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Unusual Amino Acid Usage in the Variable Regions of Mercury-Binding Antibodies

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ABSTRACT **Monoclonal antibodies (mAb) specific for mercuric ions were isolated from BALB/c mice injected with a mercury-containing, hapten-carrier complex. The antibodies reacted by enzyme-linked immunosorbent assay with bovine serum albumin-glutathione-mercuric chloride (BSA-GSH-HgCl) but not with BSA-GSH without mercury. Nucleotide sequences from polymerase chain reaction products encoding six of the antibody heavy-chain variable regions and seven light-chain variable regions revealed that all the antibodies contained an unpaired cysteine residue in one hypervariable region, which is unusual for murine antibodies. Mutagenesis of the cysteine to either tyrosine or serine in one of the Hg-binding antibodies, mAb 4A10, eliminated mercury binding. However, of two influenza-specific antibodies that contain cysteine residues at the same position as mAb 4A10, one reacted with mercury, although not so strongly as 4A10, whereas the other did not react at all. These results suggested that, in addition to an unpaired cysteine, there are other structural features, not yet identified, that are important for creating an appropriate environment for mercury binding. The antibodies described here could be useful for investigating mechanisms of metal-protein interactions and for characterizing antibody responses to structurally simple haptens. Proteins 1999;37:429–440.** © 1999 Wiley-Liss, Inc.

Key words: monoclonal antibodies; ELISA; nucleotide sequence; site-directed mutagenesis; pComb3 phagemid

INTRODUCTION

The humoral immune system responds to immunogenic challenge by producing antibodies that react specifically with the inducing immunogen. Beginning with the pioneering work of Landsteiner,[1] considerable effort has been spent to define the concept of immunogenicity. Two important characteristics involved in immunogenicity are size and structural complexity.[2] Molecules generally must have a molecular mass of 5,000–10,000 Da before they can induce formation of specific antibodies. Molecules smaller than this can induce antibody formation, but they are usually conjugated as haptens to large molecular weight carriers to do so.

The importance of structural complexity is suggested by the vigorous immune responses induced by large molecular weight proteins. Even haptens, though, seem to have some structural requirements, as shown by the fact that the most common ones are derivatized benzene rings, such as dinitrophenol, or polar molecules, such as phosphorylcholine.[3] Molecules with less structural complexity, such as simple inorganic compounds, are usually considered incapable of inducing formation of specific antibodies.[2]

In an apparent contradiction to these size and structural requirements, our laboratory has produced monoclonal antibodies (mAbs) that react specifically with mercuric ions.[4,5] The antibodies were initially identified by their reactivity in an enzyme-linked immunosorbent assay (ELISA) with bovine serum albumin-glutathione-mercuric chloride (BSA-GSH-HgCl) and their lack of reactivity with BSA-GSH without mercury. They were subsequently shown to react specifically with free mercuric ions in solution.[5] The antibodies bound mercuric ions with a high affinity but did not react with other metals tested or with the carrier to which the metal had been conjugated for injection.[5]

These antibodies are interesting for a number of reasons. First, they indicate that antibodies capable of reacting with such relatively simple molecules as metals are either encoded in the primary repertoire or can arise by somatic mutation or other mechanisms that generate antibody diversity, such as combinatorial diversity in V, D, and J gene joining and in heavy- and light-chain combination. Second, they constitute a model system that is amenable to investigating the interactions of metals with proteins. Finally, they could form the basis for simple, convenient immunoassays to detect mercury in various matrices. Their use for detection of mercury in environmen-

Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid); GSH, glutathione; HAU, hemagglutinating unit; KLH, keyhole limpet hemocyanin; OD, optical density; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PhAb, antibody expressed on a phage surface; RT, room temperature.

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tal soil samples and fish tissue has already been demonstrated.[6,7]

We describe the reactivity of additional mercury-binding antibodies and report the nucleotide sequences of their heavy- and light-chain variable regions. Each antibody contains an unpaired cysteine residue in one of its hypervariable regions, which is the least common amino acid in the antigen-binding sites of antibodies of mice and other vertebrates. We also demonstrate that the cysteine is an absolute requirement for mercury binding.

MATERIALS AND METHODS

Mercury-Specific Hybridoma Antibodies

Mercury-specific hybridoma antibodies were produced as described previously.[5] Antibodies were considered mercury-specific if they reacted in an ELISA with BSA-GSH-HgCl but not with BSA-GSH.

Enzyme-Linked Immunosorbent Assay

Mercury-specific antibodies were assayed as described previously.[5] Mercuric nitrate was added to microtiter plates containing adsorbed BSA-GSH, and the plates were then used as the immunoadsorbent in an ELISA. Reactivity with influenza virus hemagglutinin was performed as described.[8] Briefly, PR8 virus was diluted in phosphate-buffered saline (PBS) to a concentration of 1 hemagglutinating unit (HAU)/μL, and 50 μL of the virus suspension was dried in each well of a microtiter plate.

In both the mercury and influenza ELISA, the plates were blocked with 1% polyvinyl alcohol in PBS for 1 hour, and then the appropriate monoclonal antibody was added and incubated for 2 hours at room temperature (RT). After the plates were washed with PBS/0.1% Triton X-100, 100 μL of goat anti-mouse serum conjugated to horseradish peroxidase was added, and the plates were incubated for 1 hour at RT. After washing, 100 μl of 2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid) (ABTS) was added to each well, and the absorbance at 405 nm was measured after 15–30 minutes. For isotype analysis, a rabbit antiserum specific for a single mouse isotype (BioRad, Hercules, CA) was used, followed by peroxidase-conjugated, goat anti-rabbit serum.

PCR Amplification of Antibody Variable Regions

Initially, the sequence of the first six amino acids of the heavy and light chains of mAb 4A10 and the light chain of mAb 1F10 were determined as described.[9–11] The most probable nucleotide sequences for these residues were determined from Kabat et al.,[12] and the corresponding primers were synthesized by the Oligonucleotide Synthesis Facility at the University of Florida. Each heavy-chain, variable-region primer contained an *Xho* I site at its 5′ end, and each light-chain, variable-region primer contained a *Sac* I site at its 5′ end.

