JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Preparation of Specific Monoclonal Antibodies (MAbs) against Heavy Metals: MAbs That Recognize Chelated Cadmium Ions

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Monoclonal antibodies (MAbs) were produced against chelated Cd^{2+}. Since Cd^{2+} ions are too small to elicit an immune response, the metal was coupled to protein carrier (keyhole limpet hemocyanin, KLH) using a bifunctional chelator 1-(4-isothiocyanobenzyl)ethylenediamine *N*,*N*,*N*′,*N*′-tetraacetic acid (ITCBE). Several mice were immunized with this Cd^{2+}–ITCBE–KLH immunoconjugate. Spleen cells of two immunized mice were fused with myeloma cells, and the resulting hybridomas were screened using protein conjugates with covalently bound metal-free ITCBE (ethylenediamine tetraacetic acid) or Cd^{2+}–ITCBE. Four hybridoma cell lines that produced MAbs with high selectivity and sensitivity (Aa4, Aa6, Ac4, and Ba2) were expanded for further study. Cross-reactivities with other metals were below 1% except for Hg^{2+}, which showed a slight cross-reactivity in competitive ELISA. These antibodies were used to construct competitive ELISAs for ionic cadmium; the IC_{50} of the four antibodies (Aa4, Aa6, Ac4, and Ba2) were 10.59, 4.19, 29.45, and 6.63 μg/L, respectively. The detection range and the lowest detection limit for cadmium, using the Aa6 antibody, were 2.19–86.38 μg/L and 0.313 μg/L, respectively. Spike-recovery studies in tap and stream water showed that the most sensitive antibody can be used for cadmium detection in drinking water.

KEYWORDS: Monoclonal antibodies; cadmium; immunoassay; ELISA

INTRODUCTION

The environmental pollution by heavy metals such as cadmium, mercury, lead, and chromium has become a worldwide public health hazard (*1*, *2*). Contamination with cadmium is particularly worrisome because of its persistence in the environment and very long biological half-life in humans, between 10 and 40 years. Ingested and inhaled cadmium accumulates primarily in the kidneys and ultimately leads to kidney failure (*3*). Increased levels of dietary cadmium intake may also cause other functional disturbances, especially in the young developing organisms, and recent evidence has linked high serum cadmium levels to early onset pancreatic cancer (*4*). Thus, the ability to rapidly and inexpensively monitor environmental cadmium is a prerequisite for minimizing human and animal exposure.

Current analytical methods involving inductively coupled plasma atomic emission spectroscopy (ICPAES) and graphite furnace atomic absorption spectroscopy (GFAAS) for the detection of heavy metal ions residues are sensitive and reliable (*5*–*8*). However, they require expensive instrumentation, a highly qualified analyst, and time-consuming sample preparation steps. Therefore, there is a growing demand for more rapid and more economical methods for determining heavy metal residues. The development of immunoassays to detect metal ions has been a promising trend (*9*–*15*).

While the most vigorous immune responses are induced by large molecular weight, structurally complex molecules with a high degree of foreignness from the stimulated recipients, heavy metals, under appropriate conditions, can also induce specific antibody responses. Monoclonal antibodies directed against glutathione–, dithiocarbamate–, EDTA (ethylenediamine tetraacetic acid)–, and DTPA (diethylenetriamine pentaacetic acid)–metal complexes of Hg^{2+}, Cd^{2+}, Pb^{2+}, and In^{3+} have been reported (*10*, *16*–*19*). Reardan et al. first demonstrated the use of an EDTA–chelate immunogen to generate a monoclonal antibody, CHA255, that bound with subnanomolar affinity to an In^{3+}–EDTA complex but not with metal-free EDTA (*9*). In this study, we report the isolation and characterization of cadmium-specific monoclonal antibodies.

MATERIALS AND METHODS

All chemicals were ultrapure grade. Cadmium foil (99.999%) was obtained from Aldrich Chemical Co. (Milwaukee, WI); other metals

