Metal Binding Properties of a Monoclonal Antibody Directed toward Metal-Chelate Complexes

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Diane A. Blake‡§, Pampa Chakrabarti¶, Mehraban Khosraviani‡, Frank M. Hatcher‡, Connie M. Westhoff§, Peter Goebel, Dwane E. Wylie§, and Robert C. Blake II**

From the ‡Department of Ophthalmology, Tulane University School of Medicine, New Orleans, Louisiana 70112, the ¶Department of Microbiology, Meharry Medical College, Nashville, Tennessee 37208, the §Department of Biology, University of Nebraska, Lincoln, Nebraska 68588, and the **Department of Basic Pharmaceutical Sciences, Xavier University of Louisiana, New Orleans, Louisiana 70125

A monoclonal antibody that recognizes cadmium-EDTA complexes has been produced by the injection of BALB/c mice with a metal-chelate complex covalently coupled to a carrier protein. The ability of purified antibody to recognize 16 different metal-EDTA complexes was assessed by measuring equilibrium binding constants using a KinExa™ immunoassay instrument. The antibody bound to cadmium- and mercury-EDTA EDTA complexes with equilibrium dissociation constants of 21 and 26 μM, respectively. All other metal-EDTA complexes tested, including those of Mn(II), In(III), Ni(II), Zn(II), Co(II), Cu(II), Ag(I), Fe(III), Pb(II), Au(III), Tb(III), Ga(III), Mg(II), and Al(III) bound with affinities from 20- to 40,000-fold less than that determined for the cadmium-EDTA complex. With the exception of mercury and magnesium, the binding of divalent metal-chelate complexes was well-correlated with the size of the metal ion. The amino acid sequences of the heavy and light chain variable regions were deduced from polymerase chain reaction-amplified regions of the corresponding genes and subsequently used to construct molecular models of the antigen binding region. The key residue for cadmium binding in the model for 2A81G5 appeared to be histidine 96 in the heavy chain.

Monoclonal antibodies directed toward metal chelating agents or metal-chelate complexes have many potential uses both in medicine and in environmental analysis. In medicine, chelator-linked antibodies have been used in vivo to transport and deliver radioisotopes to specific target sites, such as tumors (1–3). The availability of monoclonal antibodies to the chelating agent has permitted researchers to investigate the biodistribution of these “magic bullets” in cancer chemotherapy (4). In environmental analysis, the availability of monoclonal antibodies that can distinguish among different metals provides a basis for rapid, sensitive immunoassays that can be used on-site to assess heavy metal contamination (5, 6). Monoclonal antibodies that bind to chelators such as diethylenetriaminepentaoacetic acid and 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid have been reported (7, 8); however, these antibodies were directed primarily toward the chelate portion of the molecule and did not demonstrate the ability to differentiate among various metals bound in the chelate complex. Two hybridomas that synthesized monoclonal antibodies showing metal ion specificity have also been reported. The first, CHA255, was the result of immunization with a derivative of In(III)-EDTA coupled to a carrier protein by a thioureido-t-benzyl group (4). The monoclonal antibody synthesized by CHA255 bound to a variety of chelate complexes but bound to indium-chelate complexes more tightly than it did to other chelated metals (4, 9). Monoclonal antibodies directed toward mercuric ions have also been elicited by immunization of animals with a glutathione-Hg derivative (10). These antibodies, which bind to mercury with high affinity, have been used to detect mercuric ions in aqueous samples (6) and are the basis for a commercially available immunoassay to monitor mercury contamination in environmental samples.

The molecular features responsible for an antibody’s ability to differentiate among metals have been explored in some detail with CHA255 (11). Binding studies have shown that CHA255 was highly specific for the indium chelate; the antibody’s affinity decreased from 24- to 20,000-fold when other metals were substituted for In(III) in the chelate complex. The structural basis for this fine discrimination was investigated by x-ray crystallographic analyses of the antigen-binding fragment complexed with either In(III) or Fe(III) chelates of thioureido-t-benzyl-EDTA. A notable feature of the antibody-In(III)-EDTA complex was the additional coordination of the chelated metal by a histidine residue from the heavy chain’s third complementarity determining region. This histidine coordination did not occur in the corresponding Fe(III) complex, due to a slightly different hapten coordination that reduced access to the metal. It was concluded that the absence of the histidine ligation was largely responsible for the 24-fold lower binding of the iron hapten to CHA255 relative to that of the indium hapten. The larger differences in the binding affinities observed with other metals were attributed to different configurations of the EDTA moiety around individual metal ions.

The present study describes the preparation and characterization of a metal-specific monoclonal antibody generated in a manner analogous to that used for CHA255. Equilibrium binding and kinetic studies revealed two principal features of the binding of metal chelates to this antibody. (i) The binding was metal-specific; of 16 metal ions tested, the greatest affinity was reserved for Cd(II) and Hg(II). (ii) The binding affinity in-
creased with the complexity of the organic chelator, indicating that the ureido-L-benzyl portion of the hapten participated in the binding reaction. The sequence of the light and heavy chain variable regions were deduced from the corresponding cDNA produced from PCR-amplified regions of the corresponding genes. Given the similarities in the functional properties of 2A81G5 and CHA255, a structural model of the metal-chelate binding pocket was constructed from the primary structure and the analogous x-ray crystallographic structure of the CHA255 binding pocket. A histidine in the heavy chain’s third complementarity determining region may be implicated in coordination of the metal ion. These studies suggest that it is possible to generate immunological reagents to a variety of metal ions.

