Site-specific conjugation on Serine→Cysteine Variant Monoclonal Antibodies*

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*Invention was subject of International Patent WO 96/14339, filed May 17, 1996 and published in November 1997.

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Running Title: Site-specific Conjugation of Monoclonal Antibodies
SUMMARY

We have engineered a cysteine residue at position 442 (EU/OU numbering) in the third constant domain (C_H3) of the heavy chain of several IgGs with different specificities, isoforms, and variants with the intent to introduce a site for chemical conjugation. The variants were expressed in NS0 mouse myeloma cells, where monomeric IgG is the major form and formation of aggregate was minimal. Monomeric IgG contained no free thiol; however, it was discovered that the engineered thiols were reversibly blocked and could be reduced under controlled conditions. Following reduction, reactive thiol was conjugated with a cysteine-specific bifunctional chelator, bromoacetyl-TMT to a humanized 323/A3 IgG4 variant. Conjugation had no significant effect on antibody affinity. To prove that the conjugation was site-specific, an antibody-TMT conjugate was labeled with lutetium-177 and subjected to peptide mapping followed by sequence analysis. Glu-C digestion demonstrated that 91% of the label was recovered in the C-terminal peptide fragment containing the engineered cysteine.

Keywords: site-specific, engineered cysteine, monoclonal antibody, bromoacetyl-TMT, antibody therapy, peptide mapping,
INTRODUCTION

Monoclonal antibodies and fragments are valuable diagnostic and therapeutic agents (1). Their utility derives both from their unique antigen specificity and their ability to interact with components of the immune system. Antibodies’ utilities can be enhanced by chemically conjugating agents that add new functions such as a reporter, a drug, an enzyme, or a chelator-radionuclide for radioimmunotherapy. Antibody isotype (2) and linkage chemistry (3) affect the pharmacokinetic properties of the conjugate.

Conjugation reactions typically exploit the functional groups on a residue such as the tyrosines (4-6), the ε-amino side chain of lysines either directly (7-9) or indirectly (10,11), or the carboxyl side chain of aspartic and glutamic acids (12,13). One disadvantage of this approach is that it is random. For example, lysine residues occur throughout the structure, and therefore, the natural properties that these residues help convey such as antigen recognition, complement activity, or effector cell interaction can be compromised by conjugation.

Conjugation to a specific residue conveys the advantage of a homogeneous structure. The site may be selected to be spatially removed from areas known to affect antigen-binding properties. For example, additions to the C_H3 domain of the heavy chain may avoid compromising antigen-binding properties (variable region) and effector and complement functions (C_H2 domain).

Examples of site-directed conjugation procedures include conjugation to the carbohydrate portion of antibodies (14,15), which for human IgGs at N-297 (EU/OU notation (16) used throughout) presents the risk of altering carbohydrate that might be required for antigen or effector interaction (17-19). Conjugation to intrinsic cysteines
requires reduction of S-S bonds that risks protein fragmentation (20,21). Antibodies naturally contain no free sulphydryl groups and 16 or more highly conserved cysteine residues whose thiols form disulfide bridges.

It is hypothetically attractive to engineer an antibody to possess a cysteine whose thiol is not oxidized. However, when applied at Cys-444 in C_H3, dimerization and structural aberrations were common (22,23). In another example, a Ser→Cys variant in C_H1 of the heavy chain generated dimers and an interchain structural variant or “tethered” antibody (24). Where molecular modeling was employed in C_H1, a free thiol was observed when the cysteine was introduced into a molecular pocket but not on the solvent-accessible surface (25,26). Solvent-accessible cysteines were reversibly blocked. However, the authors were not able to generate reactive thiol without reduction of hinge disulfides, and these variants were not pursued further.