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10.1021/jf071025l CCC: $37.00
Published on Web 08/23/2007

ions, including Fe^{3+}, Zn^{2+}, Cu^{2+}, and Ni^{2+}, were purchased from Merck Chemical Co. (Darmstadt, Germany). 1-(4-Isothiocyanobenzyl)ethylenediamine *N,N,N′,N′*-tetraacetic acid (ITCBE) was a product of Dojindo Laboratories (Gaithersburg, MD). Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), Freund's complete and incomplete adjuvants, 3,3′,5,5′-tetramethylbenzidine (TMB), cadmium atomic absorption standard metals (1000 μg/mL in 2% HNO_3), and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO). Reagents for monoclonal antibody production included in the ClonaCell-HY kit were purchased from StemCell Technologies (Vancouver, British Columbia, Canada). Ultrapure hydrochloric and nitric acid were purchased from Shanghai Chemical Reagents Co. (Shanghai, China). Fetal bovine serum (FBS) was the product of Hangzhou "Sijiqing" Co. (Hangzhou, China). Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG and IgM were purchased from Sino-American Biotechnology Co. (Luoyang, China). ELISA high-binding microplates and tissue culture plates were obtained from Corning Co. (Cambridge, MA); ImmunoPure IgM Purification Kit was purchased from Pierce Co. (Rockford, IL); water used for reagent preparation was purified by filtration through a Millipore (MUL-TYPE9000) water purification system. All glassware was treated with mixed-acids (concentration HCl:concentration HNO_3 = 1:1, v/v) for 4 h and liberally rinsed with ultrapure water; all plasticware was soaked in 3 M HCl overnight and rinsed liberally with purified water before use (*20*). Amicon Centricon 30 concentrator tubes (Millipore Co., Bedford, MA) were treated with 100 mM EDTA and liberally rinsed with ultrapure water before use. BD Cell serum free mAb medium and CELLine Device were the products of BD Biosciences (San Jose, CA). A Wellwasher Plus microplate washer from Thermo Electron Co. (Marietta, OH) was used to wash ELISA plates. A Multiskan Ascent reader for microtiter plates was from Thermo Electron Co. (Marietta, OH); this device was controlled by a personal computer containing the standard software package EasySoftware.

SP2/0 cells were obtained from College of Veterinary Medicine, Nanjing Agricultural University, and BALB/c mice were from Shanghai Experimental Animal Center. All animal studies were performed in accordance with institutional guidelines.

Preparation of Metal–ITCBE–Protein Conjugates. For the preparation of cadmium immunoconjugates, Cd^{2+} was coupled to carrier protein using ITCBE according to the procedures previously described (*16*). KLH or BSA (10 mg) was reconstituted into 0.5 mL of HEPES buffer (0.01 M, pH 9.0). ITCBE (10 mg) was dissolved in 1 mL of dimethyl sulfoxide (DMSO) to make a 23 mM solution. Cadmium metal (99.999%, 53.7 mg) was dissolved in 100 μL of concentrated HNO_3, and then ultrapure water was added to a final volume of 1 mL to make a 477.8 mM solution.

Conjugate synthesis was initiated by adding 78 μLof the ITCBE solution (1.8 μmol) dropwise under gentle stirring to 0.5 mL of KLH or BSA solution (20 mg/mL). If necessary, the pH was adjusted to 9.0 with 10 M KOH. After 24 h at room temperature the ITCBE–KLH conjugate was purified by removing the unreacted low-molecular-weight reagent (ITCBE) with a Centricon 30 device that had been previously treated with EDTA and washed profusely with Hepes-buffered saline (HBS, 137 mM NaCl, 3 mM KCl, 10 mM HEPES, pH 7.4). The conjugate was washed four times with metal-free HEPES buffer (0.1 M, pH 9.0) and twice with metal-free HEPES buffer (0.1 M, pH 7.4).

The cadmium solution (18.8 μL, 8.9 μmol) was added dropwise with gentle stirring to the purified ITCBE–protein conjugate; the pH was adjusted to 7.0 to avoid precipitation. The binding reaction proceeded for 3 h at room temperature. Finally, the reaction mixture was purified to remove unbound cadmium as described above.

