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Dwane E. Wylie
University of Nebraska-Lincoln, dwylie1@unl.edu

Di Lu
BioNebraska Inc.

Larry D. Carlson
BioNebraska Inc.

Randy Carlson
BioNebraska Inc., rrcarlson11@gmail.com

K. Funda Babacan
University of Marmara

See next page for additional authors

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Monoclonal antibodies specific for mercuric ions

(hybridomas/metals)

DWANE E. WYLIE*†‡, DI LU‡, LARRY D. CARLSON‡, RANDY CARLSON‡, K. FUNDA BABACAN§, SHELDON M. SCHUSTER‡¶, AND FRED W. WAGNER‡‖

†School of Biological Sciences, University of Nebraska, Lincoln, NE 68588; ‡BioNebraska, Inc., 3940 Cornhusker Highway, Lincoln, NE 68504; §Department of Microbiology, University of Marmara, Istanbul, Turkey; ¶Department of Biochemistry and Molecular Biology, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610; and ‖Department of Biochemistry, University of Nebraska, Lincoln, NE 68583

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ABSTRACT **Monoclonal antibodies (mAbs) that react with soluble mercuric ions have been produced by injection of BALB/c mice with a hapten–carrier complex designed to maximize exposure of the metal to the immune system. Three hybridomas producing antibodies that reacted with bovine serum albumin (BSA)–glutathione–HgCl, but not with BSA–glutathione, were isolated from the spleen of a mouse given multiple injections with glutathione–HgCl conjugated to keyhole limpet hemocyanin. Stable subclones were established from two of these antibodies, designated mAb 4A10 and mAb 1F10. The binding of both antibodies to immobilized BSA–glutathione–HgCl was inhibited by soluble $HgCl_2$, and dissociation constants for mercuric chloride binding were 2.3 and 3.7 nM for mAbs 4A10 and 1F10, respectively. Both antibodies bound mercuric acetate with similar affinities, demonstrating that the antibodies were capable of binding to mercuric ions in the presence of a different counterion than the one used in the immunogen. Reactions were not observed with other metal cations by either antibody. These data demonstrate the successful induction of antibodies that react very specifically with mercuric ions in solution regardless of the presence of a carrier.**

The most vigorous immune responses are induced by large molecular weight, structurally complex molecules with a high degree of foreignness for the stimulated recipient. An immune response to a small, relatively simple compound, known as a hapten, can also be induced by linking it to an immunogenic carrier, usually a protein, before injection. Even then, there appear to be limits to the types of molecules that can induce specific responses, in that most of the commonly used haptens for classical immunological studies have contained aromatic rings or charged, polar regions, as exemplified by nitrophenyl derivatives and phosphocholine. Smaller, less complex molecules, such as metals or metallic ions, have not been considered to be of sufficient size or complexity to induce the formation of specific antibodies. This belief has been held despite the fact that small molecules in general, and metals in particular, have long been known to induce cell-mediated immunity, as in contact hypersensitivity (1), and that other proteins, such as enzymes, can demonstrate specificity with respect to metal binding. Furthermore, exposure to heavy metals, such as lead and mercury, can induce autoimmune disease in genetically predisposed strains of mice and rats (2), and observations by several investigators have suggested that metal ions affect the specificity of antibodies produced against proteins or haptens to which the metals are bound (3–9). Finally, it was noted several years ago that myeloma patients with hypercupremia and hypercalcemia produced antibodies that bound copper and calcium (10, 11).

These observations indicate that metals can at least influence antibody specificity and, rarely, in immunopathological conditions, can serve as haptens toward which an antibody response can be directed. This report describes the production of monoclonal antibodies (mAbs) specific for soluble mercuric ions by immunization of BALB/c mice with a complex consisting of mercuric chloride–glutathione–keyhole limpet hemocyanin (KLH).

MATERIALS AND METHODS

Mice. BALB/c mice were obtained from the animal colony of the School of Biological Sciences, University of Nebraska, which was established with breeders from The Jackson Laboratory.

Antigen Preparation. Glutathione was first conjugated to KLH or bovine serum albumin (BSA) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride as described (12). After dialysis against phosphate-buffered saline (PBS), 3 ml of glutathione–BSA or glutathione–KLH was added dropwise to 25 ml of 1 mM $HgCl_2$ in water while stirring. The solution was then dialyzed overnight against one change of PBS and two changes of water. Atomic absorption analysis revealed 38 mol of mercury per 400 kDa of KLH and 8 mol of mercury per mol of BSA.