**Experimental Procedures**

**Materials**—BALB/c AnNTacBR inbred mice were purchased from Taconic Laboratory Animals and Services, Germantown, NY. Cadmium foil (99.99%) was from Aldrich. (S)-4,4-(2,3-Bis(bis(carboxymethyl)amino)propyl)phosphoethanolamine and (S)-1-p-toluenesulfonyl-L-lysine ethylenediamine tetracetic acid were generously provided by Hybritech Inc. (San Diego, CA). Bovine serum albumin (BSA) (fatty acid ultrafree) was a product of Boehringer Mannheim. Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem. Atomic absorption standard metals were purchased from Perkin-Elmer. EZ SE Ig Agpurifying medium was a product of Middlesex Sciences (Foxborough, MA). TRIZol reagent for RNA isolation was purchased from Life Technologies, Inc. Fluorescein isothiocyanate conjugate of affinity purified goat-antimouse IgG was a product of Jackson ImmunoResearch Laboratories (West Grove, PA). Polymethacrylate beads (140–170 mesh) were obtained from Bangs Laboratories (Carmel, IN). Reagents for tissue culture and ELISA were obtained as described previously (5). Glassware was mixed acid-washed (12), and plasticware was soaked overnight in 3 x HCl and rinsed liberally with purified water before use.

**Preparation of Protein-Chelate Conjugates**—Protein-chelate complexes were prepared as described previously (5) and loaded with cadmium from a stock solution prepared by dissolving cadmium foil in warm ultrapure concentrated nitric acid. Purification and characterization of the conjugates were as described previously (5). Extent of substitution of free lysine groups was 33.2% for the BSA-thiourea-L-benzyl-ethylenediaminetetraacetic acid-cadmium conjugate (Cd-EDTA-BSA) and 15% for KLH-thiourea-L-benzyl-ethylenediaminetetraacetic acid-cadmium conjugate (Cd-EDTA-KLH). A second Cd-EDTA-BSA conjugate with 54.7% lysine substitution was used for determination of equilibrium binding constants.

**Immunization of Mice, Hybridoma Production, and Purification of Monoclonal Antibody**—Three mice were intraperitoneally injected biweekly with 50 μg of the Cd-EDTA-KLH conjugate emulsified in Ribi adjuvant (Ribi Immunocchemicals, Hamilton, MT). Third and fourth injections were given 30 and 45 days after the second boost. After the fourth injection, blood was collected from the tail vein, and antibody response was determined by indirect ELISA (described below). A final boost of 50 μg of Cd-EDTA-KLH conjugate in phosphate-buffered saline (PBS, 157 mM NaCl, 3 mM KCl, 10 mM sodium phosphate buffer, pH 7.4) was given intraperitoneally 4 days prior to fusion. Mouse spleen cells were fused with myeloma cells (X63-Ag8.653) using polyethylene glycol (13). Monoclonal antibody producing clones were selected by assaying the reactivity of culture supernatants in competitive ELISA format (5) using a cadmium-EDTA complex as the inhibiting antigen.

Ascites fluids were produced in mice by intraperitoneally injecting each of four mice with 1.27 x 10⁸ hybridoma cells. BALB/c mice were primed with incomplete Freund’s adjuvant 7 days before injection of cells. Ascites fluids were obtained after 10–15 days of injection. Heavy chain specificity of the monoclonal antibody was determined by sandwich ELISA using the subisotyping kit produced by Hyclone Laboratories (Logan, UT). The immunoglobulin G fraction of ascites was isolated using EZ SEP IgG partitioning medium according to the manufacturer’s instructions. Further purification of monoclonal antibody was achieved by chromatography on a Protein G-Superose column (Pharmacia Biotech Inc.). Protein concentration of the purified monoclonal antibody was determined by the method of Bradford (14). The concentration of IgG in the purified product and the light chain specificity of the antibody was determined by sandwich ELISA.

**Determination of Equilibrium Dissociation Constants and Association Rate Constants**—Equilibrium and kinetic measurements of the interactions of chelated metals with the 2A81G5 monoclonal antibody were performed with a KinExA™ automated immunoassay system (Sapidyne Instruments, Inc., Boise, ID). Briefly, the KinExA™ system is comprised of a capillary flow/observation cell (inner diameter = 1.6 mm) fitted with a microporous screen through which various solutions are passed under negative pressure. Uniformly dispersed in each mixture the average pore size of the screen (53 μm) were precoated with antigen and deposited above the screen in a packed bed. Individual solution mixtures of antigen and antibody either at or approaching equilibrium were then drawn through the packed bed of particles. Only those antibodies in each mixture with unoccupied binding sites were available to bind to the antigen coated on the solid phase. Quantification of primary antibody then captured was achieved by the brief exposure of the particles to a fluorescently labeled anti-species secondary antibody, followed by measurement of the resulting fluorescence from the particles after removal of excess unbound reagents.

For either equilibrium or kinetic measurements, polymethacrylate-ylate beads (85 ± 8) were coated with antigen by suspending 200 μl of beads (10 mg/ml each) of PBS containing Cd-EDTA-BSA. After 1.0 h of gentle agitation at 37 °C, the beads were centrifuged, and the supernatant solution was decanted. Any nonspecific protein binding sites remaining on the beads were blocked by the subsequent incubation of the beads with 1.0 ml of 10% (v/v) goat serum in PBS for an additional h at 37 °C. The blocked beads were stored at 4 °C in the blocking solution until further use.