Mouse Immunization and Serum Screening Procedures. Immunization of BALB/c mice was carried out according to the method described by Kishiro et al. (*21*). The antigen Cd^{2+}–ITCBE–KLH conjugate was diluted into HBS, mixed with an equal volume of Freund's complete adjuvant, and stirred to prepare a water-in-oil emulsion. The emulsion (200 μg conjugate per mouse) was injected into the peritoneal cavity of 6-week-old BALB/c female mice. The same injection with incomplete Freund's adjuvant was repeated 3 weeks later and at 2-week intervals thereafter. One week after the fifth immunization, a drop of blood was collected from the tail vein of each mouse, and the sera was tested for antibody titer and for analyte recognition by indirect ELISA (enzyme-linked immunosorbent assay). ELISAs were carried out according to Voller et al. with slight modifications (*22, 23*). All the reactions were performed in polystyrene plates as follows: microwells were incubated overnight at 4 °C with equivalent concentrations of Cd^{2+}–ITCBE–BSA or ITCBE–BSA diluted into HBS. The coating solution was removed, and unoccupied sites were blocked for 90 min at 37 °C with 100 μL/well of 1% gelatin diluted in PBST (phosphate-buffered saline, 137 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4, PBS with 0.05% (v/v) Tween-20). The blocking solution was decanted and 50 μL/well antiserum diluted 1:2000 in PBST was added to wells, followed by a 1 h incubation at 37 °C. The plates were washed six additional times with PBST to remove unbound antibodies. HRP-labeled goat antimouse IgG/IgM diluted in PBST (1:1000) was then added to each well, followed by incubation at 37 °C for 1 h. After an additional eight washes with PBST, 50 μL/well of TMB (3,3′,5,5′-tetramethylbenzidine) substrate solution (3.3 μL of 30% H_2O_2, 200 μL of 1.2% TMB in DMSO per 25 mL of acetate buffer, pH 5.5) was added to each well. After 20 min at room temperature, the reaction was stopped with 2 M H_2SO_4, and the absorbance at 450 nm was measured. All experiments were conducted in triplicate.

Cell Fusion and Hybridoma Selection. The two mice showing highest serum reactivity were given two tail vein injections of 100 μg of Cd^{2+}–ITCBE–KLH conjugate in 50 μL of HBS at 1-week intervals. Four days after the last injection the two donor mice were sacrificed. Hybridomas were produced by the fusion of the immunized spleen cells of two mice with SP2/0 myeloma cells in logarithmic growth phase, according to the protocol of the ClonalCell-HY kit. In the first day the fused cells were incubated in a 250 mL of cell flask in 5% CO_2 for 16 h at 37 °C. Then the fused cells were transferred into ten 100 mm tissue culture plates containing the methylcellulose-based selection medium. After 10–14 days, individual hybridoma clones growing in the semisolid medium were transferred into wells of 96-well tissue culture plates and incubated at 37 °C in 5% CO_2 for an additional 1–4 days. Supernatants from these clones were collected and initially screened for antibodies by the indirect ELISA method as described above. Culture supernatants that passed the initial screen were subsequently tested for antibody specificity by competitive ELISA. To investigate whether the antibodies were reacting with chelated cadmium ions or EDTA, the first competitive ELISAs were based on the competition of microwell-bound Cd^{2+}–ITCBE–BSA conjugate and soluble EDTA at the following concentrations: 0.5, 1, 5, 10, 20, and 50 mM. These experiments identified those antibodies that not bound primarily to metal-free EDTA and also allowed us to select an optimal concentration of EDTA in subsequent screenings for anti-cadmium antibodies. In screening for anti-cadmium antibodies, atomic absorption standard cadmium (10-fold dilutions, 1000–0.001 μg/L) was diluted into a constant concentration of EDTA, and these solutions were used to inhibit the antibody binding to Cd^{2+}–ITCBE–BSA adsorbed to the wells of the microplate. This screen yielded positive hybridoma cell lines that were subsequently subcloned by limiting dilution.

Production, Purification, and Isotyping of Monoclonal Antibodies. In vivo, ascites fluid was produced in BalB/c mice primed with incomplete Freund's adjuvant by intraperitoneal (Ip) injection of 2×10^7 hybridoma cells. Ascites fluid was harvested 10–14 days after the hybridoma cell injection. In vitro, hybridoma cell lines secreting monoclonal antibodies were maintained in BD Cell serum free Mab medium in a membrane-based disposable cell cultivation system incubated in a CO_2 incubator at 37 °C using a 7/21 day harvest protocol recommended by the manufacturer.

The antibodies were partially purified using precipitation with 50% ammonium sulfate (*24*). The partially purified MAbs fractions were pooled and then dialyzed against Tris buffer (Tris 20 mM, sodium chloride 1.25 M, pH 7.4) to remove phosphate ions. Antibodies in the dialyzed buffer Ig M was subsequently purified using ImmunoPure IgM Purification Kit according to the manufacturer's recommended protocol. The fractions with OD (optical density) greater than 0.02 at 280 nm were pooled, concentrated, and dialyzed against PBS. The concentration of protein in the purified antibody preparations was determined by the Bradford method (*25*).

Determination of Affinity Constants (K_{aff}) for Cd^{2+}–ITCBE–BSA. Beatty et al. (*26*) described a method based upon the law of mass action. The use of serial dilutions of purified antibody results in a sigmoid curve of optical density (OD) vs logarithm of amount of antibody added to the wells. The OD_{50} of sigmoid curve permitted us to estimate each monoclonal antibody's affinity for the immobilized Cd^{2+}–ITCBE–BSA conjugate. We used this method with two different coating concentrations of Cd^{2+}–ITCBE–BSA (2.5 and 1.25 μg/mL diluted in HBS, pH 7.4) plus serial dilutions of monoclonal antibodies in HBS. The indirect ELISA was performed as described above.