Mouse Immunization and Hybridoma Production. BALB/c mice were given five intraperitoneal injections at 2-week intervals with 50 μg of KLH–glutathione–HgCl per injection. Antigen was emulsified in complete Freund's adjuvant for the first two injections and in either incomplete Freund's adjuvant or PBS for all subsequent injections.

One week after the fifth injection, a drop of blood was collected from the tail vein of each mouse into 0.45 ml of PBS. This was considered a 1:10 dilution, which was diluted 10-fold further in PBS containing 2% BSA prior to ELISA as described below.

Before fusion for hybridoma production, a mouse was injected intraperitoneally with 10 μg of KLH–glutathione–HgCl in PBS. Four days later, its spleen was used for fusion as described (13).

For production of ascites fluid, 5×10^6 hybridoma cells were injected into the peritoneal cavity of a BALB/c mouse that had been primed with pristane 7–60 days earlier. Ascites fluid was harvested 1–2 weeks after injection.

ELISA. Microtiter plates to which either BSA–glutathione or BSA–glutathione–HgCl had been adsorbed were blocked by incubation for 2 hr at room temperature with 5% nonfat dry milk in PBS. One hundred microliters of culture fluid or serum was added to the appropriate well and incubated for 2 hr at room temperature. The plate was then washed three times with ELISA wash (0.1% Nonidet P-40 in PBS), fol-

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Abbreviations: BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody.

*To whom reprint requests should be addressed.

lowed by addition of 100 μl of goat anti-mouse antiserum (Cappel Laboratories). The plate was incubated and washed as described above; then 100 μl of rabbit anti-goat antiserum conjugated with alkaline phosphatase (Sigma) was added. After a 2-hr incubation at room temperature, the plate was washed three times with ELISA wash followed by 5–10 rinses under gently running tap water. One hundred microliters of 2 mM *p*-nitrophenyl phosphate in 1 M diethanolamine (pH 9.6) containing 25 mM $MgCl_2$, was added, and the A_{405} of each well was measured after incubation at room temperature for 15–30 min.

Inhibition of Antibody Binding by Soluble Mercuric Chloride. Fifty microliters of various concentrations of mercuric chloride from 20 mM to 20 pM in PBS, in addition to 50 μl of diluted ascites fluid containing either mAb 4A10 or mAb 1F10, was added to appropriate wells of a microtiter plate to which BSA–glutathione–HgCl had been adsorbed. The plate was incubated at room temperature for 30 min, then rinsed three times with ELISA wash. One hundred microliters of horseradish peroxidase-conjugated goat antiserum specific for mouse IgM, IgG, and IgA (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added, and the plate was processed through the ELISA procedure described above.

The inhibition of antibody binding for each mercuric chloride concentration was calculated according to the following formula:

$$\% \text{ inhibition} = \{1 - [(A_{405} \text{ of exp} - A_{405} \text{ of neg})/(A_{405} \text{ of pos} - A_{405} \text{ of neg})]\} \times 100.$$

The negative (neg) control consisted of wells containing a mAb specific for dinitrophenol instead of mAb 4A10 or mAb 1F10, while for the positive (pos) control, the PBS added to the wells contained no mercuric chloride.

Determination of the Dissociation Constant of Mercury-Specific Antibodies. The dissociation constants of mAb 4A10 and mAb 1F10 for mercuric chloride and other metals were determined by the procedure of Friguet *et al.* (14). Two-fold dilutions of each metal salt from 10 μM to ≈10 pM were used in these experiments. The wells of a microtiter plate containing no antigen were blocked with nonfat dry milk. Fifty microliters of diluted ascites fluid was added to 50 μl of various concentrations of the metal salt being tested for antibody binding. The metal and antibody mixture was incubated for 1 hr at room temperature and then transferred to a microtiter plate containing adsorbed BSA–glutathione–HgCl.

The metal/antibody solution was incubated in this plate for 60 min, after which the plate was washed as described above, and 100 μl of horseradish peroxidase-conjugated goat antiserum specific for mouse IgM, IgG, and IgA was added. After incubation for 15 min at room temperature, the A_{405} was measured. Preliminary experiments demonstrated that no readjustment of the antigen–antibody equilibrium in the liquid phase occurred in the coated wells with the conditions under which these experiments were carried out (data not shown). The dissociation constants of the antibodies for each metal were then calculated from the slopes of the Klotz plots, as described (14).

