel Scientific). Cross-reactivity was calculated as (I_{50} carbaryl/I_{50} compound) × 100.

RESULTS

Characterization of Mice Sera. Apart from bleedings during the immunization schedule, mice sera were collected immediately before cell fusion. These sera, referred to as fusion sera, were characterized by determining their titer and affinity to carbaryl and 1-naphthol. The titers of the fusion sera CNH-3 and CNH-4 were 1/90000 and 1/300000, respectively, and these antisera dilutions were subsequently used in the competitive assays, in which both sera behaved almost identically. Figure 2 shows the inhibition curves for carbaryl and 1-naphthol (curves A and B, respectively) obtained by using the CNH-3 fusion serum. I_{50} for carbaryl was about 30 μM, whereas for 1-naphthol it was about 250 μM. From these data, the calculated cross-reactivity for 1-naphthol was 12%.

Cell Fusion Results. Eleven days after fusion, wells were visually inspected for hybridoma growth, and colonies were observed in 653 of 768 wells (85% fusion

Table 1. Cross-Reactivity of 1-Naphthol and Various *N*-Methylcarbamate Compounds with LIB/CNH-3.6 MAb

compd name	chemical structure	I_{50} (nM)	cross-reactivity (%)
carbaryl		3.6	100
1-naphthol		15300	0.02
methiocarb		12000	0.03
carbofuran		$>10^6$	$<3.6 \times 10^{-4}$
propoxur		$>10^6$	$<3.6 \times 10^{-4}$
aldicarb		$>10^7$	$<3.6 \times 10^{-5}$
methomyl		$>10^7$	$<3.6 \times 10^{-5}$

efficiency, two cell fusion experiments). The screening procedure of culture supernatants consisted of noncompetitive and competitive ELISAs performed simultaneously in the same plate and allowed us to select only those clones with a high affinity to the analyte in a simple, fast, and visual way. The sera I_{50} for carbaryl were taken into account for the competitive part of the screening, where a concentration of carbaryl clearly below sera I_{50} was assayed (1 μM). If only the noncompetitive part was evaluated, 106 wells were positives; i.e., they secreted MAbs that recognized the coating antigen (absorbance $\geq$1.0). However, not all of them were able to strongly recognize 1 μM carbaryl in solution. Among all the positive wells in the noncompetitive part, 25 also showed a signal reduction of 80% or more in the competitive part. Therefore, only these wells were considered for further cloning, expansion, and monoclonal antibody production.

MAb Characterization. The characterization data of the presumably best MAb (LIB/CNH-3.6), as judged by the screening procedure, are presented. Culture supernatants of the remaining 24 MAbs were also collected and stored as ammonium sulfate precipitates for future work. LIB/CNH-3.6 MAb was characterized by competitive homologous antigen-coated ELISA, and a characteristic inhibition curve for carbaryl is shown in Figure 2C. Depending on the assay conditions, LIB/CNH-3.6 exhibited an I_{50} of 3–5 nM, with a maximum absorbance equal to or higher than 1.0 in the absence of analyte. I_{50} values and cross-reactivities for a number of carbamate pesticides and 1-naphthol are shown in Table 1; cross-reactivities are expressed as a percentage of the I_{50} of carbaryl. None of the tested compounds were significantly recognized by the MAb, the highest cross-reactivities being 0.02 and 0.03% for 1-naphthol and methiocarb, respectively. Although other pesticides and carbaryl metabolites should be assayed, these results suggest that both the naphthalene aromatic system and the *N*-methylcarbamate group strongly participate in the MAb interaction, since both must be present for effective antibody binding and recognition.

From the inhibition ability shown by culture supernatants in the simultaneous noncompetitive/competitive screening, we cannot rule out the possibility that some of the remaining 24 MAbs could equal or improve the behavior of LIB/CNH-3.6. In fact, preliminary results suggest that two of these MAbs are able to recognize soluble carbaryl to a similar extent as LIB/CNH-3.6.

GENERAL DISCUSSION

It is generally accepted that a suitable hapten for immunization should preserve, as far as possible, the main chemical groups and structure of the target compound. With this idea in mind, the chemical strategy used to prepare the hapten consisted of the synthesis of a carbaryl-like molecule with a spacer arm attached to the methyl group of the carbamate moiety. This carbaryl derivative, which in fact preserves the carbamate function characteristic of *N*-methylcarbamate pesticides, was useful for protein conjugation, mice immunization, and MAb production. The first step in the hapten preparation was the synthesis of a chloroformate derivative by reaction of 1-naphthol with phosgene. This phosgene-based reaction, which takes advantage of one of the industrial processes applied in carbaryl production, is also used to manufacture most *N*-methylcarbamate pesticides from their corresponding hydroxyl-bearing precursors. Therefore, we consider that this hapten synthesis strategy offers the additional advantage of being a general procedure to obtain functionalized derivatives for other *N*-methylcarbamates.

