Monoclonal ELISA for 2,4-Dichlorophenoxyacetic Acid: Characterization of Antibodies and Assay Optimization

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Twelve monoclonal antibodies (MAbs) against the widely used herbicide 2,4-D were produced by hybridomas from two fusions of murine myeloma cells and spleen cells isolated from BALB/c mice immunized with hapten conjugated via the carboxyl group to thyroglobulin. To evaluate the sensitivity and selectivity of MAbs, competitive indirect ELISA was used. MAb E2/G2 exhibited the highest sensitivity toward 2,4-D (IC_{50} = 0.8 ng/mL) and a favorable selectivity toward 18 structurally related substances. Besides the expected high cross-reactivity with methyl ester 2,4-D (104.8%), cross-reactivity with MCPA (13.8%) and with 2,4,5-T (9.5%) was found. Cross-reactivity with other structural analogs did not exceed 2.7%. Optimization studies showed that in competitive ELISAs for 2,4-D coating conjugates with hapten densities of 2.3 and 3.3 mol of 2,4-D/mol of BSA were more sensitive than conjugates with hapten densities of 15.9 and 26.5 mol of 2,4-D/mol of BSA. The best dose–response curves presented in this study were almost linear in the concentration range 0.2–10 ng/mL.

2,4-Dichlorophenoxyacetic acid (2,4-D) is a broadly used herbicide for controlling weeds which could potentially contaminate groundwater and the drinking water supply. The allowable limits for pesticide residues in drinking water are becoming lower and lower. In European Community (EC) countries, the maximum admissible concentration is 0.1 ng/mL of any one substance and 0.5 ng/mL for a sum of pesticides, including metabolites (Wittmann and Hock, 1991).

Conventional methods of quantitation of polar phenoxyalkanoic acids include liquid–liquid extraction, acid–basic partitioning, chemical derivatization, and purification. This procedure is time-consuming, it involves toxic solvents and reagents, and the results are often inconsistent (Loconto, 1991). Therefore, quick, easy, and economical methods are needed which can screen a large number of samples. Thus, immunochemical methods represent an effective alternative to instrumental methods. The development of specific and sensitive antibodies has provided the reagents for the analytical tests needed. In the past, immunoassays for detecting 2,4-D using polyclonal antisera were described by different authors (Rinder and Fleeker, 1981; Knopp et al., 1985; Fleeker, 1987; Hall et al., 1989; Newsome and Collins, 1989). These antisera made the detection of 2,4-D possible in different concentration ranges of parts per trillion and parts per billion.

The present paper focuses on the preparation of monoclonal antibodies, because immunochemical analysis is limited not only by the quality of the antibody but also by its availability in large quantities. The experiments performed describe the properties of the monoclonal antibodies obtained and used in a competitive indirect ELISA. These monoclonal preparations may be used for the development of other assay formats, such as immunobiosensors.

MATERIALS AND METHODS

Reagents. Chemicals and solvents, unless stated otherwise, were purchased from Lachema (Brno, Czech Republic). Bovine serum albumin (BSA) and complete Freund's adjuvant were obtained from Difco Laboratories (Detroit, MI). Pig thyroglobulin (TG), dimethyl sulfoxide (DMSO), and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Serva (Heidelberg, Germany), and the Sephadex G-25 was from Pharmacia Fine Chemicals (Uppsala, Sweden). Additionally, the following chemicals were used: tri-*n*-butylamine and isobutyl chloroformate (Fluka, Buchs, Switzerland) and Tween 80 (Sigma, St. Louis, MO). Swine immunoglobulins against murine immunoglobulins labeled with peroxidase (SwAM-POD) were obtained from Dr. Rodák from this Institute. Lactalbumin hydrolysate tablets (LAH) were from Bioveta (Ivanovice, Czech Republic).