Ribonucleic acid (RNA) was isolated from hybridoma cells with guanidine isothiocyanate[13] and enriched for poly(A)+ RNA.[14] First-strand complementary deoxyribonucleic acid (cDNA) synthesis was catalyzed by MuLV reverse transcriptase with primers complementary to the 5′ end of the C_Hl domain of the appropriate heavy chain or to the 5′ end of the C_κ domain of the light chain. The κ-chain and heavy-chain primers contained *Xba* I and *Spe* I sites, respectively, at their 5′ ends. These same primers were used with the V-region primers mentioned above for polymerase chain reaction (PCR) amplification of all variable regions, except the 2D5 and 5B6 heavy chains. For those antibodies, V_H primer 6 of Huse et al.[15] was used. The PCR conditions were as described.[15] The products were cloned into pBluescript (Stratagene, La Jolla, CA), and their sequences were determined by the DNA Sequencing Facility of the University of Nebraska.

Site-Directed Mutagenesis

The 4A10 light chain was cloned into the pComb3 phagemid,[16] and the resulting phagemid, designated p4A, was used for cloning the Fd region of the 4A10 heavy chain and the mutants and revertants derived from it. The megaprimer method was used for site-directed mutagenesis.[17] *Pvu* II-linearized pBluescript DNA containing the heavy chain of mAb 4A10 was used as template for PCR. Two mutagenesis primers were synthesized, one replacing cysteine at position 95 with tyrosine (TGC → TAC), and the other replacing cysteine with serine (TGC → TCC) at the same position. Each of the mutagenesis primers was used with a carboxy-terminal primer for the first amplification to give a product of approximately 350 bp. This product was electrophoresed in 0.8% agarose, extracted with glass milk (Bio 101, San Diego, CA), and used as 3′ primer with the 5′, amino-terminal primer to amplify the remainder of the variable region. The nucleotide sequences of the mutagenized fragments were determined to confirm the mutations. Each mutation was reverted to wild type as above, by using primers that converted either tyrosine or serine back to cysteine at position 95.

Phab Production

Escherichia coli XL-1 Blue was transformed with the following vectors, all of which contained the gene for the 4A10 light chain with the 4A10 heavy-chain gene modified as indicated: p4A (unmutagenized mAb 4A10 heavy chain with Cys at position 95); $p4A_{cys\rightarrow tyr}$ (Tyr mutation at position 95 of the heavy chain); $p4A_{cys\rightarrow ser}$ (Ser mutation at position 95 of the heavy chain); $p4A_{tyr\rightarrow cys}$ (revertant from Tyr to Cys at position 95 of the heavy chain); and $p4A_{ser\rightarrow cys}$ (revertant from Ser to Cys at position 95 of the heavy chain). Transformants were selected on LB agar supplemented with 50 μg/mL ampicillin and 10 μg/mL tetracycline. Individual colonies from each clone were grown in 10 mL of SB medium with 50 μg/mL ampicillin at 37°C to an OD_{600} of 0.2. At this point, bacteria were infected with 10 μL of a 10^{11} pfu/mL suspension of bacteriophage M13 VCS (Stratagene, La Jolla, CA). One hour after infection, kanamycin was added to 70 μg/mL, and the culture was incubated overnight at 25°C. Phabs were precipitated with 4% PEG/3% NaCl and resuspended in PBS to a concentra-

TABLE I. Reactivity of Mercury-Specific Antibodies With BSA-Glutathione-HgCl and BSA-Glutathione†

Antibody	BSA-GSH-HgCl	BSA-GSH	Isotype
1F10	0.550	0.092	IgA
4A10	0.636	0.078	IgM
1C11	0.458	0.094	IgM
5G4	0.313	0.028	IgG_1
23F8	1.134	0.168	IgM
2D5	0.818	0.090	IgG_1
5B6	0.738	0.019	IgG_3

†One hundred microliters of antibody-containing culture fluid was screened against the indicated antigen in an ELISA as described in Materials and Methods. The numbers shown are the A_{405} obtained in the ELISA.

TABLE II. Heavy- and Light-Chain Gene Segments in Mercury-Specific Antibodies

	Heavy chain			Light chain	
Antibody	V_H	D	J_H	V_κ	J_κ
1F10	ND[a]	ND	ND	9	2
4A10	J558	FL16.2	4	1	1
1C11	J558	FL16.1	4	21	1
5G4	J558	ND	2	9	2
23F8	J558	SP2.3,4[b]	2	38C	4
2D5	7183	SP2.3,4[b]	3	12/13	2
5B6	7183	SP2.5,6,7[b]	3	9	2

[a]ND, not determined.
[b]The D gene segment could be identified only as one of the possibilities shown.

tion of 10^{12} pfu/mL. Phab concentration was determined by colony formation and by the phab-ELISA described below.

Mercury-Phab ELISA

BSA-GSH-HgCl assay plates were prepared as described previously.[5] Phabs expressing the wild-type 4A10 light chain with the Fd region of a mutated or wild-type 4A10 heavy chain were diluted and added to ELISA plates. Wells containing BSA-GSH without mercuric nitrate were used as negative controls. The plates were incubated for 1 hour at RT, followed by addition of 100 µL of a 1:10,000 dilution of rabbit anti-M13 serum in PBS/3% BSA. After incubation for 30 minutes at RT, plates were washed 10 times with PBS/0.1% sodium dodecyl sulfate (SDS), and then rinsed with water. One hundred microliters of peroxidase-conjugated, goat anti-rabbit serum was added. After a 30-minute incubation, the plates were washed, and substrate was added. The absorbance at 405 nm was measured after incubation for 30 minutes at RT.

The phab concentration was also standardized by ELISA to ensure that ELISA differences with phabs containing mutagenized heavy chains were due to differences in mercury binding, not to differences in phab concentration. Each phab preparation was diluted in PBS, and 100 µL of each dilution was incubated in a well of a microtiter plate for 1 hour at RT. Wells were washed with PBS/0.1% SDS, followed by a rinse with water. The plates were then blocked with 5% BSA, and 100 µL of a 1:10,000 dilution of rabbit anti-M13 serum in PBS/3% BSA was added. The ELISA procedure from this point was the same as described above. Phab concentration by colony formation always correlated with A_{405} in the phab-ELISA (data not shown), indicating that ELISA absorbancies reflected differences in mercury binding by phabs, not differences in phab concentration.