Antibodies Characterization and Cross-Reactivity (CR) with Other Metals. Antibodies and antigens were screened in a two-dimensional titration for the best dilution of coating antigen and supernatant culture. Then the competitive inhibition curves were measured for different antibody and antigen combinations and performed essentially as described for the determination of antibody selectivity. Cd^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, Cr^{3+}, Ni^{2+}, Mg^{2+}, Ag^{+}, Ca^{2+}, and Fe^{3+}–EDTA complexes were tested for their ability to inhibit binding to the immobilized Cd^{2+}–ITCBE–BSA. The 96-well plates coated with Cd^{2+}–ITCBE–BSA were blocked with 1% gelatin, metal solution (0.001–10000 μg/L) (in HBS containing 1 mM EDTA) and were premixed with the purified culture supernatant at desired dilution; aliquots of the mixture were added to the microplates which had been coated. After a wash step, a second antibody labeled with HRP was used for the detection of specifically bound antibody. The plates were washed six times between incubation steps. Samples and standards were generally analyzed in four replicate wells. The inhibition curves were analyzed to determine the IC_{50}, which was defined as the concentration of inhibitor r to inhibit color development by 50% compared to control wells containing no competitor.

CR was determined by dividing the IC_{50} of cadmium assigned to be 100% by the IC_{50} of another metals and multiplying by 100 to obtain a percent figure. Thus, the process of obtaining a good analytical assay becomes a function of developing an assay with the lowest possible IC_{50} and best CR profile (CR figure for analytes other than the targets are as small as possible).

Fortification of Cadmium in Water. Water samples were collected from a tap in the laboratory and from Zixia stream in Nanjing, Jiangsu. Aliquots were fortified with cadmium to 50, 20, and 5 μg/L and mixed with equal volume of 2-fold concentrated HBS (20 mM Hepes, 274 mM NaCl, 2 mM EDTA, pH 7.4) containing a predetermined limiting amount of mAb (Aa_6). The samples were analyzed with CI-ELISA.

RESULTS

Design of Antigen and Animal Response. The most vigorous immune responses are induced by large molecular weight, structurally complex molecules with a high degree of foreignness for the stimulated recipient. Cadmium was a low molecular weight hapten of insufficient size to elicit an immune response. For this reason, cadmium was conjugated to a large carrier protein (KLH) via a bifunctional EDTA derivate to elicit immune response in mice. After multiple injections of Cd^{2+}–ITCBE–KLH, blood was collected from the tail vein of each mouse and assayed by ELISA for the presence of cadmium-specific antibodies. **Table 1** shows the reactivity of each serum sample with Cd^{2+}–ITCBE–BSA and ITCBE–BSA. Sera from five of the six mice (designated as 2, 3, 4, 5, and 6 in the **Table 1**) demonstrated higher reactivity with Cd^{2+}–ITCBE–BSA than with ITCBE–BSA, suggesting that they might contain antibodies specific for either Cd^{2+} itself or to an epitope composed of both $Cd^{2+}$and ITCBE. Since mouse 3 and mouse 6 demonstrated the highest relative reactivity with Cd^{2+}–ITCBE–BSA, they were used for subsequent hybridoma production.

Hybridoma Screening and Antibody Production. Approximately 600 hybridoma clones were initially screened by indirect ELISA for their reactivities with Cd^{2+}–ITCBE–BSA and ITCBE–BSA. The results were compared to the reactivity of supernatants of myeloma Sp2/0 with the same antigens, which served as the negative control. Hybridoma cell lines such Ad9 and Df11 reacted with both ITCBE–BSA and Cd^{2+}–ITCBE–BSA, as shown in **Table 2**; the antibodies synthesized by these hybridomas were considered specific for ITCBE or ITCBE–BSA. Sixty-five hybridomas, such as Aa_6, Aa_4, Ac_4, and Ba_2, reacted strongly with Cd^{2+}–ITCBE–BSA but showed only background reactivity with ITCBE–BSA. These antibodies were presumed to be binding to Cd^{2+} or the Cd^{2+}–EDTA complex.

