Production and Characterization of DDT Antibodies and Its Application to Enzyme Immunoassay: Relation of Response and Affinity to Coating Ligand

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Received June 25, 2003

To development an immunodetection method for DDT, 1,1,1-trichloro-2,2-bis(4-chorophenyl)ethane (*p,p*'-DDT) and its metabolites (*p,p*'-DDA, *p,p*'-DDE, *p,p*'-DDD), five derivatives of DDT haptens have been synthesised and characterized as coating ligands for antibody evaluation. The appropriate lengths of linkers were introduced to investigate a matching pair of coating ligand and antibody. Among these hapten derivatives, 2,2-bis(4-chlorophenyl)acetic acid (DDA), 5,5-bis(4-chlorophenyl)-5-hydroxypentanoic acid (DDHP) and 5,5-bis(4-chlorophenyl)-5-chloropentanoic acid (DDCP) were conjugated with keyhole limpet hemocyanin (KLH) for its use as an immunogen. The bovine serum albumin (BSA) conjugates of these derivatives were prepared as a coating ligand for monoclonal antibody screening. Fifteen monoclonal antibody clones were screened using these probes. 6,6-Bis(4-chlorophenyl)-6-hydroxyhexanoic acid (DDHH) and 3-[6,6-Bis(4-chlorophenyl)-6-hydroxyhexanoylamino]propanoic acid (DDHHAP), in addition to the above hapten derivatives, were conjugated to ovualbumin (OVA) and bovine serum albumin (BSA) for their use as coating ligands to measure the titration level of the antibody and the displacement of free analytes. The indirect competitive ELISA results indicate that the titration level and free analyte displacement were greatly influenced by the DDT derivatives and carrier proteins used. Three matching pairs of monoclonal antibodies and coating ligands were selected for the DDT immunoassay: antibody clone 1A3 and coating ligand DDA-OVA, 1A1 and DDHHAP-BSA, and 1A4 and DDHP-OVA.

Key Words : DDT, Coating ligand, Antibody characterization

Introduction

DDT is an organochlorine pesticide widely used in Africa[1] since the 1940s to control malaria and typhus. DDT is a broadly toxic compound that is highly stable and insoluble in water. It tends to pass with food fats into the body and to accumulate in fat deposits in animal tissue. It slowly degrades in tissue mainly to 2,2-bis(4-chlorophenyl)acetic acid (DDA). Its two major breakdown products in the environment are 1,1-dichloro-2,2-bis(4-chlorophenyl) ethylene (DDE) and 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (DDD).[2-5] Despite the effectiveness in controlling malaria, the use of DDT was totally banned in developed countries in the 1970s because of its harmful effects on the reproductive and the nervous systems.[6-10] DDT and its breakdown products are still found in air, water, and soil samples. Urinary excretion of DDA has been reported as a sensitivity marker of DDT exposure in humans and other mammals. The urinary excretion of DDA for a normal healthy male ranges from 25 to 120 ng/mL with those reported earlier.[11] Air samples in the United States have shown levels of DDT ranging from 0.00001 to 1.56 pg/mL.[12] In the United States in the early 1970s, DDT and DDE were reported in surface waters at levels of 1 pg/mL and in soil, ranging from 0.18 to 5.86 μg/mL.[12,13] The common procedure for the analysis of DDT and its metabolites are GC/MS,[14,15] but the method requires extensive sample preparation and clean-up procedures. Immunoassay is suitable for environmental pollutants analysis due to its sensitivity, small sample volume requirements, cost-effectiveness, and rapidity.

This paper describes the competitive ELISA method for determining the presence of DDT and its metabolites.

Fifteen hybridoma cell lines were obtained by cell fusion technology of myeloma cells and mice spleen cells immunized by conjugating KLH conjugate to three haptens (DDA, DDHP, DDCP). The antibodies were screened for their detection sensitivity for DDT and to test the influence of coating ligand.[16-18] DDT and its metabolites were detected simultaneously using a matching pair of immunogen and antibody.