RESULTS

To increase the likelihood of inducing mercury-specific antibodies, a hapten–carrier complex was designed that would maximize the exposure of the mercury, making it more likely that the metal itself would be recognized as a distinct entity by the immune system. It was felt that the use of chelating agents, in which the metal is part of a multidentate complex inside a latticework with multiple interactions between it and the functional groups of the chelating agent (Fig. 1*A* and ref. 15), would lead to production of antibodies specific for the conformation of the chelating agent imparted to it by the metal. In this case, an antibody produced against a metal–chelate complex would probably recognize the chelating agent when it contains any metal that imparts a conformation similar to the one it assumed when used for antibody induction.

The strategy devised for this study involved the use of glutathione, which is a tripeptide of γ-L-glutamyl-L-cysteinylglycine, as a carrier for mercury. Because of the high affinity of mercury for sulfhydryl groups (17), no other functional groups in the peptide or protein would be required for maintenance of the cysteine–mercury complex (16). Thus, a mercuric ion in the proper molecular environment would be bound to the complex by interaction with only the -SH group of a single cysteine residue (Fig. 1*B*). The presentation of Hg^{2+} to the immune system as a monodentate ligand might allow selection of antibodies whose binding sites contain additional ligands that can interact with the remainder of the coordination sphere of the metal.

To test the feasibility of this approach, eight BALB/c mice were given five intraperitoneal injections of KLH–

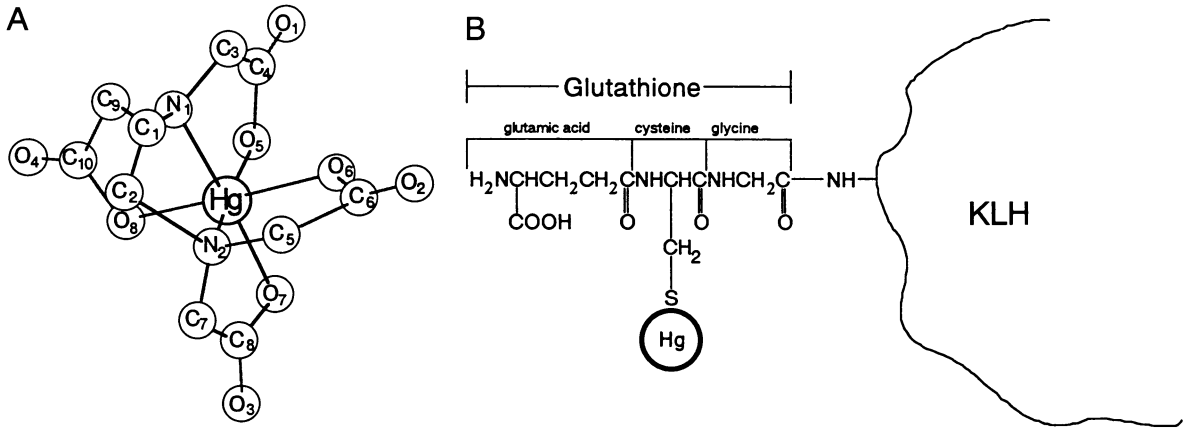

FIG. 1. Schematic diagrams of mercuric ions coordinated to EDTA and glutathione. (*A*) Representation of the coordination of mercury to EDTA as a sexadentate ligand, adapted from Mellor (15). The bonds between the atoms comprising a girdle around the mercury atom are represented by thick lines, while the other bonds are represented as thinner lines to maintain a three-dimensional effect. (*B*) Representation of mercury as a monodentate ligand coordinately bound to the sulfhydryl group of the cysteine residue of glutathione. The binding of mercury to glutathione via only the sulfhydryl group is based on data of Fuhr and Rabenstein (16).

glutathione–HgCl at 2-week intervals. One week after the fifth injection, blood was collected from the tail vein of each mouse and assayed by ELISA for the presence of mercury-specific antibodies. Table 1 shows the reactivity of each serum sample with BSA–glutathione and BSA–glutathione–HgCl, along with the difference in reactivity. Sera from five of the eight mice (nos. 2, 4, 5, 6, and 7) demonstrated higher reactivity with BSA–glutathione–HgCl than with BSA–glutathione, suggesting that they might contain antibodies specific for either Hg^{2+} itself or for an epitope composed of both Hg^{2+} and glutathione.