A bead pack approximately 4 mm high was created in the observation cell by drawing 675 μl of a suspension of the blocked beads in PBS (6.7 mg beads/ml) over the screen at a flow rate of 1.5 ml/min and washing the retained beads with sufficient PBS buffer (1.0 ml) to remove the excess goat serum. The beads were then gently disrupted with a brief backflush of PBS (50 μl at 300 μl/min), followed by a 20-s settling period to create a uniform and reproducible pack. For equilibrium measurements, the 2A81G5 monoclonal antibody (final concentration of 0.1 μg/ml) was mixed with metal-chelates in PBS or individual metal ions in PBS amended with 5.0 mM EDTA. When the metal ion was Ag(I), 0.05 mM HEPES, pH 7.4, was substituted for the PBS. BSAs were present at 0.5 mg/ml in the reaction mixture to reduce nonspecific binding of the primary antibody. Once equilibrium was achieved (less than 2 min in all cases), 500 μl of the mixture were drawn past the beads, followed by 166 μl of the PBS-EDTA buffer to wash out unbound primary antibody and excess metal chelate. One ml of fluorescently labeled goat antimouse antibody (1.0 mg/ml in PBS-EDTA containing 1 mg/ml BSA) was then drawn past the beads. All solution transfers up to this point were accomplished with a flow rate of 500 μl/min. Unbound labeled secondary antibody was subsequently removed by drawing 3.0 ml of PBS-EDTA through the bead pack over a period of 2 min.

For equilibrium measurements with soluble Cd-EDTA-BSA, the assay protocol was modified by lowering the final concentration of Cd-EDTA-BSA to 0.01 μg/ml. A signal-to-noise ratio identical to that observed with 0.1 μg/ml of antibody was achieved by drawing a 10-fold greater volume of the reaction mixture (5.0 ml) past the packed beads. Control experiments showed that the signal amplitude was independent of the concentration of the primary antibody as long as the beads were exposed to the same total number of antibody molecules.

For kinetic measurements, equal volumes (250 μl) of the primary antibody (0.2 μg/ml) and metal-chelate solutions were mixed at rates of 250 μl/min each. The binding reaction proceeded for the 11 s it took the mixture to transverse the distance from the point of mixing to the point where the bead pack was first encountered. One ml of labeled secondary antibody was then drawn past the beads (at 500 μl/min), followed by 1.5 ml (at 750 μl/min) of PBS-EDTA. Data acquisition (initiated in all cases at the introduction of primary antibody to the beads) and instrument control were accomplished via a Toshiba Satellite™ 486/33 notebook computer interfaced to the KinExA™ system via software provided by Sapidyne, Inc. Dissociation constants were obtained from nonlinear regression analyses of the equilibrium binding data using a one-site homogenous binding model and software contained in SlideWrite™ (version 2.1, Advanced Graphics, Inc., Carlsbad, CA). Association rate constants

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1 The abbreviations used are: PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; KLH, keyhole limpet hemocyanin; Cd-EDTA-BSA, bovine serum albumin-thiourea-L-benzyl-ethylenediaminetetraacetic acid-cadmium conjugate; Cd-EDTA-KLH, keyhole limpet hemocyanin-thiourea-L-benzyl-ethylenediaminetetraacetic acid-cadmium conjugate; EOTUBE, 4-(N’-2-hydroxyethyl-thiourea) L-benzyl-EDTA; r.m.s., root mean square; CDR, complementarity determining region; H, heavy chain; L, light chain.
were obtained from nonlinear regression fits of the kinetic data to single exponential functions of time also using SlideWrite.

**Amplification, Cloning, and Nucleotide Sequencing of Antibody Variable Region Genes**—RNA was isolated from hybridoma cells with TRIzol reagent and enriched for poly(A<sup>+</sup>) RNA. First strand cDNA synthesis of the variable region was carried out with primers complementary to the 5<sup>′</sup>-end of the C<sub>j1</sub> domain of the γ<sub>1</sub> heavy chain or to the 5<sup>′</sup>-end of the C<sub>k</sub> domain of the light chain. The γ<sub>1</sub>-chain primer (GGCCGCACTGAGGCGGCACCTGAGTA) was complementary to amino acids 122–136 of the C<sub>j1</sub> domain and contained an SpeI site (underlined) at the 5<sup>′</sup>-end; the k-chain primer (GAAGATCTCTAGCTTACTGACGATCGATCAG) was complementary to residues 109–114 of the C<sub>k</sub> domain and contained an XhoI site (underlined) at the 5<sup>′</sup>-end for cloning. After cDNA synthesis, PCR amplification was carried out with the primers above and with consensus variable region primers (24). The heavy chain variable region primer (AGGTCCAGCTGTCCTCGAGTCAG) contained an XhoI restriction site (underlined) and the light chain variable region primer (CCAGTTGCAACTGCTGGTTTGATGACCCCAACTCACC) contained a SacI site at the 5<sup>′</sup>-end. The products were cloned into Bluescript (Stratagene, La Jolla, CA) and sequenced from both directions by the dideoxy chain termination method.