Experimental Section

Reagents. Bovine serum albumin (BSA), ovalbumin (OVA) and keyhole limpet hemocyanine (KLH) were purchased from Pierce Chemical Co. (Rockford, IL., USA). *p,p*'-DDT, *p,p*'-DDE and *p,p*'-DDD were purchased from Chem Service Inc. (West Chester, PA, USA). *p,p*'-DDA, *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carboimide hydrochloride (EDC) and organic solvents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO., USA). Precoated preparative TLC plates (Art. 13895, PSC-Fertigplatten Kieselgel 60 F254 for preparative chromatograpy, 20×20 cm, 1 mm) were purchased from Merck Co. (Germany). ELISA substrate contained 70 mM *o*-phenylenediamine (OPD) and 5.1 mM hydrogen peroxide

in 53 mM sodium citrate buffer containing 10 mM sodium hydrogen phosphate, pH 5.3. PBST, pH 7.2, contained 10 mM phosphate buffered saline (PBS) with 0.05% Tween 20. All chemicals used were of analytical grade, and the solutions were made with deionized water, using the Milli-Q water purification system (Millipore Inc., MA, USA). A microwell module (maxisorp) was purchased from Nunc (Denmark) and the optical density of the ELISA results was measured using an Emax precision microtiter plate reader (Molecular Devices Inc., CA, USA).

Preparation of haptens, immunogens and coating ligands. Five haptens (Figure 1) (DDA: 2,2-Bis(4-chlorophenyl)acetic acid,[19] DDHP: 5,5-Bis(4-chlorophenyl)-5-hydroxypentanoic acid,[20] DDCP: 5,5-Bis(4-chlorophenyl)-5-chloropentanoic acid, DDHH: 6,6-Bis(4-chlorophenyl)-6-hydroxyhexanoic acid, DDHHAP: 3-[6,6-Bis(4-chlorophenyl)-6-hydroxyhexanoylamino] propanoic acid), three immunogens (DDA-KLH, DDHP-KLH, DDCP-KLH) and ten coating ligands (combination of five haptens and two carrier protens: BSA, OVA) were prepared according to methods described in a previous paper.[21]

Preparation of DDT monoclonal antibodies. DDT monoclonal antibody producing hybridoma cell lines were obtained by fusing myeloma and spleen cells of BALB/c mice immunized with one of the following immunogens: DDA-KLH, DDHP-KLH or DDCP-KLH. Initial immunization was performed using an immunogen with complete Freunds adjuvant emulsion (CFA), followed by twice boost injection with incomplete Freunds adjuvant emulsion (IFA). Fifteen DDT monoclonal antibodies were obtained through ELISA screening using various DDT-BSA derivatives as coating ligands.

A screening using various DDT-BSA derivatives as coating ligands.

Preparation of assay standards. PBS (10 mM, pH 7.2) was used as the working buffer for all enzyme-linked immunosorbent assay (ELISA) experiments. A stock solution of 1 mg/mL DDT in DMSO was serially diluted in PBS to 0.1 pg/mL. The same method was used to prepare standards of similar reactants at concentrations of 0.1, 1, 10, 100, 1000, 10000 and 100000 pg/mL. Standards and similar reactants were stored at 4 °C.