Since mouse 6 demonstrated the highest relative reactivity with BSA–glutathione–HgCl, it was given an intraperitoneal injection of 10 μg of KLH–glutathione–HgCl in PBS, and its spleen was used 4 days later for fusion. One hundred thirty-four hybridomas were obtained and screened by ELISA for reactivity with BSA–glutathione–HgCl and BSA–glutathione. The results were compared to the reactivity of a dinitrophenol-specific mAb with the same antigens, which served as the negative control. Four antibodies (1H11, 2A9, 3A12, and 3H9) reacted with both BSA–glutathione–HgCl and BSA–glutathione, as evidenced by A_{405} values 2–4 times above background (Table 2). Three other antibodies (1F10, 3E8, and 4A10) reacted strongly with BSA–glutathione–HgCl but showed only background reactivity with BSA–glutathione. These antibodies were considered presumptively specific for $HgCl_2$ or HgCl–glutathione. Stable subclones were established from 1F10 and 4A10 by limiting dilution for further characterization.

The reactivity of these antibodies with only BSA–glutathione–HgCl indicated that mercuric ions constituted the epitope, or at least an important part of the epitope, recognized by the antibodies. To investigate whether the antibodies would react with mercuric chloride alone, 10-fold dilutions of $HgCl_2$ from 10 mM to 10 pM were used to inhibit antibody binding to BSA–glutathione–HgCl adsorbed to the wells of a microtiter plate (Fig. 2). The binding of both antibodies to adsorbed antigen was inhibited by soluble $HgCl_2$, with 50% inhibition between 1 and 0.1 nM $HgCl_2$ for each. The specificity of the inhibition was demonstrated by the inability of the same concentrations of mercuric chloride to inhibit the binding of an asparagine synthetase-specific mAb (mAb 3D11) to asparagine synthetase. These data demonstrated that reactivity of both antibodies with $HgCl_2$ was independent of the presence of either glutathione or the carrier.

Table 1. Reactivity of serum from BALB/c mice injected with BSA–glutathione (GSH)–HgCl

Mouse	BSA–GSH*	BSA–GSH–HgCl*	% difference†
1	1.332	1.214	−8.9
2	0.808	0.886	+9.7
3	2.382	1.505	−36.8
4	0.654	0.947	+44.8
5	0.682	0.848	+24.3
6	0.453	0.756	+66.9
7	0.567	0.865	+52.6
8	1.670	1.456	−12.8
Normal serum	0.299	0.308	+3.0

One drop of blood was collected from the tail vein of each mouse into 0.45 ml of PBS, which was assumed to be a 1:10 dilution. A 10-fold dilution of this was made in 2% BSA in PBS and was used for ELISA as described. Normal serum was obtained from an unimmunized mouse and served as background reactivity for each antigen.

*A_{405} of a 1:100 dilution of serum from each mouse when assayed by ELISA against the indicated antigen. Each number represents the average of triplicate determinations.

†Calculated by the formula % difference = [(A_{405} of BSA–GSH–Hg − A_{405} of BSA–GSH)/(A_{405} of BSA–GSH)] × 100.

Table 2. Reactivity of hybridoma antibodies with BSA–glutathione–HgCl and BSA–glutathione

Hybridoma	BSA–glutathione–HgCl	BSA–glutathione
1H11	1.246	1.202
2A9	0.758	1.052
3A12	1.792	2.127
3H9	1.606	2.134
1F10	1.175	0.406
3E8	1.076	0.410
4A10	1.104	0.400
NC	0.428	0.456

Fifty microliters of culture fluid was assayed for the presence of antibody specific for the antigen shown. Each value represents the average A_{405} of triplicate determinations for each antibody. The negative control (NC) consisted of an antibody specific for dinitrophenol. The A_{405} obtained with this antibody was considered background reactivity.