Buffers and Solutions. Assay buffer, pH 8.5, contained 4.3 g of Tris and 5.84 g of NaCl in 1 L of distilled water; the resulting pH was adjusted with concentrated HCl. Phosphate-buffered saline (PBS), pH 7.2, for washing the plates and diluting the antibody contained 85 g of NaCl, 2.67 g of $Na_2HPO_4 \cdot 12H_2O$, and 0.34 g of NaH_2PO_4 with the addition of 1 mL of Tween 80 in 1 L of distilled water. The coating buffer, pH 9.6, contained 2.15 g of Tris, 0.714 mL of HCl, and 2.922 g of NaCl in 1 L of distilled water; the resulting pH was adjusted by means of 1 M NaOH. The solution LAH for preparing the detection antibody SwAM-POD was prepared by diluting 1 tablet in 50 mL of distilled water with the addition of 50 μL of Tween 80. The substrate buffer for the peroxidase (POD) was 0.1 M sodium acetate (NaAc); pH was adjusted to pH 5.5 by adding 1 M citric acid. At 37 °C substrate for POD, 100 μL of TMB (10 mg of TMB was dissolved in 1 mL of DMSO) and 10 μL of 6% H_2O_2 were added to 10 mL of substrate. The stopping solution was 1 M H_2SO_4.

Standards. Stock solutions were prepared from the weighed amount of the standard after dilution in assay buffer (0.1 mg/mL). For the calibration series and for testing cross-reactions, series of concentrations were prepared in the range 0.1-10000 ng/mL, using the following substances: 2,4-D; 2,4-D methyl ester; 2-methyl-4-chlorophenoxyacetic acid (MCPA); 2,4,5-trichlorophenoxyacetic acid (2,4,5-T); 2-chlorophenoxyacetic acid; 2-methylphenoxyacetic acid; 2-methyl-4,6-dichlorophenoxyacetic acid; 2-methyl-6-chlorophenoxyacetic acid; 4-chlorophenoxyacetic acid; 2,3-dichlorophenoxyacetic acid; 3,4-dichlorophenoxyacetic acid; 2-(2,4-dichlorophenoxy)propionic acid (2,4-DP); 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB); 2-(2-methyl-4-chlorophenoxy)propionic acid (MCPP); 4-(2-methyl-4-chlorophenoxy)butyric acid (MCPB); 2,4-dichlorophenol; 2-methyl-4-chlorophenol; 3,4-dimethylphenol; and pentachlorophenol. These standards were obtained as a present from Dr. Matoušek, ÚKZÚZ (Brno, Czech Republic), and from Dr. Eremin, Lomonosov University (Moscow, Russia).

Equipment. The measurement of absorbance on microtiter plates were carried out by means of the photometer Labsystem Multiscan MCC (Helsinki, Finland). Polystyrene microtiter plates type P, sterile, were used (Gama, České Budějovice, Czech Republic).

Hapten–Protein Conjugates. The method of mixed anhy-

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drides were used for the condensation of the carboxyl group of 2,4-D with amino groups of the carrier protein (Dean et al., 1971). 2,4-D (100 mg) was dissolved in 200 μL of tri-*n*-butylamine and 6.8 mL of dioxane. The mixture was cooled in an ice bath to 10 °C, and 50 μL of isobutyl chloroformate was then added with constant stirring. The mixture was stirred for 30 min at 10 °C. The activated mixture in volumes of 0.18, 0.7, and 3.5 mL was added to reaction vessels equilibrated to 10 °C. To each mixture were added 1.7 mL of protein solution (0.78 g of protein lyophilysate was dissolved in 20.55 mL of water), 0.78 mL of 1 M NaOH, and 13.8 mL of dioxane while stirring. The reaction solutions were kept stirring in the refrigerator at 4 °C and dialyzed twice for 24 h against 10 L of deionized water. After concentration, the dialyzed solutions were applied to the Sephadex G-25 column, using 0.05 M NH_4HCO_3 as an eluent. The purified conjugates were lyophilized.

Hapten Densities in Conjugates. The number of 2,4-D groups in 2,4-D–BSA conjugates was determined spectrophotometrically from the absorbance increment of the conjugate at λ_{max} = 278 nm using the relations $\epsilon_c - \epsilon_p = \epsilon$ and $\epsilon/\epsilon_h = H$, where ϵ_c, ϵ_p, and ϵ_h are molar absorption coefficients of the conjugate, the carrier, and the herbicide, respectively, and H is the herbicide density in the conjugate (moles of hapten per mole of protein).