RESULTS

Previous results from our laboratory have shown that hybridomas producing antibodies specific for mercuric ions can be isolated from mice injected with keyhole limpet hemocyanin (KLH)-GSH-HgCl.[5] Antibodies that reacted with BSA-GSH-HgCl but not with BSA-GSH were initially considered mercury-specific. They were subsequently shown by competitive ELISA to react with free mercuric ions.[5] The ELISA results and the isotypes for seven mercury-binding antibodies identified in this way are shown in Table I. The reactivity of each antibody with BSA-GSH-HgCl was at least 5 times higher than with BSA-GSH alone. The dissociation constants of two of these antibodies, mAb 4A10 and 1F10, have been determined to be between 10^{-8} and 10^{-9} M,[5] which is similar to that of other metal-binding proteins. Three antibodies (4A10, 1C11, and 23F8) were immunoglobulin (Ig)M, two (5G4 and 2D5) were IgG_1, one (5B6) was IgG_3, and the other (1F10) was IgA. All used a κ light chain.

Because of their unusual specificity, we sought to identify features of the antigen-binding sites that might account for reactivity with mercuric ions. Therefore, the nucleotide sequences were determined for the variable regions of the heavy and light chains. The V and J gene families of each heavy and light chain and the heavy chain D gene segments that could be identified are shown in Table II. The cDNA encoding the heavy chain of 1F10 could not be successfully amplified, although the reason for that is unknown at present.

The heavy chains of 2D5 and 5B6 used members of the V_H7183 family, whereas all the others used members of the V_HJ558 family. D gene segments from the FL16 and SP2 families were present in the antibodies. No J_H gene preference was apparent, because J_H2, J_H3, and J_H4 were each used by two of the antibodies. Three antibodies (mAbs 1F10, 5G4, and 5B6) used the same member of the $V_\kappa9$ family and were identical throughout their entire variable regions, except for the last V-region codon of 5B6. Members of the $V_\kappa1$, $V_\kappa21$, $V_\kappa38C$, and $V_\kappa12/13$ families were each used once.

The complete nucleotide and deduced amino acid sequences for the variable regions of the heavy and light chains are shown in Figures 1 and 2, respectively. The distinguishing feature of each mercury-binding antibody was the presence of an unpaired cysteine residue in one

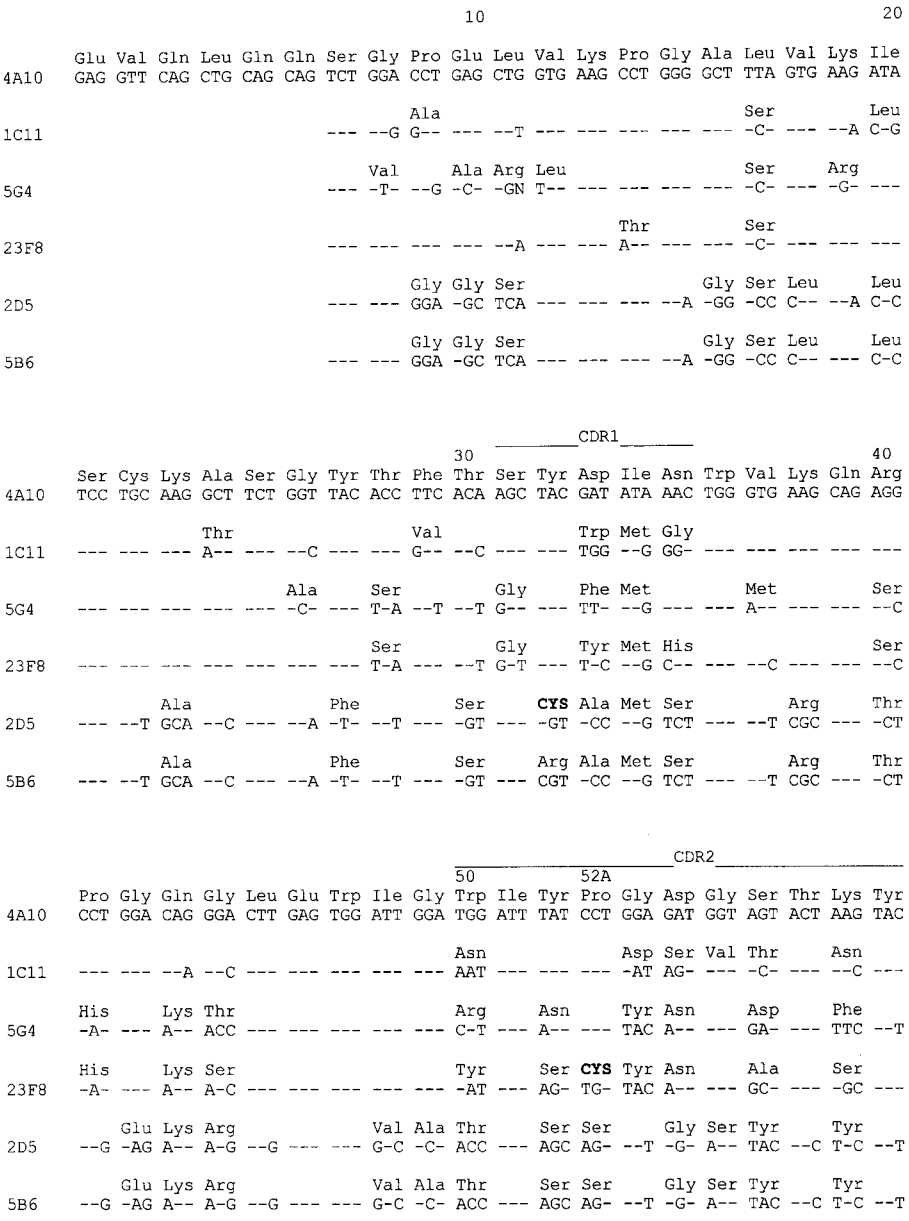

Fig. 1. Nucleotide and deduced amino acid sequences of heavy-chain variable regions of mercury-specific antibodies. The sequence for amino acids 1–6 corresponded to the PCR primers and was known with certainty only for mAb 4A10. The cysteine residues thought to be important for mercury binding are bolded in capital letters. The numbering scheme is according to Kabat et al.[12] Dashes indicate sequence identity and dots indicate gaps compared to 4A10.

hypervariable region of either the heavy or light chain. Three of the antibodies had the cysteine in their heavy chains. Specifically, it was at position 95 in complementary-determining region (CDR3) of the 4A10 heavy chain, position 52A in CDR2 of the 23F8 heavy chain, and position 32 in CDR1 of the 2D5 heavy chain (Fig. 1). The other four antibodies contained the unpaired cysteine in their light chains. In mAb 1C11 it was at position 32 in CDR1, whereas mAbs 1F10, 5G4, and 5B6 all contained cysteine at position 91 in CDR3 (Fig. 2).