These results showed clearly that antibodies capable of reacting with soluble mercuric chloride could be induced by immunization with an appropriate antigen. The existence of metal-binding proteins, however, is not unusual. In most cases, though, these proteins bind very strongly to their native metals, but other metals will bind with lower affinity. Therefore, it was of interest to determine the affinity of the antibodies for mercuric ions as well as to identify other metals that might bind. The other metal salts used for these analyses included barium chloride, cadmium chloride, chromic chlo-

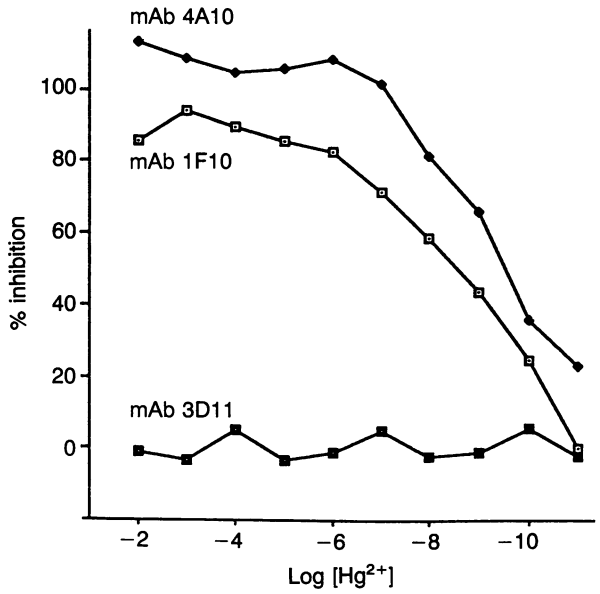

FIG. 2. Inhibition of binding of mAb 4A10 and mAb 1F10 to immobilized BSA–glutathione–HgCl by soluble mercuric chloride. Fifty microliters of each concentration of mercuric chloride and 50 μl of diluted ascites fluid containing the indicated antibody were incubated for 30 min at room temperature in the wells of a microtiter plate containing adsorbed BSA–glutathione–HgCl. The plates were then washed and horseradish peroxidase-conjugated goat anti-mouse antiserum was added. After incubation and washing as described above, 2,2′-azinobis(3-ethylbenzthiazoline sulfonate) substrate was added, and the A_{405} was measured after 15 min. Each point represents the average of triplicate determinations. Percentage inhibition was calculated as described. The positive control consisted of wells containing 50 μl of PBS without mercuric chloride, and the negative control consisted of wells containing a dinitrophenol-specific mAb instead of mAb 4A10 or mAb 1F10. mAb 3D11 is an antibody specific for bovine asparagine synthetase, whose production and characterization have been described (18).

ride, copper chloride, ferrous sulfate, gold chloride, mercuric acetate, nickel chloride, selenium oxide, silver chloride, and zinc chloride.

The dissociation constant of mAb 4A10 for $HgCl_2$ was 2.3 ± 0.8 nM, while that of mAb 1F10 was 3.7 ± 1.5 nM (Table 3). Neither antibody bound any of the other metals to a detectable extent. Since the highest concentration of each metal salt used in these experiments was 10 μM, the dissociation constants of both antibodies for the other metals was at least 3–4 orders of magnitude higher than for mercury. In addition, the antibodies did not bind to glutathione–HgCl any more strongly than they did to $HgCl_2$ (data not shown), demonstrating that glutathione does not increase the strength of the binding of mercury to the antibody and, thus, probably does not bind to the antibody.

Table 3 also includes the dissociation constants of both antibodies for mercuric acetate. As can be seen, the affinity of mAb 4A10 for mercuric acetate was 4.1 ± 0.1 nM, while that of mAb 1F10 was 8.2 ± 2.5 nM. These results clearly indicate that the mercuric ion itself is the major epitope recognized by these antibodies, regardless of the counterion originally present in the mercury-containing compound being assayed for antibody reactivity.

DISCUSSION

Although metal-binding proteins occur widely in nature, metals are generally considered incapable of eliciting a specific antibody response under normal conditions, although, as mentioned above, they can affect the specificity of antibody responses to some metalloproteins and can also elicit cell-mediated immunity. In addition, antibody light chains can be modified to coordinate zinc for use in antibody-mediated catalysis (19).

The results presented here, however, demonstrate clearly that, under appropriate conditions, mercuric ions can induce extremely specific antibodies that exhibit no detectable cross-reactivity with other metals and bind mercuric chloride with a high affinity, even when it is not conjugated to a large molecular weight carrier. The sensitivity of these antibodies has recently been demonstrated by their ability to detect mercuric ions at a concentration of 0.2 ppb in 100 μl of water (20).