Immunization. Eight female BALB/c mice, aged 6–8 weeks, were injected intraperitoneally at intervals of 2–3 weeks with 100 μg of 2,4-D–TG in 0.1 mL of PBS emulsion in complete Freund's adjuvant. One week after the fourth immunization, two mice were given a booster injection without adjuvant in the tail artery. The presence of antibodies in the antiserum was determined by means of ELISA, using 2,4-D–BSA conjugate as a coating agent.

Production of Monoclonal Antibodies. Spleen cells from the immunized mice (7.5×10^7) and myeloma cells ($Sp^2/0$, 2.2×10^7) wre mixed at the ratio of 1:3.55 and fused in 50% PEG 1000. The cells were selected after fusion with HAT (100 μmol/L hypoxanthine/0.4 μmol/L aminopterin/16 μmol/L thymidine) DMEM medium with 15% FCS in microtiter polystyrene plates. After 8 days, the hybridoma cells in ELISA positive wells were cloned by means of the method of limiting dilution. The clones grew as ascites tumors in BALB/c mice which were injected with Pristan 10 days before inoculation. In secreted antibodies, the immunoglobulin class and subclass were determined on a nitrocellulose membrane using antibodies against murine immunoglobulins (ICN Immunobiologicals, Irvine, CA).

Indirect Competitive ELISA. 2,4-D–protein conjugate (150 μL) in the coating buffer, pH 9.6, was added to each well of the microtiter plate. The plates were incubated overnight at 37 °C. After the solution was removed, each well was washed four times with 0.2 mL of PBS containing 0.1% Tween 80. One hundred microliters of standard in the assay buffer and 50 μL of antibody diluted in PBS/Tween were added into the wells. After 1 h of incubation at 4 °C, the unbound components were removed by washing (four times) with the PBS/Tween solution; 150 μL of SwAM-POD was added to each well, and the plates were incubated for 1 h at 4 °C. After the solution was removed, each well was washed four times with the washing solution; 100 μL of TMB/H_2O_2 was added to the wells. The enzymatic reaction was stopped by adding 100 μL of 1 M H_2SO_4. The intensity of the resulting yellow color was measured spectrophotometrically at 450 nm.

RESULTS

Immunogens and Coating Conjugates. The carboxyl group of the 2,4-D molecule was utilized for conjugation with proteins. Different molar ratios of the reaction components were used in the method of mixed anhydrides to achieve different degrees of hapten incorporation. The coupling was evaluated from the absorbance peak of the herbicide–BSA conjugates at 278 nm, because λ_{max} for 2,4-D used for the coupling corresponded to that wavelength. The molar ratio herbicide/protein 2.9, 11.9, 59.9, and 63.8 in the reaction mixture resulted in the formation of conjugates with incorporation densities of 2.3, 3.3, 15.9, and 26.5 2,4-D molecule, respectively, per BSA molecule.

Table 1. Characteristics of Monoclonal Antibodies

fusion	clone marking	ascites fluid dilution[a]	IC_{50}[b] (ng/mL)
I	G10[c]	1:4000	43.7
I	G10/G2/E9	1:10000	110.7
I	G10/B3/D4	1:1000	910.6
II	E2[c]	1:1000	8.2
II	E2/B5	1:10000	8.2
II	E2/G2	1:10000	2.0
II	B7	1:1000	73.0
II	F6/C10	1:20000	26.3
II	F5/E5	1:5000	287.7
II	G5[c]	1:1000	32.6
II	G5/E10	1:5000	36.1
II	G5/F7	1:1000	286.5
II	B5/C3	1:5000	43.1
II	F10	1:1000	182.9
II	C6	1:1000	2413.0

[a] Concentration of coating conjugate 2,4-D–BSA in ELISA was in all cases 50 ng/mL. [b] IC_{50} represents the concentration of 2,4-D lowering the absorbance to 50% compared to zero. [c] The starting colony of cells was further cloned.