**Metal Ion Recognition by a Monoclonal Antibody**—Heavy and light chain models were constructed by sending the protein sequence to the Swiss-Model server (Automated knowledge-based protein modeling server at ExPASy). This server created a model including the complete light chain and heavy chain from framework 1 through framework 3. This is in accordance with the canonical structure theory (15, 16) since a limited number of canonical structures have been observed for all three CDRs in the light chain and CDR1 and CDR2 in the heavy chain. Since no set of canonical structures exists for CDR3 of the heavy chain (because of its variability), the CDR3 loop was built from a known structure with closest sequence homology. The sequence from amino acid 82 to the end of framework 4 was used to find an appropriate structure, using the program SCOP at Protein Data Bank (Brookhaven National Laboratories, Brookhaven, CT). The highest score in similarity with no gaps spanning the CDR3 region was obtained with the anti-2,4-dinitrophenol antibody An02 (Protein Data Bank code 1baf). The overall sequence similarity for the entire searched peptide was 77%, with 81% similarity in the framework 3 portion, 80% in framework 4, but only 34% in CDR3. The heavy chain model was completed by superimposing the backbone atoms of conserved amino acid residues located in the framework 3 region in both structures. The superimposed residues included amino acids 86–94 with an 89% sequence similarity, resulting in a r.m.s. deviation of 0.3384 Å. Model completion and the final analysis were performed with the programs InsightII and Discover (Biosym Technologies, San Diego, CA) on a Silicon Graphics Onyx Extreme (Silicon Graphics Inc., Mountain View, CA). The initial model was energy-minimized to relax any constraints that may have been introduced during the building process. It was then superimposed onto the structure of monoclonal antibody CHA255, whose structure has been solved to 2.2-Å resolution (11). Superpositioning of the heavy chain framework regions 1–3 gave a r.m.s. deviation of 0.768 Å, whereas framework regions 1–3 of the light chain gave a r.m.s. deviation of 1.7992 Å, resulting in an overall r.m.s. deviation of 1.2838 Å. The overall sequence similarity between 2A81G5 and CHA255 is 63% for the heavy chain and 45% for the light chain. The possible antigen binding site of the 2A81G5 monoclonal antibody was examined by comparing the model with the structure of CHA255 bearing an indium-loaded EOTUBE cadmium chelate acid in the binding pocket. The key residue for indium binding in the CHA255 antigen binding pocket, His-H95 (Kabat numbering (17)), and His-H96 in the present model were less than 0.5 Å apart; residue His-H96 was therefore considered important in the antigen recognition site of 2A81G5. The EOTUBE-cadmium complex was then put into the antigen binding site of the model. Due to the somewhat unfavorable r.m.s. value between the model and CHA255, the complex was slightly repositioned. This was done by calculating a docking grid for the model with the program Delphi (Biosym Technologies, San Diego, CA) and reorienting the ligand within the binding pocket according to minimum values in electrostatic and van der Waals interactions. The model was then energy-minimized and analyzed for possible interactions between His-H96 and the cadmium ion. A dynamics calculation was performed to simulate possible movements within the amino acid side chains, and the frame with the smallest His-H96--cadmium distance was minimized to a final maximum derivative of 0.01 kca/Å.

The model showed that a second His residue, in particular His-L27D (Kabat numbering, (17)), could be in close enough proximity to the metal cation to also be involved in the antigen-antibody interaction. To evaluate this possibility, a second model was built and investigated. The principal steps in model building were identical to those described above. In order to maximize possible interactions between the antigenic thioureido tail and the binding site on the antibody, the bond between the phytic C atom and the attached N atom was rotated by approximately 180° prior to dynamics calculation.

**RESULTS**

**Design of Antigen and Animal Response**—The cadmium-chelate complex of interest in this study was a low molecular weight hapten of insufficient size to elicit an immune response. For this reason, a bifunctional EDTA derivative previously shown to elicit an immune response in mice (4) was loaded with cadmium and covalently conjugated to a large carrier protein, keyhole limpet hemocyanin. The resulting conjugate was injected multiple times into the peritoneal cavity of three BALB/c mice, and the presence of antibodies directed toward the cadmium-chelate complex was assessed by ELISA, as shown in Fig. 1. Sera from all three mice showed reactivity toward both a metal-free EDTA-BSA conjugate and a cadmium-loaded EDTA-BSA conjugate; however, only one of the three mice demonstrated a preferential binding to the cadmium-loaded conjugate. This mouse (Mouse 3) was given an additional intraperitoneal injection with Cd-EDTA-KLH in PBS, and its spleen was used 4 days later for the preparation of hybridoma cells.

**Hybridoma Screening**—The hybridomas obtained after fusion with myeloma cells were initially screened by indirect ELISA for their ability to bind to Cd-EDTA-BSA. This preliminary screen yielded 49 positive clones; these clones were subsequently screened by competitive ELISA to identify those that synthesized antibodies with specificity for the cadmium ion present in the Cd-EDTA chelate complex. Supernatants from these 49 clones were tested for their ability to bind to adsorbed Cd-EDTA-BSA in the presence of soluble inhibitors, and the 49 clones could be separated into four general subtypes, as shown in Fig. 2. Eight of the clones showed a binding response similar to that of the clone designated 1D5. Although the antibodies synthesized by hybridoma 1D5 bound to Cd-EDTA-BSA, this binding could not be inhibited by 5 mM EDTA, 50 ppm cadmium in 5 mM EDTA, or 50 ppm zinc in 5 mM EDTA. It was concluded that the clones made antibodies directed primarily toward the benzylic group present in the linkage region between the EDTA and the carrier protein, and these hybridomas were not further characterized. A majority of the clones which passed the initial
screen (33 of the original 49) showed a response in competitive ELISA similar to that of the clone designated 2E4. Antibody binding was strongly inhibited by 5 mM EDTA, as well as by soluble Cd-EDTA and Zn-EDTA complexes. Since the antibodies secreted by these clones appeared to be directed primarily toward the EDTA portion of the antigen, these hybridomas were also excluded from further analysis. Of the eight clones remaining from the original 49, three had characteristics similar to that of the clone designated 4F10. The antibodies secreted by this type of clone did not recognize metal-free EDTA even when this soluble antigen was present at concentrations as high as 5 mM. However, these clones were excluded from further analysis because their secreted antibodies could not distinguish between cadmium and zinc complexes of EDTA.

The final five clones showed characteristics similar to that exemplified by 2A8. Addition of soluble 5 mM EDTA to the antibodies secreted by three out of five of these clones actually increased the strength of their interaction with immobilized Cd-EDTA-BSA. Soluble cadmium-EDTA complexes interacted strongly with these antibodies, whereas zinc-EDTA complexes showed minimal binding. Clone 2A8 was chosen for further experiments since this hybridoma had desirable growth characteristics and its supernatant showed the highest specific titer at limiting dilution (data not shown).