In some of the antibodies, cysteine probably resulted from somatic mutation of a tyrosine codon. For example, mAb 4A10, whose cysteine residue at position 95 of the heavy chain was encoded by the D gene segment, apparently used two codons (TAC TAT) from the $D_{FL16.2}$ gene segment,[18] and the TAC codon was modified to TGC,

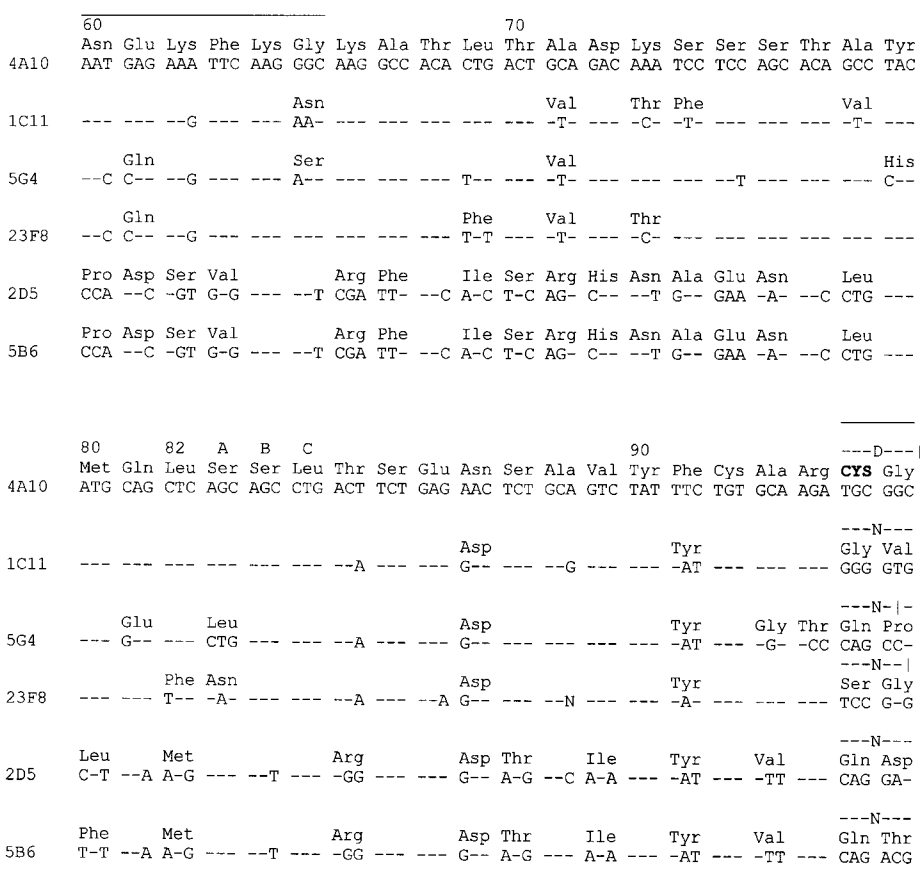

Figure 1. (Continued.)

changing tyrosine to cysteine. Somatic mutation could have also been responsible for the cysteine at position 32 of the heavy chain of mAb 2D5, because none of the V_H7183 germline genes identified thus far contain cysteine at this position.[19–25] Alternatively, this could represent a previously undiscovered V_H7183 germline gene, because it was only only 94% identical to the most closely related V_H gene from this family. Somatic mutation probably also accounted for the cysteine in the mAb 1C11 light chain, because the most closely related sequences contain tyrosine at position 32 of the light chain instead of cysteine.[26–28]

In other cases, such as mAb 23F8, the cysteine was encoded in the germline sequence of the V_H gene segment.[29] This was also true for the genes encoding the light chains of mAbs 1F10, 5G4, and 5B6, all of which used the same combination of V_κ and J_κ gene segments and contained a cysteine residue at position 91 in CDR3 of the light chain. Although none of the reported V_κ genes most similar to this one contain a cysteine codon at this position,[30–32] the presence of the same light chain in three mercury-specific antibodies from separate fusions suggests that it was germline encoded.

None of the antibodies that used a V_HJ558 gene showed more than 86% identity to each other, indicating they belong to separate subfamilies within the J558 family.[33,34] MAb 1C11 is a member of V186.2, mAb 23F8 belongs to 205.12, and mAb 4A10 is 94% identical to the MVARG2 gene, which has not been assigned to a subfamily. MAb 5G4 shows less than 86% similarity to the reported subfamilies and could represent a new one. On the other hand, mAbs 2D5 and 5B6 could have used the same member of the 7183 family, because these two V_H sequences differed from each other by only five nucleotides, which resulted in two amino acid changes. Despite the extensive similarity of 2D5 and 5B6, mercury must bind to these antibodies differently, because cysteine was present in CDR1 of the 2D5 heavy chain and in CDR3 of the 5B6 light chain.