Characterization of MAbs. One week after the fusion, the growing colonies were tested for the reaction in an ELISA. Hybridomas secreting antibodies originated from two independent fusions (Table 1). The cells were cloned by the method of limiting dilution. Antibodies were given codes according to the origin of the hybridoma. The first symbol designates the starting cell colony; the second and third symbols denote clones obtained after the first or the second cloning. Secreted antibodies were characterized in the culture medium or in the ascites fluid. All secreted antibodies being tested proved to be IgG with κ light chains. MAbs secreted by clones B5/C3, E2/B5, and F6/C10 were of the IgG1 class. The results of the cross-reactions between 2,4-D and 18 related compounds are summarized in Table 2. Chemical differences with the basic structure of 2,4-D are indicated by bold symbols. Different dilutions of ascites fluid were used to achieve an absorbance in the ELISA of 1.0–1.5 at zero concentration of 2,4-D. The sensitivity of antibodies in the ELISA was evaluated by means of IC_{50} values. As shown in Table 1, there are great differences in sensitivity among the individual antibodies. MAb E2/G2 exhibited the highest sensitivity among the individual antibodies. MAb E2/G2 exhibited the highest sensitivity of all antibodies (IC_{50} = 2.0 ng/mL at dilution 1:10000) and a high degree of selectivity toward the homologous hapten. Besides the expected high cross-reactivity with 2,4-D methyl ester (104.8%), a cross-reactivity of 13.8% was found with MCPA and of 9.5% with 2,4,5-T (Table 2). The immunoassay thus distinguishes between 2,4-D and MCPA, which can, under certain circumstances, appear together in groundwater. From Table 2 it is evident that the cross-reactions between 2,4-D and 15 other tested compounds did not exceed 2.7%. The subclone E2/B5 produced an antibody exhibiting a relatively high sensitivity (IC_{50} = 8.2 ng/mL) and having a specificity comparable with that of E2/G2, with the difference that the cross-reactivity was higher with MCPA (29.4%) but lower with 2,4,5-T (2.0%). The third most sensitive antibody, E2, exhibited a somewhat higher cross-reactivity toward 2,4-dichlorophenol (6.3%). Compared with the aforementioned antibodies, MAb F6/C10 displayed lower sensitivity (IC_{50} = 26.3 ng/mL) and somewhat different specificity. In this case a higher cross-reactivity was found with 2,4-DB (11.5%), which has a longer alkanoic moiety (structure 13, Table 2), and with 2,4-dichlorophenol (7%), where the side chain is missing altogether. The cross-reactivities with other components were below 5%. The specificities of the other antibodies are shown in detail in the remaining part of Table 2. Interestingly enough,

antibody G5 and MAb G10/G2/E9 had a relatively high cross-reactivity between 2,4-D and 2,4-DB. High cross-reactivity was exhibited by antibodies G5/E10 and G10 with 2,4-dichlorophenol.

ELISA Optimization. The dependence of the IC_{50} values obtained from the dose–response curves on the degree of hapten incorporation in the conjugates used for plate coating was confirmed. The curves were determined according to the standard protocol for different conjugate concentrations used in the coating and for different antibody concentrations in the reaction mixture.

The effect of 2,4-D concentration in the coating conjugates on the displacement curves for medium sensitive MAb B7 is illustrated in Figure 1. The coating of microtiter plates was carried out at a conjugate concentration of 200 ng/mL; the antibody dilution was 1:1000. The IC_{50} values using the slightly substituted conjugates 2,4-D–BSA I (IC_{50} = 14.4 ng/mL) and 2,4-D–BSA II (IC_{50} = 4.8 ng/mL) were about 1 order of magnitude lower than when using conjugates with a high degree of substitution, i.e., 2,4-D–BSA III (IC_{50} = 173.7 ng/mL) and 2,4-D–BSA IV (IC_{50} = 69.2 ng/mL). The comparison of the curve sensitivity in Figure 1 is optimal from the point of view of maximum sensitivity, because the values of IC_{50} correspond to different absorbance values at zero concentration of 2,4-D. Therefore, a subsequent experiment was performed so that the binding without 2,4-D in the reaction mixture, at calibration zero, was approximately the same for all tested conjugates. This can be achieved for the given concentration of antibody and at different hapten densities by changing the coating concentration of the conjugate. In our case the concentration of the conjugates used for coating was in the range 33–800 ng/mL. From the results presented in Figure 2, it is evident that the displacement curves displayed a trend in sensitivity similar to those in Figure 1, but differences in sensitivity using differently substituted conjugates were not so apparent. The sensitivity of ELISAs in which low hapten density conjugates were used was about 5 times greater than that of ELISAs employing high-density conjugates (Figure 2).