Clone 2A8 was subcloned by limiting dilution; the subclone of interest (2A81G5) was found to secrete an antibody of the IgG1 kappa type. Monoclonal antibody purified from ascites fluid produced by mice injected with clone 2A81G5 contained 465 mg/ml protein and 404.5 mg/ml IgG1. The purified antibody (>85% pure) had a specific titer of 253,150/mg IgG1. The purified antibody was then determined from measurement of the unbound antibody such as those illustrated in A, was expressed as a fraction of the concentration of total antibody binding sites and plotted versus the concentration of the free antigen in solution. Each determination was performed in duplicate; in some cases the error in the data was less than the diameter of the plotted points. The parameters for the curve drawn through the data points were determined by nonlinear regression analysis using a one-site homogeneous binding model.

**Fig. 2.** Representative results of hybridoma screening by competitive ELISA. Tissue culture supernatants from hybridomas that had passed a preliminary screen were diluted into PBS + 3% BSA. Aliquots of each monoclonal antibody were mixed with metal-free EDTA, cadmium in EDTA, or zinc in EDTA to yield a final EDTA concentration of 5 mM and a final metal concentration of 50 ppm. After 1 h at 25 °C, the incubation mixtures were added to Microwell plates coated with Cd-EDTA-BSA, and the ELISA was completed as described (5). Color formation is reported as a percentage of absorbance observed in the absence of added soluble antigen.

**Fig. 3.** Determination of equilibrium binding constants for the 2A81G5 monoclonal antibody. A, time courses of individual fluorescence responses in the KinExA™ instrument when different equilibrium mixtures of antibody, Cd-EDTA, and antibody-Cd-EDTA complex were exposed to beads coated with an excess of immobilized Cd-EDTA-BSA. The order and rates of reagent mixing are described under “Experimental Procedures.” Initial concentrations of Cd-EDTA were as follows: a, 0; b, 10 nM; c, 20 nM; d, 50 nM; and e, 50 µM. Primary and fluorescein isothiocyanate-labeled secondary antibody concentrations of 0.45 nM (0.9 nM in binding sites) and 1.0 µg/ml, respectively, provided acceptable signal-to-noise characteristics. B, example of equilibrium binding data. The concentration of the occupied antibody binding sites, determined from measurements of the unbound antibody such as those illustrated in A, was expressed as a fraction of the concentration of total antibody binding sites and plotted versus the concentration of the free antigen in solution. Each determination was performed in duplicate; in some cases the error in the data was less than the diameter of the plotted points. The parameters for the curve drawn through the data points were determined by nonlinear regression analysis using a one-site homogeneous binding model.
approximated a square wave corresponding to the fluorescence of the labeled secondary antibody during its transient passage past the beads in the observation cell. The signal failed to return to that of the background, indicating a nonspecific binding of the fluorescein-labeled antibody to the beads of 0.5%. When ligand was omitted from the equilibrium mixture (curve a), the instrument response from 90 to 190 s reflected the sum of two contributions: the fluorescence of the unbound labeled antibody in the interstitial regions among the beads and that of the labeled secondary antibody that had bound to primary antibody captured by the immobilized ligand on the beads. Binding of the secondary antibody was an ongoing process that produced a positive slope in this portion of the curve. When the excess unbound label was removed from the beads, the signal that remained was the sum of that from the nonspecifically bound antibody plus that of the labeled antispecies antibody specifically bound to the 2A81G5 monoclonal antibody captured on the beads. Equilibrium mixtures comprised of soluble ligand present at concentrations intermediate between those of zero and saturation thus provided intermediate instrument responses (curves b–d) from which the concentration of free 2A81G5 antibody in each mixture could be determined.

In principle, the concentration of free antibody could be calculated from the slopes of the curves in the 90–190-s interval, from the average value of a portion of the plateau in the 190–300-s interval, or from the corresponding integrals of the areas under selected portions of the curves. The latter alternative was chosen herein. Accordingly, the fraction of occupied binding sites on the 2A81G5 monoclonal antibody was taken as:

\[ \frac{I_0 - I_{\text{exp}}}{T_0 - I_{\text{exp}}} \]

(Eq. 1)

where \( I \) represents the integral of each curve over the interval of 220 to 300 s, and the subscripts 0, exp, and \( T \) refer to experimental traces corresponding to a ligand concentration of zero, an intermediate ligand concentration, and a saturating ligand concentration, respectively.

A plot of the fraction of occupied binding sites on the 2A81G5 monoclonal antibody as a function of the total ligand concentration is given in Fig. 3B. The data in Fig. 3B were typical of those cases where the concentration of bound ligand was insignificant compared with that of the total ligand (i.e., where the value of the dissociation constant was greater than that of the antibody concentration). The value of the apparent dissociation constant, \( K_d \), was obtained from a nonlinear regression fit of the following rectangular hyperbola to the data:

\[ \text{fraction of occupied binding sites} = \frac{X}{X + K_d} \]

(Eq. 2)

where \( X \) represents the free ligand concentration.

The structure of the chelator had an influence on the avidity of the 2A81G5 monoclonal antibody, as illustrated in Fig. 4. The data in Fig. 4 were plotted according to a rearranged form of Equation 2:

\[ \log(\text{free Cd(II) chelate}) = \frac{\varphi K_d}{1 - \varphi} \]

(Eq. 3)

where \( \varphi \) is the fraction of occupied binding sites on the population of antibody molecules. This presentation permitted the comparison of binding curves for different metal-chelator complexes over a wide range of ligand concentrations. The lower the curve on the abscissa axis, the greater the binding affinity. The value of the negative logarithm of the \( K_d \) in evident from the abscissa of the graph at the point where the fraction of occupied binding sites equals 0.5. Ionic cadmium alone was recognized by the 2A81G5 antibody with an apparent \( K_d \) of 80
of chelated metal ions that reacted for 11 s before the mixture encountered the packed beads. The primary data, which resembled those illustrated in Fig. 3A, now represented the concentration of unbound antibody plucked from a mixture of binding partners that was only 11 s old and far from equilibrium. Since technical difficulties prevented a wide range of reaction times between the point of mixing and separation of the unbound antibodies on the beads, the extent of the reaction was controlled by varying the concentration of the reagent in excess, the metal-chelate complex. Fig. 6 shows two examples of the concentration of unbound antibody remaining at 11 s after mixing on the concentration of the metal-chelator complex. A single exponential function of time was fit to each set of data:

\[ A = A_0 e^{-k_{obs} t} \]  