We describe monoclonal antibodies that contain an engineered cysteine at position 442 in heavy chain C_H3. Cells expressing the novel variants produced a majority of monomeric IgG and minimal aggregate forms. Monomeric IgG was readily purified by conventional means and was stable upon long term storage. We demonstrate that the engineered Cys-442 was reduced under controlled conditions and conjugated with a bifunctional chelator. We show that the antigen binding properties were indistinguishable from the parent antibody. Finally, we show that the conjugation was in fact site-specific by peptide mapping of a radiolabeled conjugate.
EXPERIMENTAL PROCEDURES

Reagents and Supplies - Metal-free plasticware (polypropylene) or plasticware that had been soaked in 3 M HCl overnight and rinsed thoroughly with Milli-Q (18 MΩ) water was used throughout this work to reduce metal contamination (27). All buffers and reagents were dissolved in Ultrex water (JT Baker). The reagents were of analytical grade or better. All prepared buffers were treated with Chelex-100 resin (Na+ form, 100-200 mesh, Bio-Rad Laboratories) according to the manufacturers instructions and passed through a 0.22 µM filter (Nalgene) prior to use.

Bromoacetyl-TMT (28) was prepared in the Wellcome Research Laboratories according to literature protocols with minor modifications.

Reverse-phase HPLC was performed on a LDC (now Thermoquest) system consisting of a model CM3100 pump and a model SM400 spectrometer.

Lutetium-177 was obtained from University of Missouri Research Reactor using the protocol previously described (29).

Nomenclature, Modification of the Human G4 Constant Region and Antibody Expression – Antibodies were humanized and isotypes were generated by grafting the indicated specificities and constant regions. The G4 constant region that had been altered to limit Fc interaction (2) was further modified for site-directed conjugation. A Ser to Cys change was engineered at amino acid position 442 (EU/OU) in pUC18 (GW3811W94). Plasmid DNA was digested at the C-terminal Nsi I and Eco RI sites releasing a 65 base pair fragment that was agarose (0.8%) gel purified. Positive and negative strand oligonucleotides were synthesized (Oligos Etc.; Watsonville, OR) with the Ser to Cys change at 442 (EU/OU). The synthetic oligomers were annealed and ligated into the Nsi
I site (5’) and the Eco RI site (3’) of the construct. The Cys at position 442 (EU/OU) was confirmed by DNA sequencing. This G4 variant served as the scaffold to which various specificities were grafted.

GW3811W94 is the humanized G4 variant of an anti-Ep-CAM monoclonal antibody (30, 31). GW3811W94 conjugated with TMT was designated GW3811W94-TMT or GW1209W95. GW2838W94 is the G4 variant of Campath-1H (2). Native G4 Campath-1H served as a control. G4 variant anti-digoxin was also produced.

The cDNA constructs were sub-cloned into mammalian expression vectors, transfected and selected for stable expression in NSO mouse myeloma cell lines; the respective recombinant monoclonal antibody products were purified from conditioned cell culture supernatant as described previously (2).

**Protein Preparation** - If necessary, the protein was purified further by semi-prep gel filtration HPLC using a Diol 200 size exclusion column (YMC, Inc.). Purified protein was treated with 100 mM sodium DTPA prior to concentration by ultrafiltration (Amicon). For random conjugation and site-specific conjugation with Reduce-Imm™ reduction, the proteins were concentrated in 0.1 TMAP pH 8.2, 25 µM DTPA. For site-specific conjugation with MEA reduction, the proteins were concentrated into 0.1 M sodium phosphate pH 6.0, 5 mM DTPA. The final concentration of purified protein was typically between 400-600 µM.

**Site-specific Reduction with Mercaptoethylamine (MEA)** - MEA (Pierce) was dissolved in 0.1 M sodium phosphate pH 6.0, 5 mM DTPA at a concentration of 50 mM. MEA was added to the solution in a 10-fold excess over the protein concentration (300 µM). The reduction proceeded at room temperature for 60 minutes. Following reduction, the
protein solution was passed through Bio-Spin 30 columns that had been pre-equilibrated in 0.1 M TMAP pH 8.2, 25 µM DTPA for 2 minutes at 150 x g. The protein concentration was determined by absorbance at 280 nm prior to conjugation.