The displacement curves determined for the highly sensitive MAb E2/G2 were similar, in indirect ELISA, to those obtained for MAb B7 using four different hapten densities in the conjugate (Figures 3 and 4). The most sensitive curve was obtained using coating conjugate 2,4-D–BSA I at a concentration of 200 ng/mL and an antibody dilution of 1:10000 (IC_{50} = 0.8 ng/mL) (Figure 3). The determination of IC_{50} values with similar binding at zero resulted in less difference among the displacement curves. IC_{50} values obtained for 2,4-D–BSA I and II were about 5 times lower than IC_{50} values obtained with coating conjugates 2,4-D–BSA III and IV (Figure 4), as in the case of MAb B7 in Figure 2.

DISCUSSION

One of the main problems in hapten ELISA is the preparation of antibodies with the required degree of specificity and sensitivity. The unpredictability of the results of hybridoma technology necessitate several fusions, which increases the costs of the experiments. In our experiments, in two successful fusions, 12 hybridomas secreting sensitive MAbs were obtained, which represents 0.3% of clones screened. Polyclonal as well as monoclonal antibodies formed against the 2,4-D–protein conjugate often have a higher binding affinity for the "linkage structure" than for the free 2,4-D. This phenomenon was noted in the literature as the bridge binding effect, and the structural aspects, particularly those of steroid haptens, have been discussed in detail (Fránek, 1987). Thus, low ELISA sensitivity can be caused not only by the low affinity of the antibody for the homologous ligand but also by the existence of the bridge binding of the immobilized hapten in the competitive system. On the other hand, the high sensitivity of MAb E2/G2 (IC_{50} = 0.8 ng/mL) can be due to a minimal bridge binding effect and a high affinity for the free herbicide. In structural terms, this means a small or negligible interaction of the $CONH(CH_2)_x$ bridge moiety with a binding site of the antibody.

The shape and size of the binding site determine the unique character of monoclonal specificity. The 104.7% of the cross-reactivity of 2,4-D methyl ester when using MAb E2/G2 confirmed the known fact that the antibody is not able to recognize chemical changes at the site of conjugation. Therefore, interpretation of the assay results must take into consideration high cross-reactivity with other 2,4-D esters besides the methyl ester. The degree of cross-reactivity with the derivatives modified at the site of conjugation can, however, differ significantly, even between two different antibodies belonging to the same type of immunogen. Newsome and Collins (1989) found an extremely high cross-reactivity with their antibody between 2,4-D and the methyl ester, 22 times higher than ours. To avoid forming even very small amounts of ester, these workers kept the standards as dilute solutions in methanol/NaOH to prevent this undesirable conversion. Potential assay users should also be aware that most of the phenoxy acid residues in plant tissue are in the form of amide conjugates and hydroxylated glycosides. Even this group of metabolites will exhibit considerable cross-reactivity with the MAbs. To obtain high specificity at the terminal carboxyl, it is necessary to synthesize new haptens connected to protein via the benzene moiety.

In comparison with many condensed steroid skeletons, the side OCH_2COOH chain in the small and rigid conformation 2,4-D is an important antigen subdeterminant. A good characterization of the specificity toward this moiety is desirable, because in conventional agriculture compounds related to the herbicide 2,4-D are often used (Table 2) which differ from 2,4-D only in the structure of the side chain and can thus be the cause of assay interferences. Differences in recognizing this moiety can be demonstrated particularly by MAbs G10/G2/E9, B7, F6/C10, G5, and B5/C3 by means of the cross-reactivity between 2,4-DB, structure **13** in Table 2 with a linear side chain, and 2,4-DP, structure **12**, with a branched side chain. The antibodies' inability to discriminate between the two compounds can be ascribed to a free fit of the binding site around the aliphatic chain rather than to a nonspecific interaction with this part of the structure. This idea is supported by the higher degree of specificity of all the antibodies for the phenoxypropionic structure of 2,4-DP.