To further study whether these antibodies would recognize Cd^{2+}, antigen inhibition ELISA was performed. As shown in **Figure 1A**, concentrations of EDTA less than 5 mM did not affect the binding of MAbs Aa6 or Ba2 to the immobilized Cd^{2+}–ITCBE–BSA conjugate. A concentration of 1 mM EDTA was chosen for subsequent experiments. When Cd^{2+} was serially diluted into 1 mM EDTA and incubated with the immobilized Cd^{2+}–ITCBE–BSA conjugate in the presence of hybridoma culture supernatant, the cadmium ions inhibited binding in a dose-dependent fashion, as shown in **Figure 1B**. Of these, 43 showed reactivity to metal-free EDTA, and 22 showed specificity for the chelated cadmium. This result demonstrated that reactivity of antibodies with cadmium ions was dependent of the presence of EDTA, although not inhibited by EDTA.

The stable monoclonal antibodies established from Aa4, Aa6 Ac4, and Ba2 were expanded for further study. The MAbs were

Table 1. Reactivity of Serum from BALB/c Mice Injected with Cd^{2+}–ITCBE–KLH[a]

mouse	ITCBE–BSA	Cd^{2+}–ITCBE–BSA	difference (%)
1	1.332 ± 0.108	1.214 ± 0.120	−8.9
2	0.808 ± 0.063	0.886 ± 0.053	9.7
3	0.564 ± 0.045	0.947 ± 0.022	67.9
4	0.567 ± 0.027	0.865 ± 0.055	52.6
5	0.682 ± 0.030	0.848 ± 0.052	24.3
6	0.483 ± 0.050	0.756 ± 0.025	56.5
normal serum	0.299 ± 0.022	0.308 ± 0.039	3.0

[a] Serum was collected from the tail vein of each mouse, diluted into PBS, and used for ELISA as described. Normal serum was obtained from an unimmunized mouse and served as background reactivity for each antigen. Each value represents the mean of three replicates ±S.D. Calculated by the formula % difference= (A_{450} of Cd^{2+}–ITCBE–BSA − A_{450} of ITCBE–BSA)/(A_{450} of ITCBE–BSA) × 100.

Table 2. Reactivity of Hybridomas Antibodies with Cd^{2+}–ITCBE–BSA and ITCBE–BSA[a]

hybridoma designation	A_{450} with Cd^{2+}–ITCBE–BSA	A_{450} with ITCBE–BSA
Aa4	1.137 ± 0.073	0.419 ± 0.021
Aa6	1.259 ± 0.101	0.400 ± 0.021
Ac3	0.935 ± 0.083	0.593 ± 0.059
Ac4	1.028 ± 0.125	0.438 ± 0.036
Ac5	1.207 ± 0.087	0.678 ± 0.050
Ae12	0.867 ± 0.055	0.405 ± 0.033
Af9	0.923 ± 0.085	0.437 ± 0.047
Ba2	1.217 ± 0.137	0.409 ± 0.049
Be5	0.735 ± 0.260	0.433 ± 0.021
Dd2	0.958 ± 0.059	0.425 ± 0.025
Ad9	1.687 ± 0.148	2.054 ± 0.131
Df11	1.858 ± 0.126	1.839 ± 0.082
NC	0.407 ± 0.035	0.429 ± 0.027

[a] Culture supernatants were assayed for the presence of antibody specific for antigen shown. Each value represents the average A_{450} of triplicate determinations for each antibody. The negative control (NC) is from the supernatant of myeloma SP2/0. The A_{450} with this supernatant was considered background reactivity. Each value represents the mean of three replicates ±S.D.

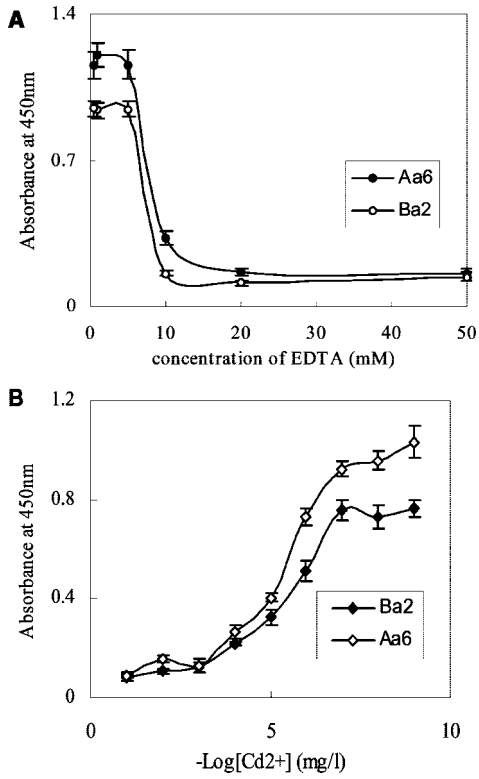

Figure 1. Competitive ELISA with Mabs Aa6 and Ba2: (**A**) inhibition by soluble EDTA; (**B**) inhibition by when Cd^{2+} was serially diluted into 2 mM EDTA. Each point represented the mean of triplicate determinations.

produced from supernatant of CELLine (10–100 mg) or ascites fluid in mouse (3.10 mg/mL), purified and stored at −20 °C for characterization studies.