*Reduction with Reduce-Imm™ gel (Pierce)* - Antibody solutions (100 µM; degassed 100 mM sodium phosphate, pH 8) consisting of GW2838W94 and native G4 Campath-1H control were mixed with a fixed volume containing either buffer or increasing amounts of Reduce-Imm™ gel. (Reduce-Imm™ is a solid phase reductant. The reductant capacity was assumed from the manufacturer to be 30 µmole/mL packed gel.) The mixture was shaken for 1 hr at room temperature and centrifuged. The solution was subjected to free thiol determination by conventional means using Ellman’s reagent.

*Protein Conjugation: Preparation of GW1209W95 (GW3811W94-TMT) by Reduction with Mercaptoethylamine* - During the reduction, a 10 mM stock solution of the bifunctional chelator, bromoacetyl-TMT, was prepared in 0.1 M tetramethylammonium phosphate, pH 8.5 with no DTPA, and a small amount of this stock solution was trace-labeled with lutetium-177 for a minimum of 20 minutes at room temperature. Following reduction, two protein conjugation reactions were prepared: a small-scale analytical conjugation that used the trace-labeled chelator, and a large-scale conjugation that used unlabeled chelator. The small-scale conjugation was used to monitor the extent of the conjugation reaction (32). In both conjugations the antibody (200 µM) was incubated with a 10-fold excess of bromoacetyl-TMT for 24 hours at room temperature. The analytical reaction was evaluated by thin-layer chromatography to monitor the extent of protein conjugation (see below). Unreacted reagents in the large scale conjugation were removed by centrifugation using Bio-Spin 30 columns that had been pre-equilibrated
with 0.1 M ammonium acetate, pH 6.5 for 2 minutes at 150 x g. The centrifugation was repeated until all small molecular weight materials were removed. This was typically accomplished after 2-3 centrifugations. GW1209W95 was evaluated for binding affinity and ability to accept metal (post-conjugation efficiency).

Efficiency of Conjugation - The efficiency of the conjugation reaction was evaluated by thin-layer chromatography on the analytical scale conjugation. Trace labeling of bromoacetyl-TMT was evaluated by adding an aliquot to 10 µL of 0.1 M sodium phosphate, pH 6.5. An aliquot (5 µL) of the small scale conjugation reaction was added to 10 µL 0.1 M ammonium citrate, pH 6.5. An aliquot (5 µL) of this solution was challenged for 5 minutes with 2 µL of 100 mM sodium DTPA, pH 6.5. An aliquot (5 µL) of each sample was spotted at the origin on a thin-layer chromatography plate (Whatman 4685 821) and allowed to air dry. The plate was eluted with 10% ammonium acetate : methanol (1:1). A radioanalytical image of the plate was obtained using an A-MK2 scanner (AMBIS, Inc.) Samples treated with phosphate resulted in free metal remaining at the origin and chelated metal migrating to the solvent front. In the case of the samples treated with citrate, chelated metal remained at the origin and free metal migrated to the solvent front.

Post-conjugation Efficiency - The protein concentration of the conjugates was determined prior to performing this assay (typically 150-200 µM) using a BCA assay (Pierce), because TMT absorbs at 280 nm. With the knowledge of the protein concentration and the number of chelators/antibody, the concentration of the chelator in solution can be calculated. This will be used to determine the final concentration of metal in the assay that was two-fold greater than the chelator concentration. To a metal-free vial were
added equivolumes of conjugate solution and metal solution that had been trace-labeled. The vial was incubated at room temperature for 20 minutes, and then 20 µL of 0.1 M ammonium citrate pH 6.5 was added. An aliquot (5 µL) was challenged with 2 µL of 100 mM NaDTPA pH 6.5. The vials were incubated for a minimum of 5 minutes. Five µL were spotted on ITLC SG plates and developed with 0.1 M sodium citrate pH 6.0. The % radioactivity chelated by the conjugate was determined by radioanalytical imaging. The number of chelators that accept metal or efficiency can be calculated.