From the results presented in Figures 1–4, it is evident that the properties of the antigen conjugate used for coating significantly affected the sensitivity of the displacement curves in the indirect ELISA. The hapten density on BSA was determined by using molar absorption coefficients of 2,4-D and BSA. Although herbicide–BSA conjugates were extensively purified by dialysis and gel filtration before UV measurement, the values obtained indicate probable hapten density, measuring not only covalently linked but also noncovalently bound hapten. Recently, Wengatz et al. (1992) determined hapten densities in various pesticide–protein conjugates by UV spectra and compared them with those based on the MALDI (matrix-assisted UV laser desorption ionization mass spectrometry) method. The values determined by MALDI were considerably lower

Table 2. Cross-Reactivity of 2,4-D and Related Structures with MAbs against 2,4-D–Thyroglobulin

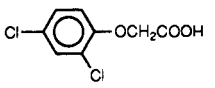
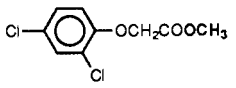
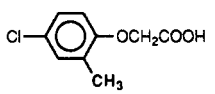
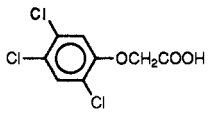
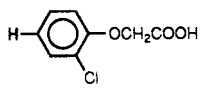
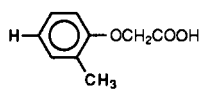
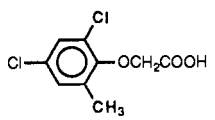
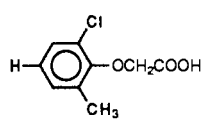
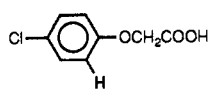
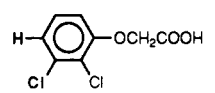
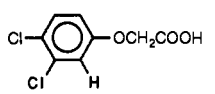
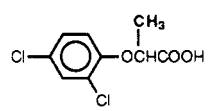
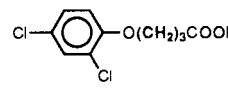
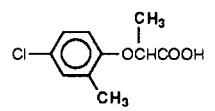
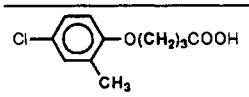
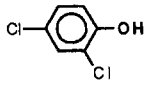
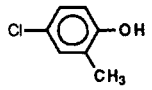
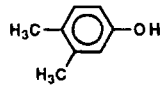
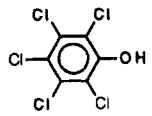
substance	clone											
	G10	G10/G2/E9	E2	E2/B5	E2/G2	B7	F6/C10	F6/E5	G5	G5/E10	B5/C3	F10
 1	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
 2	70.8	100.0	92.5	70.8	104.8	100.0	104.7	107.9	76.6	75.6	160.0	29.2
 3	22.7	13.6	25.7	29.4	13.8	32.6	26.3	14.6	14.7	8.6	47.6	6.2
 4	15.8	<1.0	4.3	2.0	9.5	7.3	12.9	7.4	8.6	7.9	6.3	14.5
 5	1.8	1.7	0.6	0.4	0.9	0.5	2.6	<2.8	1.3	1.2	0.6	<1.8
 6	1.3	<1.0	0.2	<0.2	<0.2	<0.7	<0.2	2.9	0.7	0.7	<0.4	<1.8
 7	1.6	<1.0	0.2	0.2	<0.2	0.7	<0.2	<2.8	0.9	0.6	1.1	<1.8
 8	0.4	2.9	1.0	1.1	<0.2	<0.7	<0.2	<2.8	0.1	0.5	<0.4	<1.8
 9	1.1	2.5	1.0	1.9	0.9	1.3	1.3	<2.8	0.5	4.1	1.0	<1.8
 10	1.0	1.9	0.9	1.2	1.6	1.9	2.3	<2.8	3.4	5.4	2.9	<1.8
 11	2.3	2.9	3.6	2.7	2.7	2.7	5.8	<2.8	4.7	7.3	3.9	<1.8
 12	2.6	1.7	0.1	0.3	0.4	<0.7	4.3	<2.8	2.1	1.6	<0.4	<1.8
 13	2.1	15.8	1.5	2.8	1.5	6.8	11.5	6.3	19.9	2.9	4.6	6.8
 14	<0.4	5.4	<0.1	0.2	<0.2	<0.7	2.6	<2.8	0.5	<0.3	<0.4	<1.8