The presence of an unpaired cysteine residue in one of the hypervariable regions of every mercury-specific antibody suggested that this was important for mercury binding. To verify this, the cysteine in CDR3 of mAb 4A10 was modified by site-directed mutagenesis to either tyrosine, which is the residue encoded at this position in the germline D gene, or to serine, because of its structural

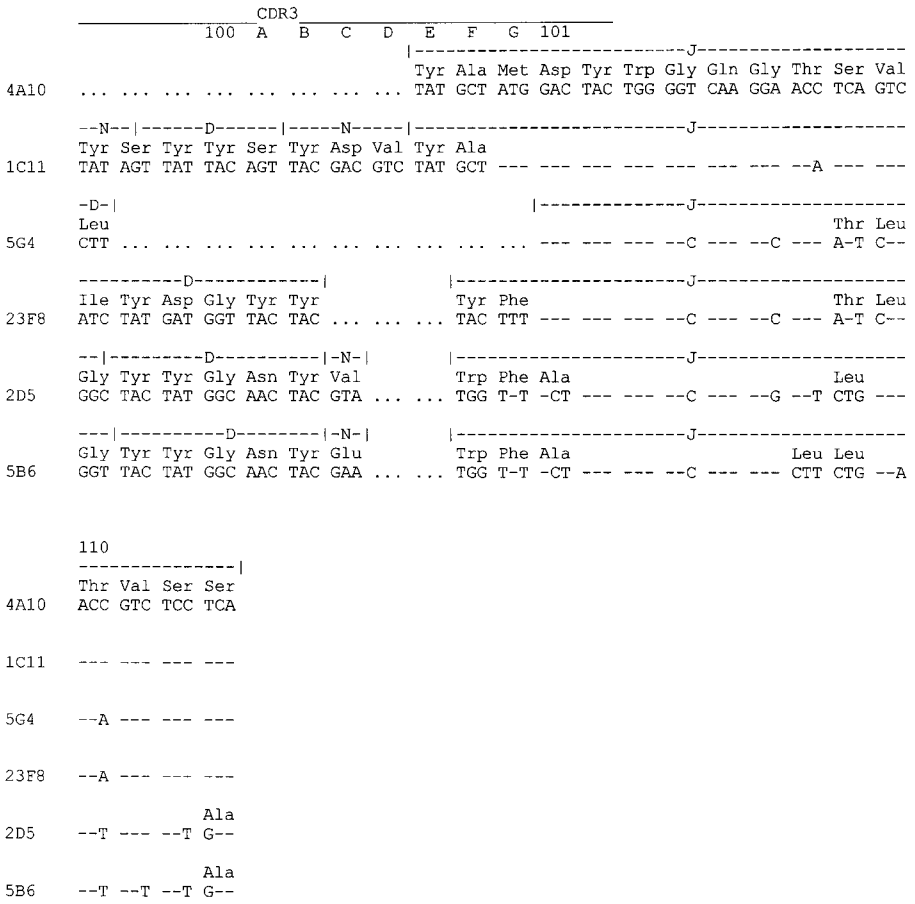

Figure 1. (Continued.)

similarity to cysteine. When the cysteine was changed to either tyrosine ($p4A_{cys\rightarrow tyr}$) or serine ($p4A_{cys\rightarrow ser}$), reactivity with BSA-GSH-Hg was the same as reactivity with BSA-GSH (Fig. 3). The background binding in this experiment, as demonstrated by binding of phage expressing 4A10 to BSA-GSH or binding of the mutated 4A10 to BSA-GSH-Hg, was higher than the results shown for binding of 4A10 to BSA-GSH because of "stickiness" of the phage (data not shown).

To ensure that the effect on mercury binding was due only to the intended amino acid modification, the tyrosine and serine were converted back to cysteine. In both cases, binding to mercury was restored (compare reactivity to $p4A_{tyr\rightarrow cys}$ and $p4A_{ser\rightarrow cys}$ with BSA-GSH-Hg and BSA-GSH in Fig. 3).

These results clearly demonstrated that cysteine was required for mercury binding by mAb 4A10 and most likely by the other antibodies. They also raised the question of whether the relative structural simplicity of mercuric ions compared with other antigens might enable any antibody with an unpaired cysteine residue in one of its hypervariable regions to bind mercury. To address this, two influenza hemagglutinin-specific antibodies, H37-24 and H37-88,[35] were tested for mercury binding. These two antibodies used V_H genes of the 36–60 family, but, like mAb 4A10, contained an unpaired cysteine residue at position 95 in CDR3 of their heavy chains. The amino acid sequence comparisons of mAbs 4A10 with H37-24 and H37-88 are shown in Figure 4. The two influenza-specific antibodies were identical in CDR1 and differed by only three amino acids in both CDR2 and CDR3 of their heavy chains. The light chains of the influenza antibodies showed only one difference in CDR1 and one in CDR3. The heavy chains of H37-88 and H37-24 showed extensive differences with mAb 4A10 heavy chain throughout the variable region, including all the amino acids of heavy chain CDR3 except cysteine at position 95. Overall, the heavy-chain variable regions of the two influenza-specific antibodies were 92% identical to each other at the amino acid level, but were only 64–67% identical to 4A10. Likewise, the light-chain variable regions of the influenza-specific antibodies were 95% identical but were only 63–64% identical to 4A10.

When tested in the mercury ELISA, mAb H37-24 showed some reactivity with BSA-GSH-HgCl, although not so much as mAb 4A10, whereas mAb H37-88 did not react at all (Fig. 5). $HgCl_2$ was required for mAb H37-24 reactivity,