(Eq. 4)

where \( A \) is the concentration of free, unbound antibody at ligand concentration \([L]\), \( A_0 \) is the total concentration of free antibody, \( t \) is 11 s, and \( k_{obs} \) is the observed pseudo-first order rate constant for the concentration-dependent change. For the reversible bimolecular combination of an antibody with a molar excess of antigen, the value of \( k_{obs} \) equals \( k_1([L]) + k_2 \), where \( k_1 \) and \( k_2 \) are the bimolecular association and unimolecular dissociation rate constants, respectively. Since time was fixed in these experiments at 11 s, the \( k_2 \) term in the argument of the exponent became a constant that could be separated mathematically, and the \( k_{obs} \) term in Equation 4 reduced to \( k_1([L]) \). Values for the association rate constants were thus obtained from the nonlinear regression fits of exponential curves to data such as those presented in Fig. 6. Although values for the dissociation rate constant could in principle be extracted from the \( k_2 \) term factored from the exponential, in practice the values thus obtained exhibited unacceptably large standard errors. Accordingly, the value of the unimolecular dissociation rate constant was simply obtained from the identity \( K_d = k_2/k_1 \) in those instances where both \( K_d \) and \( k_1 \) were determined independently. Values for the association and dissociation rate constants for selected antibody-chelated metal interactions are also summarized in Table I.

**Nucleic Acid and Deduced Amino Acid Sequence of Light and Heavy Chain Variable Regions**—cDNA was prepared from clone 2A81G5 poly(A)+ mRNA by using primers complementary to the 5′ end of the CH1 domain of the \( \gamma_1 \) heavy chain or to the 5′ end of the C\(_\varepsilon\) domain of the light chain. After first strand synthesis, PCR amplification was carried out with the primers above and with consensus variable region primers (described under “Experimental Procedures”). Products of the correct molecular size were cloned and sequenced. The nucleic acid sequence and deduced amino acid sequence of the variable region of the light chain are shown in Fig. 7. The 2A81G5 antibody uses a \( V_{\varepsilon}1 \) and \( J_{\varepsilon}1 \) gene segment. The nucleic acid sequence and deduced amino acid sequence of the heavy chain variable region are shown in Fig. 8. The heavy chain sequence starts with amino acid number 6. The antibody uses a member of the \( V_{\varepsilon}Q52 \) gene family and the \( J_{\varepsilon}2 \) gene segment. Numbering of amino acids is according to the Kabat system (17).

**Molecular Modeling of Heavy and Light Chains**—Heavy and light chain models of the 2A81G5 antibody were constructed from the deduced amino acid sequences determined above and correlated to the known structure of antibody CHA255. The

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**TABLE I**

| Chelated metal          | Binding of chelated metals to 2A81G5 monoclonal antibody |
|-------------------------|-----------------------------------------------------------|
|                         | Chelated metal complex | Equilibrium dissociation constant | Association rate constant | Dissociation rate constant |
|                         |                         | \( M \)                         | \( M^{-1} \) s\(^{-1} \) | \( s^{-1} \) |
| EDTA-metal complexes    | AI(III)                  | 8.2 ± 2.0 \times 10\(^{-4} \)   | 2.2 ± 0.2 \times 10\(^{6} \) | 5.7 ± 0.7 \times 10\(^{-2} \) |
|                         | Mg(II)                   | 2.2 ± 0.5 \times 10\(^{-4} \)   | 5.4 ± 0.6 \times 10\(^{5} \) | 5.3 ± 0.6 \times 10\(^{-2} \) |
|                         | Ca(III)                  | 1.9 ± 0.2 \times 10\(^{-4} \)   | 5.0 ± 0.6 \times 10\(^{5} \) | 5.2 ± 0.6 \times 10\(^{-2} \) |
|                         | Ti(III)                  | 7.7 ± 1.2 \times 10\(^{-5} \)   | 4.9 ± 0.9 \times 10\(^{-5} \) | 5.1 ± 0.9 \times 10\(^{-5} \) |
|                         | Au(III)                  | 7.5 ± 0.6 \times 10\(^{-5} \)   | 2.7 ± 0.3 \times 10\(^{-6} \) | 2.5 ± 0.3 \times 10\(^{-6} \) |
|                         | Pb(II)                   | 7.4 ± 1.3 \times 10\(^{-5} \)   | 2.5 ± 0.2 \times 10\(^{-6} \) | 2.4 ± 0.2 \times 10\(^{-6} \) |
|                         | Fe(III)                  | 5.4 ± 0.6 \times 10\(^{-5} \)   | 2.1 ± 0.2 \times 10\(^{-6} \) | 2.0 ± 0.2 \times 10\(^{-6} \) |
|                         | Ag(II)                   | 5.0 ± 0.6 \times 10\(^{-5} \)   | 6.2 ± 1.3 \times 10\(^{-7} \) | 6.2 ± 1.3 \times 10\(^{-7} \) |
|                         | Cu(II)                   | 4.9 ± 0.9 \times 10\(^{-5} \)   | 1.0 ± 0.3 \times 10\(^{-7} \) | 1.0 ± 0.3 \times 10\(^{-7} \) |
|                         | Co(II)                   | 2.6 ± 0.3 \times 10\(^{-6} \)   | 2.2 ± 0.1 \times 10\(^{8} \) | 5.7 ± 0.7 \times 10\(^{-2} \) |
|                         | Mn(II)                   | 2.1 ± 0.3 \times 10\(^{-6} \)   | 2.5 ± 0.1 \times 10\(^{8} \) | 5.3 ± 0.6 \times 10\(^{-2} \) |
|                         | Hg(II)                   | 2.1 ± 0.3 \times 10\(^{-6} \)   | 2.5 ± 0.1 \times 10\(^{8} \) | 5.3 ± 0.6 \times 10\(^{-2} \) |
|                         | Cd(II)                   | 2.1 ± 0.3 \times 10\(^{-6} \)   | 2.5 ± 0.1 \times 10\(^{8} \) | 5.3 ± 0.6 \times 10\(^{-2} \) |
|                          | Phospho-EDTA             | 8.1 ± 1.1 \times 10\(^{-8} \)   | 2.5 ± 0.1 \times 10\(^{8} \) | 5.3 ± 0.6 \times 10\(^{-2} \) |
|                          | p-Nitrobenzyl-EDTA       | 5.6 ± 0.3 \times 10\(^{-10} \)  | 2.1 ± 0.1 \times 10\(^{7} \) | 1.2 ± 0.1 \times 10\(^{-2} \) |
|                          | BSA-thioureido-L-benzyl-EDTA | 7.2 ± 1.6 \times 10\(^{-11} \) | 4.6 ± 0.3 \times 10\(^{7} \) | 3.3 ± 0.7 \times 10\(^{-3} \) |
monoclonal antibody CHA255, whose crystal structure has been solved to 2.2-Å resolution (11), was raised to a hapten structurally related to the Cd-EDTA-KLH antigen used in the present study. The possible antigen binding site of the 2A81G5 antibody was examined by comparing the model with the structure of CHA255 bearing an indium-loaded EOTUBE tetraacetic acid in the binding pocket. The key residue for indium binding in the CHA255 antigen binding pocket (His-H95, Kabat number) and His-H96 in the model for 2A81G5 were less than 0.5 Å apart; residue His-H96 was therefore considered as being important in the antigen recognition site. The model also revealed a possible stacking interaction between tryptophan H52 and the phenyl moiety in the cadmium-loaded EOTUBE. This stacking, together with possible hydrogen binding with the nitro group of the antigen, may account for the higher affinity (37-fold) of the antibody for the $p$-nitrobenzyl-EDTA complex of cadmium as compared with a cadmium-EDTA complex. Fig. 9 shows molecular models of the heavy and light chains of the 2A81G5 monoclonal antibody. The heavy chain sequence starts with amino acid number 6. The number scheme is according to Kabat (17), and the CRD regions are indicated by a line over one-letter amino acid code. The antibody uses a member of the V$\gamma$452 gene family and the J$\gamma$2 gene segment. The dotted lines in the CDR3 region have been added to bring the numbering in the heavy chain into agreement with the Kabat numbering system.