Evaluation of Specific Binding – 1209W95 was evaluated for quantitative binding to its antigen, Ep-CAM, using an ELISA assay. The protein concentration was determined by nephelometry (33). Ideal values should be 0.7 – 1.3 KU/mg indicating that the binding affinity of the antibody had not been affected (34).

Identification of the site of conjugation in 1209W95 – The principles of lutetium labeling have been recently described (29). Briefly, non-radioactive lutetium-176 (1 mM) stock was prepared in 0.1 M ammonium acetate pH 6.7 and added to 1209W95 (24.13 mg/mL, 1 mg) to give a final concentration of 100 µM lutetium. The chelation reaction was allowed to proceed for 60 minutes at room temperature. Simultaneously, 1209W95 (400 µg) was trace-labeled with radioactive lutetium-177 using the same conditions. To remove unchelated lutetium, ammonium DTPA (50 mM) was added to the chelation solutions to give a final DTPA concentration of 1 mM, and each solution was incubated at room temperature for 30 minutes. During the chelation incubation, Bio-Spin 30 columns were pre-equilibrated with 0.1 M Tris-HCl pH 8.6, 5 mM EDTA. Then, Lu-1209W95 was purified by passing each solution through a Bio-Spin 30 column for 2 minutes at 150 x g. The spin column purification was repeated to give a total of two
spins. The protein concentration of Lu-1209W95 was determined by BCA analysis and the purity of the labeled protein was determined by TLC analysis.

Protein reduction and alkylation was accomplished using DTT and iodoacetamide (Aldrich) in 0.1 M Tris-HCl pH 8.6, 5 mM EDTA, 0.01 % SDS. DTT was added to Lu-1209W95 at a final concentration that was 50-fold greater than the thiol concentration of 1209W95 (16 -SH/Mab). The reduction was incubated at 37°C for 4 hrs., exchanged into bicarbonate buffer containing 0.1% SDS and concentrated using a Microcon ultrafiltration device. Aliquots of the radiolabeled and nonradioactive antibody were combined for digestion. Pooled antibody was digested with a 1:25 weight:weight ratio of either S. aureus V-8 protease (Glu-C, Boehringer Mannheim) or Achromobacter Lys-C (Wako) for 16 h at 37°C. Following initial HPLC chromatography as described in the figure legends, radioactive fractions were mixed with 1/5 volume of 8 M urea and 4/5 volume of 20 mM potassium phosphate prior to rechromatography.
RESULTS

Rationale for Selection of Site of Cysteine Modification and Production of Novel Cysteine Containing Variants - Our selection of potential mutation sites focussed on the C\text{H3} domain because the C\text{H2} domain was glycosylated and residues in this domain were involved in effector function. Consideration of residue types for substitution was limited to serines, which provided the most reasonable sites for mutation to cysteine on the basis of side chain similarity. Individual serine residues were evaluated for solvent accessibility and molecular environment using the three-dimensional structure of IgG1 (35) as a model of IgG4. Serine 442 was selected on the basis of solvent accessibility and its position in a stable portion of the immunoglobulin fold, as part of a \(\beta\)-pleated sheet. The subsequently determined structure of IgG4 (36) was consistent with that analysis. The position of serine 442 in the IgG4 structure is illustrated in Figure 1.

When the novel Cys-442-containing constructs were expressed in NS0 mouse myeloma cells, only a small percentage of the antibody was in an aggregated form. Monomeric IgG was readily purified by conventional chromatography and was stable upon long term storage. The monomeric IgG was found to possess no free thiol (Tables 1 and 2). The engineered thiols were blocked with cysteine and glutathione (not shown).