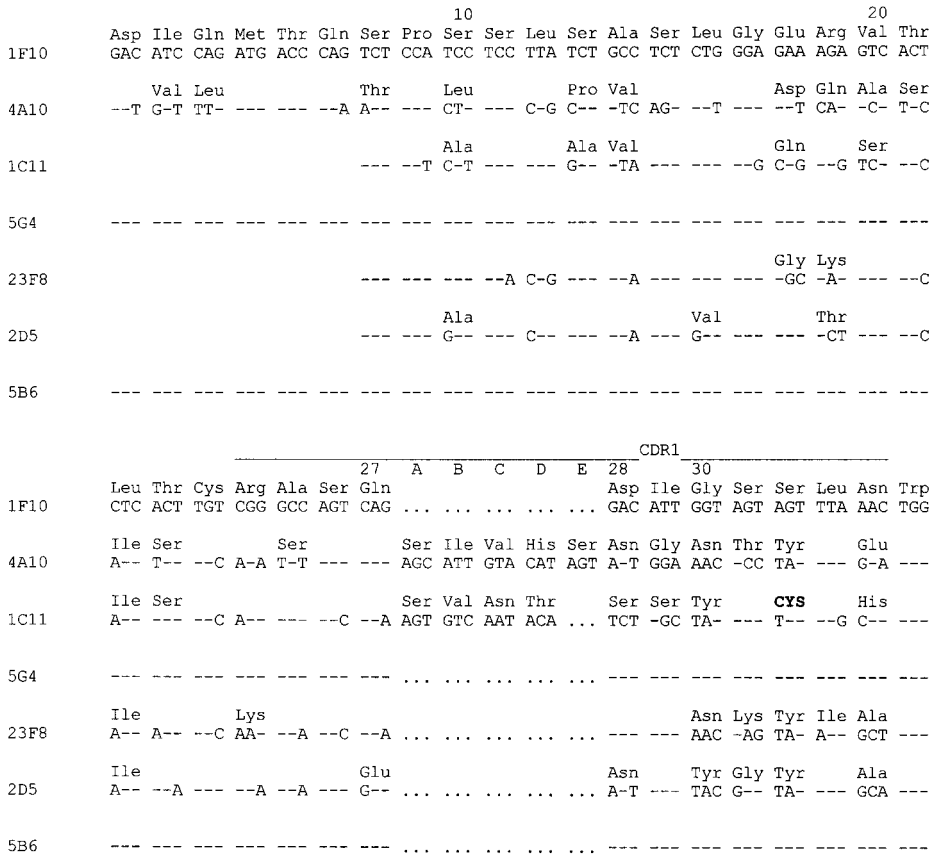

Fig. 2. Nucleotide and deduced amino acid sequences of light-chain variable regions of mercury-specific antibodies. The nucleotide sequences of the region encoding amino acids 1–6 were not included for mAbs 1C11, 23F8, and 2D5, because they corresponded to the PCR primers. The cysteine residues thought to be important for mercury binding are bolded in capital letters. The numbering scheme is according to Kabat et al.[12] Dashes indicate sequence identity, and dots indicate gaps compared with 1F10.

because it did not react with BSA-GSH alone (data not shown). Both HA-specific mAbs reacted with PR8 (data not shown). These results, along with those in Figure 3, indicate that an unpaired cysteine residue in a CDR is required for Hg binding, but other structural features that have yet to be defined are also important.

DISCUSSION

The nucleotide sequences of the variable regions of mAbs that bind mercuric ions are reported here. All the antibodies contained an unpaired cysteine residue in one of the hypervariable regions of the heavy or light chain. Cysteine is abundant in proteins that bind mercury and other metals,[36] but it is the least common amino acid in the CDRs of antibodies of mice and other vertebrates.[37,38] Examination of more than 90 germline V_HJ558 sequences from both the IgH^a and IgH^b murine haplotypes revealed only two with cysteine codons in either of the hypervariable regions.[29,33] One of these genes, H26-6,[29] is identical to that used by the mercury-binding mAb 23F8. The use of a V_κ germline gene by three of the Hg-binding antibodies also indicates that some germline light-chain genes can encode antibodies that bind mercury. The cysteine residues in the other Hg-binding antibodies were probably introduced by somatic mutation.

In proteins that bind mercury as part of their normal physiological function, such as metallothionein,[39] phytochelatins,[40] and proteins encoded by the *mer* operon in bacteria,[41] mercury is bound as a bithiolate or higher complex. For example, in metallothioneins (MT), in which 30% of the amino acids are cysteine, molecular modeling of the Hg-MT complex suggests that all the mercuric ions are bound as tetra-thiolate complexes involving both bridging and terminal cysteines.[42] In the proteins encoded by the *mer* operon, which are probably the best characterized of the naturally occurring Hg-binding proteins, Hg is bound in bithiolate or trithiolate complexes. The *merA* gene encodes the enzyme, mercuric ion reductase, which reduces mercuric ions to metallic mercury so it can be released from the bacterial cell by vaporization.[41] This enzyme is a homodimer with an active site in which a cysteine and a tyrosine from each monomer contribute to formation of a tetrahedral complex with mercury.[43] The *merR* gene encodes a DNA-binding protein that, in the

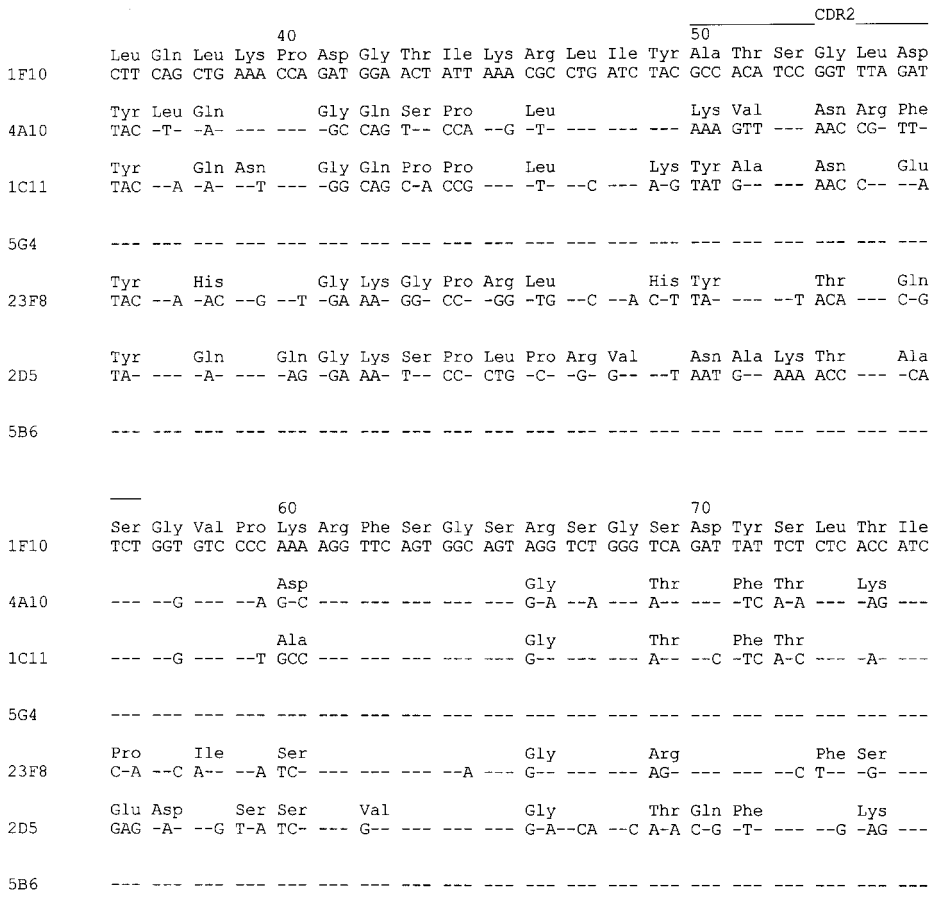

Figure 2. (Continued.)