**DISCUSSION**

This report represents the second instance in which metal-specific antibodies have been prepared to a particular metal-chelate complex and demonstrates that this procedure may provide a general method for the isolation of antibodies with binding affinities for individual di- and trivalent metals. Metal ions have affinity for amino acid side chains containing sulfur, nitrogen, and oxygen atoms (19), and antibodies that contain these amino acids in their complementarity determinants have been shown to bind metal ions with high affinity. The antibody response to metal-chelate complexes may be useful in the development of new diagnostic and therapeutic agents for metal-related diseases.
ing regions might be expected to have the ability to bind tightly to metal ions or metal-chelate complexes. The high affinity for metals demonstrated by these antibodies is not without precedent in other metal-protein interactions; the copper binding site of human albumin (20), the calcium binding loop of thermolysin (21), and zinc liganding of carbonic anhydrase (22) all demonstrate metal ion binding affinities greater than or equal to that of antibodies to metal-chelate complexes.

Although the CHA255 antibody with specificity for indium chelate complexes and the 2A81G5 antibody reported herein were generated by very similar procedures, the two antibodies show significant differences in their affinities for their primary ligand (4). CHA255 bound to indium chelates with highest affinity and approximately 20-fold less tightly to Fe(III)-chelate complexes. The values were not well-correlated with either atomic radii or metal-chelate stability constants. The 2A81G5 antibody bound most tightly to chelates of cadmium and mercury and less tightly to other mono-, di-, and trivalent metals. With the exception of Hg(II) and Mg(II), the binding of seven other divalent metal ion-chelate complexes was well-correlated with the size of the divalent metal ion, as shown in Fig. 10. In this analysis, the negative logarithm of the equilibrium dissociation constant for each metal-EDTA complex was plotted versus the absolute value of the difference between the atomic volume of the cadmium ion and that of the individual metal ion. Magnesium bound to the antibody less tightly than would be expected for its molecular size. Mg(II) has been reported to form a heptacoordinate complex with EDTA and one molecule of water, rather than the usual hexacoordinate complexes formed by EDTA and other divalent metal ions (18), and this heptacoordinate complex might be predicted to bind differently.

**Fig. 9. Molecular models of the heavy and light chains of the anti-cadmium antibody.** All photographs show the heavy chain in red and the light chain in blue. Residues of special interest are colored by atom (carbon, green; oxygen, red; nitrogen, dark blue; hydrogen, white; cadmium, magenta). Panel A, hydroxethyl-thioureido-L-benzyl-EDTA-cadmium complex in the antibody binding pocket. Closest distance between cadmium and the Nε in His-H96 is 2.72 Å. Panel B, close-up of the cadmium complex and two residues (His-H96 and Trp-H52) that may be involved in binding interactions. Panel C, space filling model of antibody variable region and the cadmium complex. Panel D, close-up of the cadmium complex and the histidine residue (His-L27D) that was implicated in the cadmium-protein interaction in the second model.