The antibodies presented here constitute, to our knowledge, the first MAbs reported for a *N*-methylcarbamate pesticide. Both affinity and specificity data of the LIB/CNH-3.6 MAb greatly contrast with the same parameters found for fusion sera. As shown in Figure 1 (curves A and C), I_{50} for carbaryl was decreased 4 orders of magnitude by using the MAb, as compared with its corresponding fusion serum. Likewise, the cross-reactivity for 1-naphthol was lowered from 12% with the serum (curves A and B) to 0.03% with the MAb (curves C and D), which should reasonably allow the MAb to be used for carbaryl determination without significant interferences from 1-naphthol. Comparisons between the characteristics of MAbs and those of rabbit polyclonal sera have been occasionally reported (Deschamps et al., 1990; McAdam et al., 1992; Huang and Chu, 1993), with variable results. In the few cases in which MAbs have been compared with the serum from the mice used to derive the MAbs (Karu et al., 1991), an improvement in the I_{50} for the analyte of 1–2 orders of magnitude has been found. Consistent with this finding, our experience indicates that improvements of 2–3 orders of magnitude can be achived in most cases simply by including a competitive assay in the screening procedure. Otherwise, the risk of choosing MAbs with high affinity for the inmobilized conjugate but low affinity for the free analyte is a real possibility. Furthermore, we are convinced that the testing of supernatants by competitive ELISA is also advisable during cloning steps.

The features displayed by the LIB/CNH-3.6 MAb make it a very promising immunoreagent for the future development of a quantitative immunoassay for carbaryl. In fact, the pesticide I_{50} found in this work is lower than that described by Marco et al. (1993; 2–5

ng/mL ≡ 10–25 nM) when a heterologous antigen-coated assay based on polyclonal antibodies was used. The influence of factors such as coating and MAb concentrations, temperature, incubation times, pH, and ionic strength on the parameters of the LIB/CNH-3.6 inhibition curve is currently under study to optimize the assay. Furthermore, it is known that ELISA format and/or the use of heterologous haptens can modulate the sensitivity of the assay (Schneider and Hammock, 1992; Marco et al., 1993). In this sense, different haptens are currently being synthesized for coupling to proteins and enzymes, and these eventually obtained conjugates will be used in different ELISA formats with LIB/CNH-3.6 MAb. Further evaluation of the new conjugates as immunogens will also be performed to study the influence of the hapten structure on the affinity of the obtained MAbs.

ABREVIATIONS USED

GC, gas chromatography; HPLC, high-performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; MAb, monoclonal antibody; DMF, *N,N'*-dimethylformamide; BSA, bovine serum albumin; OVA, ovalbumin; OPD, *o*-phenylenediamine; DMEM, Dulbecco's modified Eagle's medium; NMR, nuclear magnetic resonance; EIMS, electron impact mass spectra; UV, ultraviolet; CNH, 6-[[(1-naphthyloxy)carbonyl]-amino]hexanoic acid; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.05% Tween 20; I_{50}, analyte concentration required for 50% inhibition; DEAE, diethylaminoethyl.

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LITERATURE CITED

Abad, A.; Manclús, J. J.; March, C.; Montoya, A. Comparison between a Monoclonal-Based Enzyme-Linked Immunosorbent Assay and Gas Chromatography for the Determination of Nicotine in Cigarette Smoke Condensates. *Anal. Chem.* **1993**, *65*, 3227–3231.

Argauer, R. J.; Shimanuki, H.; Alvarez, C. C. Fluorometric Determination of Carbaryl and 1-Naphthol in Honeybees. *J. Agric. Food Chem.* **1970**, *18*, 688–691.

Barcelo, D. Environmental-Protection-Agency and Other Methods for the Determination of Priority Pesticides and Their Transformation Products in Water. *J. Chromatogr.* **1993**, *643*, 117–143.

Branch, R. A.; Jacqz, E. Subacute Neurotoxicity Following Longterm Exposure to Carbaryl. *Am. J. Med.* **1986**, *80*, 741–745.

Carpenter, F. H.; Gish, D. T. The Application of p-Nitrobenzyl Chloroformate to Peptide Synthesis. *J. Am. Chem. Soc.* **1952**, *74*, 3818–3821.

Casale, G. P.; Vennerstrom, J. L.; Bavari, S.; Wang, T. L. Inhibition of Interleukin-2 Driven Proliferation of Mouse CTLL2 Cells, by Selected Carbamate and Organophosphate Insecticides and Congeners of Carbaryl. *Immunopharmacol. Immunotoxicol.* **1993**, *15*, 199–215.