presence of Hg(II), activates transcription of the genes involved in mercury resistance.[44] The form of this protein that binds Hg(II) is also a dimer, although the Hg(II) binds as a trithiolate complex to two cysteine residues from one monomer and one from the other.[45–47] MerP, which binds mercury in the periplasmic space and transfers it to merT for transport across the membrane into the cytoplasm,[48] is monomeric and binds mercury as a bithiolate complex.[49] Site-directed mutagenesis of either cysteine residue eliminates its ability to bind Hg at a thiol concentration similar to that of the periplasm.[50]

Only one cysteine residue is present in the binding site of each antibody, so they all bind Hg(II) as monothiolate complexes. Despite this, the K_d values of 10^{-8}–10^{-9} M for 4A10 and 1F10[5] are similar to those reported for binding of Hg(II) by the merR protein.[51] Other amino acids, such as aspartic acid, glutamic acid, histidine, and methionine, can participate in interactions between metals and proteins,[52] and they are commonly found in CDRs of antibodies.[12] Computer modeling suggests that in mAb 4A10 the carbonyl oxygens of Ala-100F, Tyr-100E, Gly-96, and Cys-95, and the carboxyl oxygens of Asp-33, all in the heavy chain (Fig. 1), are within the appropriate distance to interact with Hg bound to the sulfhydryl of Cys-95 (P. Goebel, unpublished observation).

The absolute requirement for an unpaired cysteine was shown by the loss of mercury binding by mAb 4A10 when its cysteine in H-CDR3 was changed to either tyrosine or serine. Also, the absence of mercury reactivity by one of the HA-specific antibodies (H37-88) and the low reactivity of the other (H37-24), despite their high degree of similarity to each other and the presence of a cysteine residue at the same position as mAb 4A10, indicates that differences in a small number of amino acid residues can have a profound effect on Hg binding, even if cysteine is present in the antigen-binding site.

The structural differences between mAbs 4A10, H-37-24, and H37-88 that account for their varying capacities to bind Hg(II) are currently not understood. Difference in solvent accessibility of their cysteine residues is probably not the reason, because they all contain cysteine at position 95 in H-CDR3, which is one of the most solvent-exposed positions in the antigen-binding site.[53] Morea et al.[54,55] defined H-CDR3 as the region between Cys-92 and Gly-104 and divided this region into a torso, exhibiting the hydrogen-bonding pattern of a β-sheet, and a head, which makes up the tip of the CDR loop. According to this scheme, the Cys at position 95 would be in the torso. However, computer modeling of these three mAbs suggests that the presence of Gly at position 96 in mAb 4A10

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                                                             _________CDR3_______
                           80                                      90
          Asn Ser Leu Glu Ser Glu Asp Phe Val Asp Tyr Tyr Cys Leu Gln CYS Ser Asn Ser Pro
1F10      AAC AGC CTT GAG TCT GAA GAT TTT GTA GAC TAT TAC TGT CTA CAA TGT TCT AAT TCT CCG

          Ser Arg Val     Ala         Leu Gly Val             Phe     Gly     His Val Arg
4A10      -G- --A G-G --- G-- -AG --- C-G -G- -TT --- --- --C T-T -A- G-- --A C-- GT- -G-

          His Pro Val     Val         Ser Ala Thr     Phe     Gln His Ser Trp Glu Ile
1C11      C-T CCT G-G -A- GTG --- --- AG- -C- ACA --- -T- --- -A- --C A-- -GG G-G AT- --T

                                                                      CYS
5G4       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

          Ser Asn         Pro         Ile Ala Thr                     Tyr Asp         Leu
23F8      -G- -A- --G --- C-- --- --- A-- -C- ACT --- --T --- --- --G -A- GA- --- ... CTG

          Arg Thr     Gln Pro             Gly Thr             Gln His His Tyr Gly Thr
2D5       -GG -CA T-G C-- C-- --- --- --- -GG ACT --- --- --- -A- --T CA- -A- GG- A-- ---

                                                                      CYS
5B6       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---



          _______
                           100
         |-------------------------J-------------------
          Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
1F10      TAC ACG TTC GGA GGG GGG ACC AAG CTG GAA ATA AAA