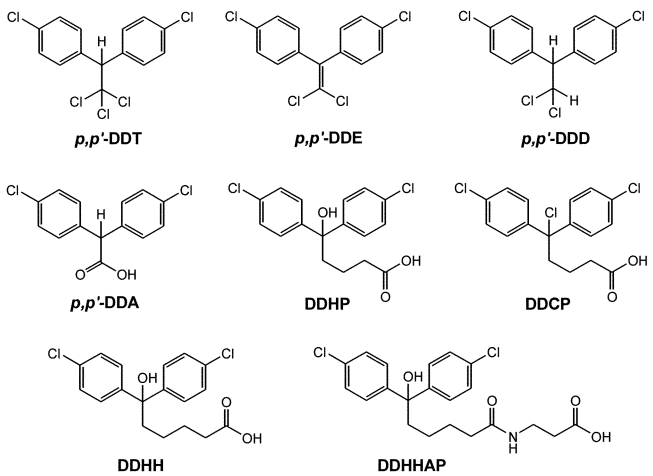

Figure 1. Chemical structures of DDT analytes and prepared haptens

Assessment of titration levels of the antibodies by ELISA. A microtiter plate was coated with 50 μL (10 μg/mL) of DDA-BSA coating ligand in 50 mM carbonate buffer (pH 9.6) for 12 hours at 4 °C, and then washed three times with 200 μL PBST. The wells were blocked using 150 μL of 1% BSA in PBS for 2 hours at room temperature and washed three times. The DDA-BSA coated microwells were incubated for 2 hours with 50 μL of serially diluted MAb culture supernatant in PBS-1% BSA solution at room temperature and washed. HRP-conjugated anti-mouse-IgG (200 μL of 1/1000 diluted solution) was added to each well and incubated for 2 hours at room temperature, and then washed five times with PBST. After incubating for 10 minutes with 100 μL OPD substrate, the color reaction of the enzyme substrate was stopped with 50 μL of 2 N H_2SO_4. The optical density was read at 490 nm. The titer level of antibodies was chosen by the level of antibody dilution to produce approximately 70% of maximum absorbance. The titer levels of antibodies for the other coating ligands were obtained in the same way. All assays were run in duplicate.

Dose-response curve for DDT analytes by ELISA. A dose-response curve for each DDT analyte (*p,p'*-DDT, DDA, *p,p'*-DDE, *p,p'*-DDD) was established based on the assessment of the titer level of the antibody. The antibody coating step was the same as that in the antibody titration method. To construct a dose-response curve of DDT analytes, 180 μL free DDT analyte standard and 180 μL antibody were mixed and pre-incubated for 2 hours at room temperature. Aliquots (100 μL) of the resulting mixture were added to the hapten-protein coated wells. The remaining steps were then performed as described above. All assays were run in duplicate.

Results and Discussion

Five kinds of appropriate haptens resembling the chemical structure of DDT were synthesized for immunodetection of DDT and its decomposition products. DDT derivatives (DDA, DDHP and DDCP) that have high similarity to DDT haptens were coupled with keyhole limpet hemocyanin (KLH) to use as an immunogen to produce antibodies. The bovine serum albumin (BSA) conjugates of these derivatives were prepared as a coating ligand for the monoclonal antibody screening. Fifteen monoclonal antibody clones were screened using these probes. Five kinds of synthetic haptens (DDA, DDHP, DDCP, DDHH and DDHHAP) were coupled with carrier proteins, BSA or OVA, for use as a coating ligand. The monoclonal antibodies (MAb) were characterized for their affinities and specificities to DDT analytes with the ten coating ligands above. The absorbance values in Table 1 show the degree of binding ability between the antibodies and coating ligands under the same reaction procedures. The titer level of each antibody was determined by competitive ELISA, using coating ligands. The antibody

Table 1. Effect of coating ligand on the titration curves

Immunogen	MAb clone	Titer level[a]									
		BSA-coating ligand					OVA-coating ligand				
		DDA-BSA	DDHP-BSA	DDCP-BSA	DDHH-BSA	DDHHAP-BSA	DDA-OVA	DDHP-OVA	DDCP-OVA	DDHH-OVA	DDHHAP-OVA
DDA-KLH	1A1	3.22	3.11	3.18	2.97	2.97	0.44	0.31	0.30	0.30	0.23
	1A2	0.78	0.57	0.44	0.33	0.44	0.86	2.24	1.00	0.99	1.06
	1A3	0.07	0.08	0.07	0.19	0.13	0.66	0.78	0.57	0.50	0.53
	1A4	0.08	0.08	0.06	0.20	0.10	0.39	0.47	0.37	0.41	0.48
	1B6	0.18	0.16	0.15	0.24	0.20	0.55	0.69	0.58	0.62	0.64
DDHP-KLH	2A1	0.36	0.22	0.09	0.32	0.19	0.14	0.11	0.12	0.12	0.23
	2A2	0.15	0.15	0.09	0.29	0.19	0.14	0.10	0.11	0.15	0.26
	2A6	0.33	0.17	0.09	0.53	0.24	0.14	0.17	0.21	0.13	0.30
	2B1	0.31	0.43	0.13	0.24	0.26	0.13	0.14	0.11	0.22	0.60
	2B3	0.08	0.09	0.09	0.34	0.43	0.60	0.54	0.46	0.43	1.01
DDCP-KLH	3A1	3.42	3.39	3.38	3.36	3.35	3.16	3.23	3.13	3.17	3.26
	3A2	0.64	0.56	0.78	0.61	0.56	1.60	1.95	1.86	1.82	1.88
	3A3	0.22	0.15	0.11	0.14	0.13	0.59	0.58	0.48	0.66	0.59
	3A4	0.51	0.37	0.15	0.16	0.18	0.59	0.61	0.51	0.53	0.55
	3A5	1.39	1.07	1.03	1.52	0.24	0.91	1.00	0.73	0.71	0.69