Table 2 (Continued)

substance	clone											
	G10	G10/G2/E9	E2	E2/B5	E2/G2	B7	F6/C10	F6/E5	G5	G5/E10	B5/C3	F10
 15	0.6	6.8	0.7	0.2	0.9	1.1	2.6	3.9	1.9	1.6	1.0	<1.8
 16	21.5	<1.0	6.3	1.6	1.6	12.8	7.0	<2.8	6.3	11.6	5.3	7.9
 17	<0.4	<1.0	0.1	0.3	0.5	<0.7	0.8	<2.8	<0.3	<0.3	0.5	<1.8
 18	<0.4	<1.0	<0.1	<0.2	<0.2	<0.7	<0.2	<2.8	<0.3	<0.3	<0.4	<1.8
 19	2.9	1.8	0.2	<0.2	0.5	<0.7	3.1	<2.8	1.3	1.0	2.1	<1.8

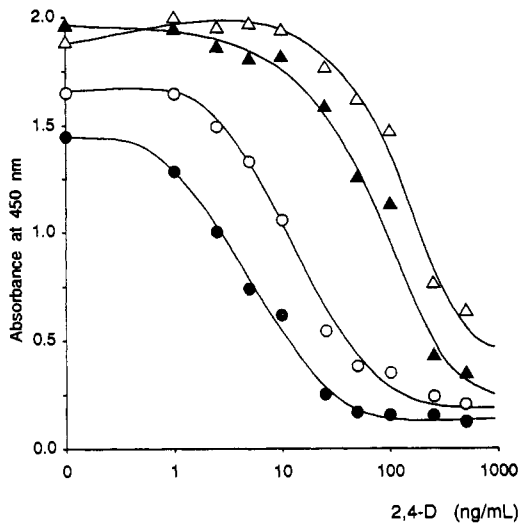


Figure 1. Displacement ELISA curves for 2,4-D using MAb B7 and coating conjugates with hapten densities 2.3 (○), 3.3 (●), 15.9 (△), and 26.5 (▲) mol of 2,4-D/mol of BSA. The coating of wells in microtiter plates was carried out in all tested conjugates at a concentration of 200 ng/mL. The dilution of MAb B7 in the reaction mixture was 1:1000.

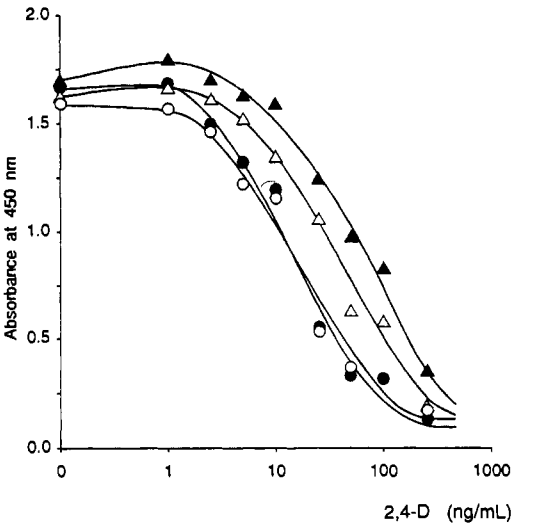


Figure 2. Displacement ELISA curves for 2,4-D using MAb B7 under different coating concentrations in conjugates with hapten densities 2.3 (○), 3.3 (●), 15.9 (△), and 26.5 (▲) mol of 2,4-D/mol of BSA. The coating concentrations of 2,4-D–BSA I (○) and II (●) were 800 ng/mL; that of 2,4-D–BSA III (△) was 33 ng/mL, and that of 2,4-D–BSA IV (▲) was 50 ng/mL. The dilution of MAb B7 in the reaction mixture was 1:1000.

than those obtained by spectrophotometry. According to the authors, this is most probably due to the fact that MALDI detects only covalently bound haptens, while haptens bound by adsorption (noncovalent binding) may also contribute to the UV signal. It may be noted that the data obtained by UV analysis can better reflect antigen–antibody interactions, because desorption of 2,4-D from BSA during assay procedure is not probable. The assay data obtained are in accordance with the findings of van de Water and Haagsma (1990) and Manning (1991), who examined the effect of different degrees of hapten incorporation in conjugates on ELISA sensitivity using monoclonal antibodies and serum. In the studies carried out by Manning, reducing the degree of substitution in the coating antigen resulted in a proportional increase in sensitivity within about 2 orders of magnitude. The highst sensitivity and slope of the curves were attained using 1.1 and 2.4 mol of indole-3-acetic acid/mol of BSA, whereas for the hapten values about 9.9 mol/mol of BSA there was a conspicuous decrease in sensitivity, as well as a cooperative allosteric binding appearance (Manning, 1991). A marked decrease in the sensitivity of the system when using a highly substituted conjugate was ascribed to the nonspecific interaction of MAb with those immobilized haptens which for steric reasons could not participate in the competition for the antibody at the binding site (van de Water and Haagsma, 1990). The size of the antibody macromolecule and its potential binding with part of the conjugate is obviously only one of the relevant factors of the final assay sensitivity. Use of this system allows us to propose an additional view concerning the competition