4A10      ... --- --- --T --A --C --- --- --- --- --C ---

          Pro
1C11      CCG --- --- --T --A --C --- --- T-- --- --C ---

5G4       --- --- --- --- --- --- --- --- --- --- --- ---

          Phe             Ser
23F8      -T- --- --- --C TC- --- --A --- T-- --- --- ---

2D5       --- --- --- --- --- --- --- --- --- --- --- ---

                                                  Asn
5B6       --- --- --- --- --- --- --- --- --- --- --- --C
```

Figure 2. (Continued.)

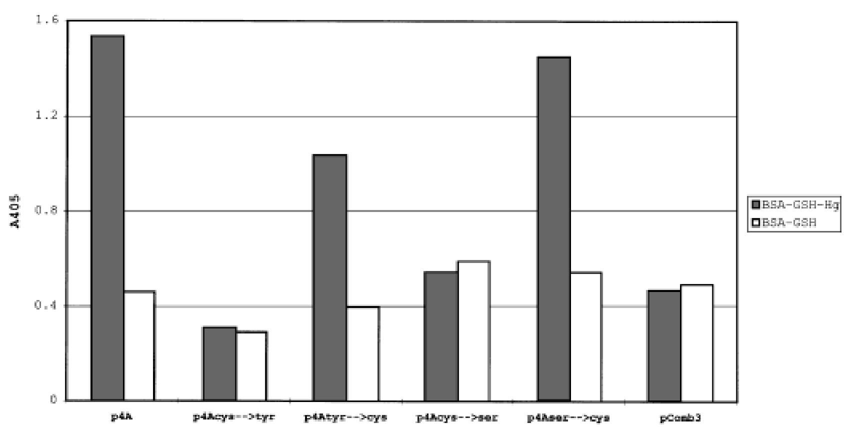

Fig. 3. ELISA results of mAb 4A10 modified by site-directed mutagenesis. The cysteine residue at position 95 in CDR3 of mAb 4A10 was then changed to either tyrosine or serine by the megaprimer PCR method.[17] Phagemids containing the modified heavy-chain and native light-chain genes were assayed for reactivity with BSA-GSH and BSA-GSH-HgCl in a modified ELISA as described in Materials and Methods. The results indicated that modification of cysteine to either serine or tyrosine eliminated mercury binding.

induces a turn in the H-CDR3 loop, so that Cys-95 is situated near the tip of a loop instead of being farther down the torso, as it is in H37-24 and H37-88 (P. Goebel and D. Wylie, unpublished observations). This might adversely affect the accessibility of the cysteine residues in H37-24 and H37-88 to Hg when it is presented on BSA-GSH.

Fig. 4. Comparison of deduced amino acid sequences of the heavy and light chains of cysteine-containing HA-specific mAbs with mercury-specific mAb 4A10. The amino acid sequences of mAb 4A10 heavy and light chain are the same as in Figures 1 and 2, respectively, whereas the sequences of the HA-specific mAbs H37-24 and H37-88 were reported by Clarke et al.[35] Dashes indicate sequence identity, and dots indicate gaps compared with 4A10. The cysteine at position 95 in the heavy chains of all three antibodies is enclosed within the box.

Not all amino acid positions in CDRs of the antigen-binding site are equally likely to make direct contact with antigen. Those positions most often involved in antigen contact have been identified by Padlan et al.[56] using average structural dissimilarity (ASD) scores and by MacCallum et al.[53] using mean fractional burial values for antibodies of known structure. Because most of the residues in CDR3 of the heavy and light chains are highly variable and exposed to solvent, the cysteine residues in these CDRs (mAbs 4A10, 1F10, 5G4, and 5B6) are in positions normally involved in antigen contact. However, in mAbs 23F8, 1C11, and 2D5, the cysteine is located at positions that have low ASD values[56] and, even though accessible, are rarely involved in direct contact with small haptens.[53] None of the Hg-binding antibodies had the unpaired cysteine in L-CDR2. This probably reflects the fact that this CDR is the most removed from the center of the antigen-binding site and infrequently participates in antigen binding[57] so that, even with a small hapten like Hg(II), there would not be enough coordinating ligands provided by other, nearby residues to stabilize the Hg-antibody interaction.

Using the strategy described previously by our laboratory,[5] Yang and Merritt[58,59] produced antibodies to GSH complexes of chromium, cobalt, and nickel, which can be elevated in the blood and tissue of orthopedic patients after implantation of metallic, prosthetic devices. Other investigators have modified antibodies with non-metal specificities by site-directed or random mutagenesis so they coordinate metals.[60–62] In addition, there are several reports of antibodies to metal-chelate complexes,[63–68] but none of these have been shown to bind the unchelated metals. In fact, the crystal structure of an antibody specific for an indium-ethylenediaminetetraacetic acid (EDTA) complex[63] has revealed that most of the interactions are between EDTA and the antigen-binding site.[69] The only direct interaction between indium and the antibody is via a histidine residue at position 95 in the heavy-chain CDR3. Computer modeling suggests this is also the case for an mAb specific for a Cd-EDTA complex.[68]

Heavy metal exposure in humans and experimental animals can result in a number of immunopathological conditions, primarily autoimmune disease[70] and hypersensitivity reactions.[71] The role of antibodies in these disease processes is uncertain, although antibodies to metal-protein complexes have been detected in patients with hard metal asthma and chronic beryllium disease.[72,73] Mercury has been associated with both type I and type IV hypersensitivity reactions in humans,[71] although our laboratory could not detect mercury-binding antibodies in individuals suffering from mercury hypersensitivity.[74] Mercury-induced autoimmune disease does, however, lead to antibodies to fibrillarin and laminin in rodents.[75,76] Whether the antibodies reported here have a biological role related to their mercury-binding capability is yet to be determined.

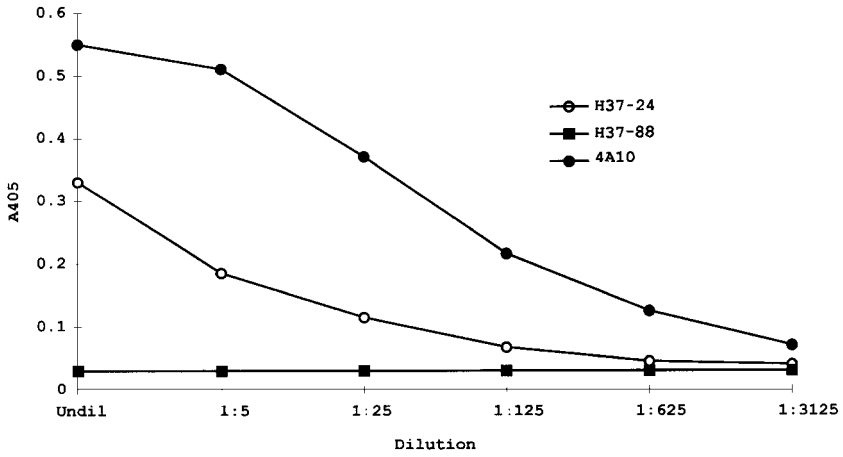

Fig. 5. Reactivity of HA-specific mAbs with BSA-GSH-HgCl. Dilutions of two HA-specific monoclonal antibodies (H37-24 and H37-88) and one mercury-specific mAb (4A10) were assayed for reactivity with BSA-GSH-HgCl as described in Materials and Methods. Each antibody solution was at an initial protein concentration of 1 µg/mL. Fivefold dilutions were made from this stock solution in 0.1 M HEPES, pH 7.1, containing 3% BSA. Each dilution was tested in duplicate.

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