Apparent Affinity Constant (K_{aff}) of MAbs for Immobilized Cd^{2+}–EDTA–BSA. The affinity constants (K_{aff}) were measured by indirect ELISA using serial dilutions of monoclonal antibodies and two concentrations of Cd^{2+}–ITCBE–BSA for coating of the microwell plates (2.5 and 1.25 μg/mL). **Figure 2** shows the primary data for the four MAbs tested. The general equation used to calculate the affinity constant, adapted from Beatty et al. was $K_{aff} = n - 1/n(Ab') - (Ab)$, where (Ab) and (Ab′) were the measurable total antibody concentrations in the wells at OD_{50} and OD_{50}' for plates coated with (Ag) and (Ag′), respectively, with (Ag′) = (Ag)/n.

The K_{aff} values of Mabs (Aa6, Ba2, Aa4, and Ba2) calculated in **Table 3** were 1.52×10^{10}, 3.28×10^{9}, 7.23×10, and 6.07×10^{8} L/mol, respectively, which showed that monoclonal antibody Aa6 bound most tightly to the immobilized Cd^{2+}–ITCBE–BSA conjugate.

Characteristics of Immunoassay. The ELISA standard curves for cadmium were performed, and the results are presented in **Figure 3**. Quantification of Cd^{2+} was carried out by CI-ELISA with the four new MAbs isolated in this study. The IC_{50} of the four antibodies (Aa_6, Ba_2, Aa_4, and Ac_4) were 4.19, 6.63, 10.59, and 29.45 μg/L, respectively. The lowest detection limit (defined as 2 standard deviations above the lowest detectable level) and the detection range (IC_{20-80}) were estimated as 0.313 μg/L and 2.19–86.38 μg/L, respectively, when antibody Aa6 was used in the competitive ELISA. These results illustrated that mAb Aa6 showed the highest affinity for the Cd^{2+}–EDTA complex.

Cross-Reactivity Studies. Several metals were tested for cross-reactivity. **Table 4** shows the cross-reactivities that were found by CI-ELISA, expressed in percentage of the IC_{50} of cadmium. It was noteworthy that all antibodies showed a small cross-reactivity to Hg^{2+}, which was understandable because the three-dimensional structures of Cd^{2+}–EDTA and Hg^{2+}–EDTA have been shown to be very similar (*27*).

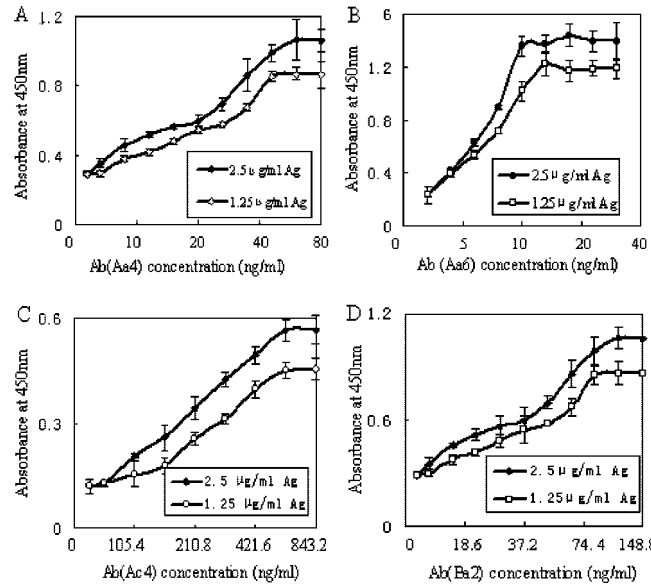

Figure 2. Apparent affinity constant was measured as described in Materials and Methods: (**A**) affinity for mAb Aa4 (7.23×10^{9} L/mol); (**B**) affinity for mAb Aa6 (1.52×10^{10} L/mol); (**C**) affinity for mAb Ac_4 (6.07×10^{8} L/mol); (**D**) affinity for mAb Ba2 (3.28×10^{9} L/mol). Each point represented the mean of triplicate determinations.