Chiba, M. A Rapid Colorimetric Method for the Analysis of Carbaryl Spray Deposits on Fruit Tree Foliage. *J. Agric. Food Chem.* **1981**, *29*, 118–121.

Dekok, A.; Hiemstra, M. Optimization, Automation, and Validation of the Solid-Phase Extraction Cleanup and on-Line Liquid Chromatographic Determination of N-Methylcarbamate Pesticides in Fruits and Vegetables. *J. AOAC Int.* **1992**, *75*, 1063–1072.

Dekok, A.; Hiemstra, M.; Brinkman, U. A. T. Low ng/L-Level Determination of 20 N-Methylcarbamate Pesticides and 12 of Their Polar Metabolites in Surface Water via Off-Line Solid-Phase Extraction and High-Performance Liquid Chromatography with Post-Column Reaction and Fluorescence Detection. *J. Chromatogr.* **1992**, *623*, 265–276.

Deschamps, R. J. A.; Hall, J. C.; McDermott, M. R. Polyclonal and Monoclonal Enzyme Immunoassays for Picloram Detection in Water, Soil, Plants, and Urine. *J. Agric. Food Chem.* **1990**, *38*, 1881–1885.

Farage-Elawar, M.; Blaker, W. D. Chick Embryo Exposure to Carbamates Alters Neurochemical Parameters and Behavior. *J. Appl. Toxicol.* **1992**, *12*, 421–426.

Farber, H.; Scholer, H. F. Gas Chromatographic Determination of Carbamate Pesticides After Flash-Heater Methylation with Trimethylsulfonium Hydroxide. *J. Agric. Food Chem.* **1993**, *41*, 217–220.

Feng, P. C. C.; Wratten, S. J.; Horton, S. R.; Sharp, C. R.; Logusch, E. W. Development of an Enzyme-Linked Immunosorbent Assay for Alachlor and Its Application to the Analysis of Environmental Water Samples. *J. Agric. Food Chem.* **1990**, *38*, 159–163.

Gallignani, M.; Garrigues, S.; Martínez-Vado, A.; Delaguardia, M. Determination of Carbaryl in Pesticide Formulations by Fourier Transform Infrared Spectrometry with Flow Analysis. *Analyst* **1993**, *118*, 1043–1048.

Hodgson, E. Pesticides—Past, Present and Future. *Rev. Pestic. Toxicol.* **1991**, *1*, 3–12.

Huang, X.; Chu, F. S. Production and Characterization of Monoclonal and Polyclonal Antibodies Against the Mycotoxin Cyclopiazonic Acid. *J. Agric. Food Chem.* **1993**, *41*, 329–333.

Karu, A. E.; Harrison, R. O.; Schmidt, D. J.; Clarkson, C. E.; Grassman, J.; Goodrow, M. H.; Lucas, A.; Hammock, B. D.; Van Emon, J. M.; White, R. J. Monoclonal Immunoassay of Triazine Herbicides—Development and Implementation. In *Immunoassays for Trace Chemical Analysis*; Vanderlaan, M., Stanker, L. H., Watkins, B. E., Roberts, D. W., Eds.; ACS Symposium Series 451; American Chemical Society: Washington, DC, 1990; pp 59–77.

Kearney, J. F.; Radbruch, A.; Liesegang, B.; Rajewsky, K. New Mouse Myeloma Cell Line that has Lost Immunoglobulin Expression but Permits the Construction of Antibody Secreting Hybrid Cell Lines. *J. Immunol.* **1979**, *123*, 1548–1551.

Kendrick, P. N.; Trim, A. J.; Atwal, J. K.; Brown, P. M. Direct Gas Chromatographic Determination of Carbaryl Residues in Honeybees (Apis-Mellifera L) Using a Nitrogen-Phosphorus Detector with Confirmation by Formation of a Chemical Derivative. *Bull. Environ. Contam. Toxicol.* **1991**, *46*, 654–661.

Krause, R. T.; Liquid Chromatographic Determination of N-Methylcarbamate Insecticides and Metabolites in Crops. I. Collaborative Study. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 726–733.

Kumaran, S.; Tranminh, C. Determination of Organophosphorous and Carbamate Insecticides by Flow Injection Analysis. *Anal. Biochem.* **1992**, *200*, 187–194.

Lambrech, J. A. Naphthyl Esters of N-Alkyl-Substituted Carbamic Acids and Insecticidal Compositions Therefrom (to Union Carbide Corp.). Br. Pat. 841,141; *Chem. Abstr.* **1961**, *55*, 2591b.