Table 3. Dissociation constants of mAb 4A10 and mAb 1F10 for various metal salts

Metal salt	mAb 4A10	mAb 1F10
$HgCl_2$	$2.3 \pm 0.8 \times 10^{-9}$	$3.7 \pm 1.5 \times 10^{-9}$
$BaCl_2$	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$
$CdCl_2$	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$
$CrCl_3$	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$
$CuCl_2$	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$
$AuCl_3$	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$
$FeSO_4$	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$
$NiCl_2$	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$
SeO_2	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$
$AgNO_3$	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$
HgOAc	$4.1 \pm 0.1 \times 10^{-9}$	$8.2 \pm 2.5 \times 10^{-9}$

Two-fold dilutions of each metal salt from 10 μM to ≈10 pM were used in these experiments. The wells of a microtiter plate containing no antigen were blocked with nonfat dry milk. Fifty microliters of diluted ascites fluid was added to 50 μl of various concentrations of the metal salt being tested for antibody binding. The metal solution and the antibody were incubated for 1 hr at room temperature and then transferred to a microtiter plate containing adsorbed BSA–glutathione–HgCl and analyzed by ELISA as described. The dissociation constants of the antibodies for each metal were then calculated from the slopes of the Klotz plots as described (14). Each value represents the average derived from four separate experiments, each done in duplicate.

It seems likely that the successful induction of metal-specific antibodies was due to the method by which the metal ions were presented to the immune system. Other investigators have used metal–chelate complexes for induction of metal-specific antibodies. However, in this case, the chelating compound surrounds the metal with a cage-like structure (Fig. 1*A*; see also ref. 15). It seems likely under these conditions that the immune system would respond to the conformation of the complex imparted to it by the particular metal bound. In this scenario, the role of the metal in the antigen–antibody interaction would be an indirect one, in which it determines the shape of the complex without necessarily coming into direct physical contact with the antigen-binding site. If this were the case, an antibody specific for either a metal–chelate complex or a protein containing a highly coordinated metal would bind when the epitope contains a metal that imparts the same conformation as the one present when the complex was used for antibody induction. This prediction seems to be borne out by patterns of cross-reactivity reported for antibodies to porphyrins (21), metal–chelate complexes (9, 22), and other metal-binding proteins (3, 5). These antibodies bind most strongly with the complex containing the metal present when used for immunization, but they will cross-react when other metals are bound to the complex.

In contrast to the situation discussed above for chelating agents, peptides or proteins containing cysteine residues can be used as the carrier for mercuric salts because of the high affinity of mercury and its derivatives for sulfhydryl groups (17). Therefore, if glutathione or another cysteine-containing peptide were used as a carrier, a mercuric ion in the proper milieu could be bound to the complex by interaction with only the -SH group of a single cysteine residue. Such a situation would maximize the exposure of the mercuric ion and increase the probability that the metal would be perceived as a distinct entity by the immune system and not simply as a component of a larger epitope. This does not mean to imply that this is the only way in which mercuric ions are bound in the protein complex used for immunization. In actuality, they probably exist in all possible coordination states, but perhaps only those bound to a single sulfhydryl group can induce antibodies with the specificity described here.

From the standpoint of metalloprotein chemistry, the affinity of these antibodies for Hg^{2+} is not without precedent, since it is approximately of the same magnitude as carboxypeptidase A for its native metal Zn^{2+} (23). However, as most metalloproteases bind a multiplicity of metal ions, the singular specificity of the antibodies for Hg^{2+} is highly unusual.

From an immunological standpoint, the affinity of the interaction between the antibodies and mercuric ions is high for such a simple hapten. Generally, affinities of hapten-specific antibodies are 2–4 orders of magnitude lower than those reported here, although the affinities of some of the more tightly binding fluorescein-specific mAbs are similar to these (24).

It is difficult to envision the factors responsible for the specificity of the interaction between the antibodies and a molecule so small as a mercuric ion, since the area of the antigen-binding sites of antibodies ranges from 160 to 900 $Å^2$ (25), and the diameter of a mercuric ion is only 2.2 Å (26). The specificity of these antibodies suggests that sulfhydryl groups are not involved in the interaction, since reactivity with other sulfhydryl-reactive metals, such as silver, cadmium, and lead, was not observed. However, since mercury derivatives have an extremely high affinity for sulfhydryl groups, with dissociation constants as low as 10^{-42} (17), it is possible that a partially occluded sulfhydryl group could be present in the antigen-binding site and that only mercury, because of its affinity, can react with it. The basis of the interaction between mercuric ions and the antigen-binding site should

become more evident when the variable regions of the heavy and light chains have been characterized.

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