[a] The titer level was determined by the value of antibody dilution fold on the 70% of maximum absorbance.

concentration that showed 70% of maximum binding response (absorbance at 490 nm) was determined as the titer level. We observed that the affinities varied depending on the coating ligands that were derivatized to different structures and conjugated with different carrier proteins. MAb 1A1 and 3A5 showed higher binding affinity on the BSA coating ligands than on the OVA coating ligand. They were obtained from the DDA-KLH and DDCP-KLH immunogens, respectively. Most of the MAbs obtained from the DDHP-KLH immunogen showed low binding affinity with the exception of the pairs of MAb 2B3 and OVA coating ligands. 1A2 and 1B6 antibodies showed good affinity for OVA coating ligands. Despite its strong affinity for all coating ligands, MAb 3A1, which was produced from DDCP-KLH immunogen, could not be used to obtain good competitive curves for DDT analytes. When the antibody has a very strong affinity for the coating ligand, good assay sensitivity cannot be obtained because free analytes cannot easily bind to the antibody in competition with the coating ligand. Colbert et al.[22] also reported that increased sensitivity was achieved by reducing the antibody affinity of the tracer.

To screen a matching pair of antibody and coating ligand for the simultaneous detection of DDT and its related compounds (DDT, DDA, DDE, DDD), each antibody was investigated for the displacement of free ligand, using selected coating ligands through the method of affinity screening. Selected were four matching pairs of antibody and coating ligand that showed reactivities to DDT, DDA, DDE and DDD. Figure 2 shows the results for displacement responses of DDT analytes, using a selected pair of antibody and coating ligand. They were MAb 1A1 with coating ligand of DDHHAP-BSA, MAb 1A3 with DDA-OVA or DDHP-OVA and MAb 1A4 with DDHP-OVA. Table 2 shows the relative reactivity and sensitivity of DDT analytes using a selected pair of antibody and coating ligand. The detection limit of each analyte was obtained by the dose-response curve at the optimum condition of the respective pair of