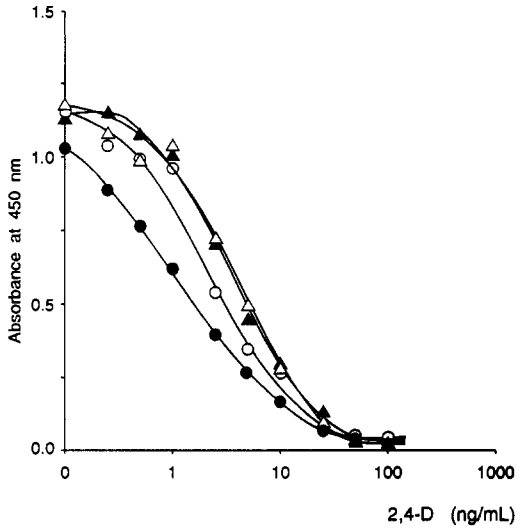


Figure 3. Displacement ELISA curves for 2,4-D using MAb E2/G2 and coating conjugates with hapten densities 2.3 (○), 3.3 (●), 15.9 (△), and 26.5 (▲) mol of 2,4-D/mol of BSA. The coating of wells in microtiter plates was carried out in all tested conjugates at the concentration of 200 ng/mL. The dilution of MAb E2/G2 in the reaction mixture was 1:10000.

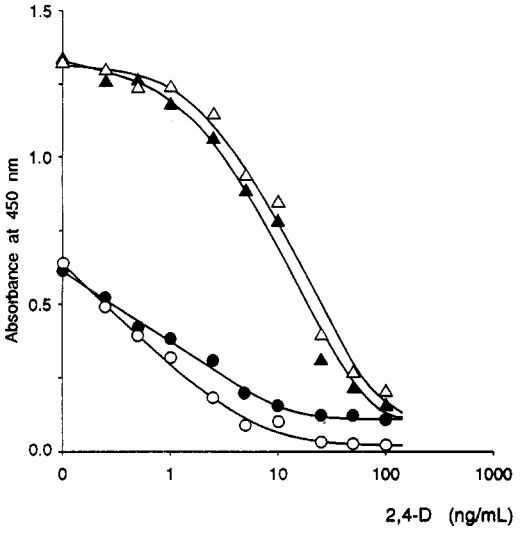


Figure 4. Displacement ELISA curves for 2,4-D using MAb E2/G2 at different concentrations of coating conjugates with hapten densities 2.3 (○), 3.3 (●), 15.9 (△), and 26.5 (▲) mol of 2,4-D/mol of BSA. The concentrations of 2,4-D–BSA I (○) and II (●) were 800 ng/mL and of 2,4-D–BSA III (△) and IV (▲) were 33 ng/mL. The dilution of MAb E2/G2 in the reaction mixture was 1:10000.

mechanism. If a small molar excess of the hapten reagent is used, the hapten binds preferentially to those groups of the protein carrier which are sterically the most accessible. Increasing the molar excess of the hapten results in more heterogeneous substitution, because hapten molecules then bind to the less accessible amino acid groups, including those partially hidden in the protein surface. In this way, it is possible to imagine that, under certain circumstances, accessible haptens, as well as sterically hindered and inaccessible haptens, all enter into the simultaneous competitive interaction with the antibody. It should be kept in mind that the primary and tertiary structures of the protein, including the potential conformation changes after the incorporation of hapten, can also be important factors for the final assay sensitivity. It is evident that predicting interactions and/or the mechanism of competition is very difficult. That is why empirical approaches in combination with immunochemical strategies represent an effective tool in the development and optimization of ELISA and related techniques.

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