Table 3. Affinity Constants of mAbs Determined by ELISA

Ab	Ag (μg/mL)	OD-50	[Ab] at OD-50 (ng/mL)	K_{aff} (L/mol)
Aa4	2.5	0.531	13.1	7.23×10^{9}
	1.25	0.430	16.9	
Aa6	2.5	0.70	5.71	1.52×10^{10}
	1.25	0.589	7.79	
Ac4	2.5	0.282	168.2	6.07×10^{8}
	1.25	0.227	207.6	
Ba2	2.5	0.64	24.7	3.28×10^{9}
	1.25	0.57	35.2	

Cadmium-Fortified Water Samples. Tap and stream water samples spiked with cadmium were analyzed by CI-ELISA. Ionic strength of the samples was adjusted by addition of an equal volume of 2-fold concentrated HBS containing a fixed amount of mAb. The recovered concentrations of cadmium by

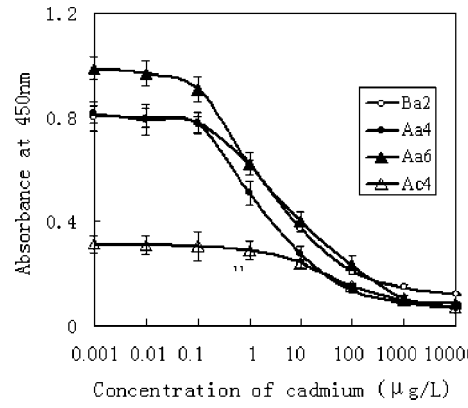

Figure 3. Standard curve for cadmium ions obtained with the antibody (Aa4, Aa6, Ac4, and Ba2). Each point represented the mean of triplicate determinations.

Table 4. Cross-Reactivity of mAbs with Other Metal Ions (Each Value Represents the Mean of Three Replicates ±S.D.)

	Aa4		Aa6		Ac4		Ba2	
metal ions	IC_{50} (μM)[a]	CR[b] (%)	IC_{50} (μM)[a]	CR[b] (%)	IC_{50} (μM)[a]	CR[b] (%)	IC_{50} (μM)[a]	CR[b] (%)
Cd^{2+}	0.0942 ± 0.02	100	0.037 ± 0.01	100	0.263 ± 0.003	100	0.0592 ± 0.01	100
Mg^{2+}	148.625 ± 14.93	0.063	121.18 ± 10.83	0.035	147.063 ± 7.81	0.179	135.42 ± 5.34	0.0437
Hg^{2+}	7.173 ± 0.09	1.310	3.039 ± 0.144	1.21	26.48 ± 1.98	0.991	2.511 ± 0.18	2.358
Zn^{2+}	81.04 ± 11.17	0.116	18.52 ± 3.70	0.200	66.485 ± 5.92	0.396	76.69 ± 8.90	0.0772
Fe^{3+}	>178.57[c]	0.053	159.50 ± 10.92	0.023	100.52 ± 7.72	0.262	0.151.20 ± 15.83	0.039
Cu^{2+}	59.54 ± 4.82	0.158	17.29 ± 1.90	0.214	>156.25[c]	<0.168	15.77 ± 1.34	0.375
Pb^{2+}	10.60 ± 1.36	0.886	5.21 ± 0.54	0.710	>48.31[c]	<0.544	>48.31[c]	<0.122
Ni^{2+}	28.233 ± 1.87	0.333	13.48 ± 1.53	0.274	74.66 ± 2.70	0.352	>153.85[c]	<0.0385
Ag^{+}	76.62 ± 5.21	0.123	>92.59[c]	<0.040	>92.59[c]	<0.284	91.625 ± 9.04	0.0646
Ca^{2+}	178.1 ± 27.17	0.0528	>250[c]	<0.015	183.17 ± 10.05	0.143	>250[c]	<0.0237

[a] IC_{50} is the concentration of metal that inhibited the color development in the competitive immunoassay by 50%. [b] Cross-reactivity. [c] Precise determination beyond limits of assay.

Table 5. Percentage Recovery of Cadmium Fortified to Tap and Stream Water Samples by Competitive Inhibition ELISA

	cadmium (μg/L)			
sample	fortified	detected	S.D.[a]	mean recovery (%, $n = 4$)
tap water	50	43.95	5.8	87.9
	20	18.21	2.5	91.1
	5	4.83	0.7	96.7
stream water	50	56	2.9	112.0
	20	21.64	1.3	108.2
	5	5.27	0.5	105.5

[a] Standard deviation.