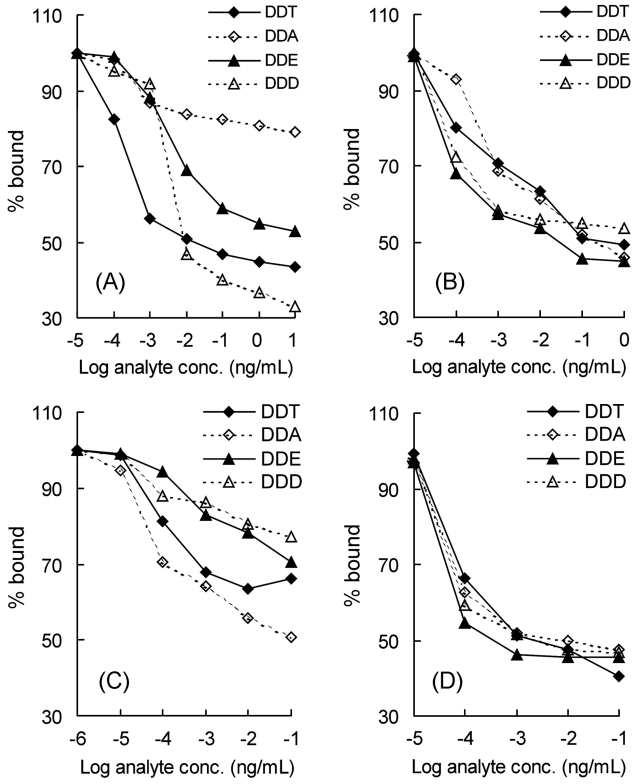

Figure 2. Dose-response curves of DDT analytes. The MAbs (50 μL of titer level of each antibody) and the coating ligand (50 μL of 10 μg/mL) that were used for the dose-response curve of DDT analytes: (A) MAb DDA 1A1 with DDHHAP-BSA coating ligand, (B) MAb DDA 1A3 with DDA-OVA coating ligand, DDA 1A4 with DDHP-OVA coating ligand.

Table 2. Relative reactivity of DDT analytes with a matching pair of antibody and coating ligand

Antibody	Coating ligand	DDT analytes	Relative reactivity[a] (%)	Sensitivity[b] (*p*g/mL)
DDA 1A1	DDHHAP-BSA	*p,p*'-DDT	100	0.12
		p,p'-DDA	4.2	-
		p,p'-DDE	3.3	5.34
		p,p'-DDD	6.0	1.82
DDA 1A3	DDA-OVA	*p,p*'-DDT	100	0.10
		p,p'-DDA	3.3	0.34
		p,p'-DDE	201	0.04
		p,p'-DDD	172	0.05
	DDHP-OVA	*p,p*'-DDT	100	0.70
		p,p'-DDA	122	0.11
		p,p'-DDE	146	475
		p,p'-DDD	129	9378
DDA 1A4	DDHP-OVA	*p,p*'-DDT	100	0.08
		p,p'-DDA	107	0.06
		p,p'-DDE	134	0.04
		p,p'-DDD	121	0.05

[a] % Relative reactivity defined as % ratio of concentration for 70% displacement by DDT concentration/ 70% displacement by other analyte. [b]Sensitivity defined as the concentration of DDT analytes that yielded 80% of the maximum response from zero concentration on the dose-response curve.

antibody and coating ligand. The relative reactivities of DDT analytes were compared with 70% inhibition concentration of DDT. Sensitivity was defined as the concentration of DDT analytes that yielded 80% of the maximum response from zero concentration on the dose-response curve. The relative reactivity values of DDT analytes when using the pair of MAb 1A1 and DDHHAP-BSA were calculated as 100, 4.2, 3.3, 6.0%. With MAb 1A3 and DDA-OVA, they were 100, 3.3, 201, 172%; with MAb 1A3 and DDHP-OVA, they were 100, 122, 146, 129%, and with MAb 1A4 and DDHP-OVA, they were 100, 107, 134, 121% (Table 2). MAb 1A4 and DDHP-OVA coating ligand exhibited high sensitivity values for the DDT analytes (DDT, DDA, DDE and DDD), but the combination of MAb 1A3 and DDA-OVA exhibited the best response, covering a wide range of DDT levels.

The results indicate that DDT was the most responsive to the pair of 1A1 and DDHHAP-BSA; DDE to the pairs of 1A3 and DDA-OVA and 1A4 and DDHP-OVA; and DDA to the pair of 1A3 and DDHP-OVA. From these observations, we conclude that the length of spacer that is attached for coupling of carrier protein in immunogen and coating ligand could significantly influence the immunoreactivity of an indirect ELISA by affecting the antibody affinity and dose-response immunoreaction.

In conclusion, it is important to screen a best matching pair of antibody and coating ligand for the immunoreactions of affinity and displacement with DDT analytes. DDA-KLH immunogen and DDA-OVA coating ligand were the best combination at a wide range of DDT analytes.

Acknowledgment. This investigation was supported by a fund of the National Research Laboratory Program (No. 2000-N-NL-01-C-045), Ministry of Science and Technology, Republic of Korea.

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