**Fig. 10. Linear free energy relationship in the binding of EDTA complexes of divalent metal ions to the 2A81G5 monoclonal antibody.** The negative logarithm of the equilibrium dissociation constant for each metal-EDTA complex was plotted versus the absolute value of the difference between the atomic volume of the cadmium ion and that of the individual metal ion. Atomic volumes were calculated from ionic radii as reported (23).
in the antibody binding site. Mercury bound more tightly than could be predicted from its reported ionic radius. The molecular basis for this tighter-than-expected binding is under investigation.

CHA255 has been reported to bind to indium complexes of EDTA with a $K_D$ of $5.9 \times 10^{-9} \text{ M}^{-1}$ (4), whereas 2A81G5 bound to the cadmium complexes of EDTA with a $K_D$ of $2.1 \times 10^{-3} \text{ M}^{-1}$. Alteration of the ligand structure to include the L-benzyl group actually involved in the interactions of 2A81G5 with the cadmium chelate increased by almost 40-fold (see Table I).

In an attempt to determine the molecular basis for these binding characteristics, the variable regions of the light and heavy chains were amplified by PCR, cloned, and sequenced. A comparison of the sequence in the complementarity determining regions that interact strongly with the L-benzyl portion of the hapten suggested an alternative conformation in which another histidine was of interest because in the crystal structure of the CHA255-In(III)-EDTA complex, this histidine provided the only direct coordination between the antibody and the chelated metal (11). Molecular models were subsequently constructed of 2A81G5 to determine if the His-96 in the CDR3 region of the heavy chain could assume the conformation necessary to provide such a coordination site for the cadmium ion. These models suggested that histidine 96 in the heavy chain may play a role in the binding of the cadmium-chelate complex to 2A81G5; tryptophan 52 of the heavy chain may also interact with the phenyl moiety of the antigen.

The availability of antibodies that bind to specific metal ions will prove useful on at least two different levels. Such antibodies have been useful in the design of immunoassays that could be used to assess environmental contamination by specific heavy metals (5, 6). These assays could provide rapid, on-site analyses of specific heavy metal contamination. In addition, the study of a series of metal-specific monoclonal antibodies may provide new information about protein-metal interactions and may ultimately generate sufficient information to permit the design of recombinant antibodies with customized metal ion specificity.

REFERENCES

1. Camera, L., Kinuya, S., Garmestani, K., Wu, C., Brechbiel, M. W., Pai, L. H., McMurry, T. J., Gansow, O. A., Paik, C. H., and Carraquillo, J. A. (1994) J. Nucl. Med. 35, 882–889
2. Kozak, R. W., Raubitschek, A., Mirzadeh, S., Brechbiel, M. W., Junghaus, R., Gansow, O. A., and Waldmann, T. A. (1989) Cancer Res. 49, 2639–2644
3. Gansow, O. A. (1991) Nucl. Med. Biol. 18, 369–381
4. Reardon, D. T., Mears, C. F., Goodwin, D. A., McTigue, M., David, G. S., Stone, M. R., Leung, J. P., Bartholomew, R. M., and Frincke, J. M. (1985) Nature 316, 265–268
5. Chakrabarti, P., Hatcher, F. M., Blake, R. C., Ladd, P. A., and Blake, D. A. (1994) Anal. Biochem. 217, 70–75
6. Wylie, D. E., Carlson, L. D., Carlson, R., Wagner, F. W., and Schuster, S. M. (1991) Anal. Biochem. 194, 381–387
7. Gillette, R. W., Singleton, J., Janowicz, A., and Gilman, S. C. (1989) J. Immunol. Methods 124, 277–282
8. Anderson, W. H. K., Reed, J. A., and Pollock, D. K. (1992) Hybridoma 12, 677–688
9. Meyer, D. L., Fineman, M., Unger, B. W., and Frincke, J. M. (1990) Bioconjugate Chem. 1, 278–284
10. Wylie, D. E., Lu, D., Carlson, L. D., Carlson, R., Babacan, K. F., Schuster, S. M., and Wagner, F. W. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 4104–4108
11. Love, R. A., Villafranca, J. E., Aust, R. M., Nakamura, K. K., Jue, R. A., Major, J. G., Radhakrishnan, R., and Butler, W. F. (1993) Biochemistry 32, 19559–19565
12. Thiers, R. C. (1957) Methods Biochem. Anal. 5, 273–335
13. Gelfter, M. L., Margulies, D. H., and Scharff, M. D. (1977) Somatic Cell Mol. Genet. 3, 211–236
14. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
15. Murzin, A. G., Brenner, S. E., Hubbard, T., and Chothia, C. (1995) J. Mol. Biol. 247, 536–540
16. Chothia, C., and Lesk, A. M. (1987) J. Mol. Biol. 196, 901–917
17. Kabat, E. A., Wu, T. T., Reid-Miller, M., Perry, H. M., Gottesman, K. S., and Foeller, C. (1991) Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda
18. Lind, M. D., Hamor, J. H., Hamor, T. A., and Hoard, J. L. (1964) Inorg. Chem. 3, 34–38
19. Regan, S. L. (1993) Annu. Rev. Biophys. Biomol. Struct. 22, 257–281
20. Kruck, T. P. A., Lau, S. J., and Šakar, B. (1976) Can. J. Chem. 5, 1300–1308
21. Toma, S., Campagnoli, S., Margarit, I., Grandi, G., Bolognesi, M., De Filippis, V., and Fontana, A. (1991) Biochemistry 30, 97–106
22. Quicho, F. A., and Lipscomb, W. N. (1971) Adv. Protein Chem. 25, 1–78
23. Lide, D. R. (ed) (1995–1996) CRC Handbook of Chemistry and Physics, 76th Ed., pp. 12–14, CRC Press, Boca Raton, FL
24. Huse, W., Sastry, L., Iverson, S., Kang, A., Alting-Mees, M., Burton, D., Benkovic, S., and Lerner, R. (1989) Science 246, 1275–1281