ELISA correlated well with the spike concentration, with a correlation coefficient of 0.975 for water and with overall mean recovery ranging from 87.9 to 112% (**Table 5**). Concerning the reproducibility, average intra-assay CVs and inter-assay CVs were 7.18% and 15.2%, respectively. The data showed that this analytical technique seemed to be adequate for effective environmental monitoring of cadmium.

DISCUSSION

The metal ion was too small and its structure was too simple to induce an immune response. Metal ions were therefore conjugated to a carrier protein (KLH) via ITCBE to increase the likelihood of eliciting cadmium-specific antibodies. In preliminary experiments, we attempted to prepare monoclonal antibodies for heavy metals according to the method described by Kishiro et al. (*21*); however, we did not obtain any usable monoclonal antibodies using this approach. If an antibody-producing cell is present at low levels in original lymphocyte population, the chances of producing a monoclonal antibody to a rare immunogen are low. The most reasonable explanation for our inability to isolate hybridomas that recognized metals in our early experiments was that lymphocytes that produced antibodies with the desired binding properties were present at low frequency in the original lymphocyte population.

In second series of experiments we immunized mice with Cd^{2+}–ITCBE–KLH conjugates via Ip injections and used hybridoma technology to create ~600 clones from a total lymphocyte population of 1×10^8 cells. In the experiments reported herein, hybridomas were generated using the ClonaCell-HY kit, which is designed to select and clone hybridomas soon after fusion. This eliminated the overgrowth of potentially valuable slow-growing clones by fast-growing clones.

There are two kinds of antigen binding epitopes: one is the linear epitope, and the other is the conformational epitope. Since the area of the antigen-binding sites of antibodies ranges from 160 to 900 Å (*28*) and the diameter of a cadmium ion is only 2.03 Å (*29*), it is not surprising that antibodies that bind to free metal ions have not been described. Therefore, metal ions cannot react directly with antibody, and reaction with the antibody is mediated by the metal–chelate complex. Experiments on the properties of metal-specificity antibodies have demonstrated that the main factors affecting antibody production were the stability of the metal–chelator complex and the three-dimensional structure of the metal–chelator complex. The antibodies appear to recognize differences in the shapes and electronic properties of distinct metal–chelate complexes (*27*).

Rapid, inexpensive, sensitive, and selective enzyme-linked immunosorbent assays (ELISAs) have been utilized in environmental science for 20 years or so. In our laboratory, many ELISAs have been developed for other toxic substances. Our goal was to develop simple analytical methods based on the formation of chelator of the metal ion to be detected. We have utilized the high affinity bifunctional chelator (ITCBE) coupled to KLH via a primary amine and linked with Cd^{2+} by binding to the carboxyl groups of the chelator. In contrast to previously reported antibody 2A81G5 (*16*), the MAbs reported herein did not show significant cross-reactivity with other metal–chelator complexes, indicating that these monoclonal antibodies are highly specific for cadmium.

This new ELISA can detect Cd^{2+} at concentrations as low as 0.313 μg/L and has an IC_{50} of only 4.19 μg/L for cadmium. In addition, to our knowledge, this optimization made it possible to detect cadmium ions which are below the threshold level regulated by WHO for cadmium in drinking water (0.01 mg/L) (*30*). These monoclonal antibodies should be very promising analytical tools for rapid and sensitive determination of metal ions in the environment.

ABBREVIATIONS USED

The following abbreviations were used in the text: AAS, atomic absorption spectroscopy; Ab, antibody; AI, additivity index; BSA, bovine serum albumin; CR, cross-reactivity; DMEM, Dulbecco's minimal essential medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetraacetic acid; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; Ep, epitope; FBS, fetal bovine serum; GFAAS, graphite furnace atomic absorption spectroscopy; HBS, Hepes-buffer saline (137 mM NaCl, 3 mM KCl, 10 mM Hepes, pH 7.4); HRP, horseradish peroxidase; ICPAES, inductively coupled plasma atomic emission spectroscopy; Ip, intraperitoneally; ITCBE, 1-(4-isothiocyanatobenzyl)ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; K_{aff}, equilibrium association constant; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; OD, optical density; PBS, phosphate-buffered saline (137 mM NaCl,

3 mM KCl, 10 mM phosphate, pH 7.4); TMB, 3,3′,5,5′-tetramethylbenzidine peroxidase substrate.

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Received for review April 8, 2007. Revised manuscript received July 27, 2007. Accepted July 30, 2007. This research was supported by National "863" Project (2007AA10Z439) of China and in part by the Office of Science (BER), U.S. Department of Energy, Grant DE-FG02–98ER62704 (D.A.B.).

JF071025L