Langone, J. J.; Van Vunakis, H. Radioimmunoassay of Nicotine, Cotinine, and γ-(3-pyridyl)-γ-oxo-N-Methylbutyramide. *Methods Enzymol.* **1982**, *84*, 628–640.

Marco, M. P.; Gee, S. J.; Cheng, H. M.; Liang, Z. Y.; Hammock, B. D. Development of an Enzyme-Linked Immunosorbent Assay for Carbaryl. *J. Agric. Food Chem.* **1993**, *41*, 423–430.

McAdam, D. P.; Hill, A. S.; Beasley, H. L.; Skerritt, J. H. Monoclonal and Polyclonal Antibodies to the Organophosphate Fenitrothion. 1. Approaches to Hapten-Protein Conjugation. *J. Agric. Food Chem.* **1992**, *40*, 1466–1470.

McGarvey, B. D. High-Performance Liquid Chromatographic Methods for the Determination of N-Methylcarbamate Pesticides in Water, Soil, Plants and Air. *J. Chromatogr.* **1993**, *642*, 89–105.

Montoya, A.; Castell, J. V. Long-Term Storage of Peroxidase-Labelled Immunoglobulins for Use in Enzyme Immunoassay. *J. Immunol. Methods* **1987**, *99*, 13–20.

Nowinski, R. C.; Lostrom, M. E.; Tam, M. R.; Stone, M. R.; Burnette, W. N. The Isolation of Hybrid Cell Lynes Producing Monoclonal Antibodies against the p15(E) Protein of Ecotropic Murine Leukemia Viruses. *Virology* **1979**, *93*, 111–126.

Rajskowsky, K. M.; Cittanova, N.; Desfosses, B.; Jayle, M. F. The Conjugation of Testosterone with Horseradish Peroxidase and a Sensitive Enzyme Assay for the Conjugate. *Steroids* **1977**, *29*, 701–713.

Schattenberg, H. J.; Hsu, J. P. Pesticide Residue Survey of Produce from 1989 to 1991. *J. AOAC Int.* **1992**, *75*, 925–933.

Schneider, P.; Hammock, B. D. Influence of the ELISA Format and the Hapten Enzyme Conjugate on the Sensitivity of an Immunoassay for s-Triazine Herbicides Using Monoclonal Antibodies. *J. Agric. Food Chem.* **1992**, *40*, 525–530.

Seiber, J. N. N-Perfluoroacyl Derivatives for Methylcarbamate Analysis by Gas Chromatography. *J. Agric. Food Chem.* **1972**, *20*, 443–446.

Sherry, J. P. Environmental Chemistry: The Immunoassay Option. *Crit. Rev. Anal. Chem.* **1992**, *23*, 217–300.

Simon, V. A.; Pearson, K. S.; Taylor, A. Determination of N-Methylcarbamates and N-Methylcarbamoyloximes in Water by High-Performance Liquid Chromatography with the Use of Fluorescence Detection and a Single o-Phthalaldehyde Post-Column Reaction. *J. Chromatogr.* **1993**, *643*, 317–320.

Strait, J. R.; Thornwall, G. C.; Ehrich, M. Sensitive High-Performance Liquid Chromatography Analysis for Toxicological Studies with Carbaryl. *J. Agric. Food Chem.* **1991**, *39*, 710–713.

Thurman, E. M.; Meyer, M.; Pomes, M.; Perry, C. A.; Schwab, A. P. Enzyme-Linked Immunosorbent Assay Compared with Gas Chromatography Mass Spectrometry for the Determination of Triazine Herbicides in Water. *Anal. Chem.* **1990**, *62*, 2043–2048.

Van Emon, J. M.; López-Avila, V. Immunochemical Methods For Environmental Analysis. *Anal. Chem.* **1992**, *64*, A79–A88.

Westlake, G. E.; Hardy, A. R.; Stevenson, J. H. Effects on Storage and Pesticide Treatments on Honey Bee Brain Acetylcholinesterase Activities. *Bull. Environ. Contam. Toxicol.* **1985**, *34*, 668–675.

Wolf, G.; Seligman, A. M. Synthesis of Naphthyl Carbonate Derivatives of Amino Acids as Chromogenic Substrates for Carboxypeptidase. *J. Am. Chem. Soc.* **1951**, *73*, 2080–2082.

Worthing, C. R.; Hance, R. J. In *The Pesticide Manual: A World Compendium*, 7th ed.; BCPC Publications: Berks, U.K., 1983.

Yess, N. J.; Gunderson, E. L.; Roy, R. R. United-States-Food-and-Drug-Administration Monitoring of Pesticide Residues in Infant Foods and Adult Foods Eaten by Infants Children. *J. AOAC Int.* **1993**, *76*, 492–507.

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