Preparation of site-directed monoclonal antibody chelator conjugates - Purified IgG did not contain free thiol. We found that the engineered thiols could be selectively reduced under controlled conditions with a mild reducing agent, MEA, or the solid-phase reductant, Reduce-Imm\textsuperscript{TM}, without reducing natural disulfide bonds as indicated in Tables 1 and 2. Using MEA to prepare chelator conjugates (Table 1), selective reduction was achieved with a 10-fold excess of reagent over the protein concentration for one hour at
room temperature. The specific example shown in Table 1 employs a humanized antidigoxin antibody that contains the same constant region as 1209W95. The results from this experiment are consistent with results from similar experiments performed on antibodies with different specificities and isotypes containing the engineered cysteine. A common pattern emerged from reductions under different conditions: the reduction reached a maximum value at one hour and did not significantly increase over the course of the experiment. There was evidence that conditions that produced greater than 5.0 moles SH/mole antibody produced free heavy chain and free light chain together with a proportional decrease in the concentration of intact IgG (data not shown). Thus, we were confident that the conditions that produced 2.0 moles SH/mole antibody did not affect disulfide linkages. The same conditions utilized for the reduction with MEA on an analytical scale were used successfully to produce 1 g of protein conjugate.

Table 2 summarizes the results obtained with solid-phase reductant and GW2838W94, a Cys-442 G4 variant of Campath-1H, and its native G4 control. Since the mutant thiols were blocked with cysteine or glutathione, 2 moles of free thiol per antibody thiol were liberated and detected upon reduction. The results show that a 30-fold molar excess of reductant selectively liberated 2-3 moles of free thiol (1-1.5 moles antibody thiol) per mutant antibody molecule. In contrast, the intrinsic disulfides of the native G4 control were not reduced. Antibody reduced for conjugation was stable upon long term storage; for example, it did not form aggregate at concentrations as high as 75 µM and remained capable of conjugation over months of storage.

The cysteine reactive reagents, bromoacetyl-TMT, iodoacetyl-TMT, and maleimide-TMT, were each evaluated in conjugation reactions with the cysteine-mutated
antibody (data not shown). These reagents were evaluated for selectivity of reaction with
cysteine-442 by monitoring incorporation of labeled reagent into both antibody heavy
and light chain polypeptides over time at room temperature and 37°C. Bromoacetyl-
TMT provided the best selectivity with essentially no incorporation into antibody light
chain polypeptides after 24 hr at room temperature and was used in conjugation protocols
with engineered cysteines as the reagent of choice.

The optimized conditions for antibody reduction and conjugation detailed in
“Experimental Procedures” have been used for greater than 50 conjugation reactions
involving small scale (< 20 mg) and large scale (> 20 mg) amounts of antibodies having
different constant regions (IgG4 and IgG2) and binding specificities containing the
engineered cysteine. Following reduction and using bromoacetyl-TMT as the
bifunctional chelator of choice, the typical incorporation of chelator into monoclonal
antibody was between 1.0 – 2.0. The metal-free conditions of the conjugation reaction
produced protein-TMT conjugates that accepted metal post conjugation. Most
importantly, the binding affinity of the antibody was not affected by the conjugation
conditions or incorporation of TMT into the introduced cysteine in the heavy chain.
1209W95 is a humanized version of mouse parent 323A/3 that contains a mutated G4
constant region with the engineered cysteine at position 442 conjugated to TMT. This
antibody conjugate was used to prove that conjugation was in fact site-specific.

Identification of radioactive peptides from a Glu-C digest of Reduced/alkylated [177Lu]-
1209W95-- 1209W95 was radiolabeled with lutetium-177 and TLC analysis indicated
that 100% of the radiolabel was associated with the antibody (data not shown). Prior to
peptide mapping, the trace-labeled protein conjugate was reduced and alkylated
according to standard protocols and a sample was analyzed by non-reducing gel electrophoresis (Figure 2). As expected, a majority of the radioactivity was associated with the heavy chain polypeptides (HC) following reduction and alkylation. Importantly, there is no radioactivity associated with the light chain polypeptides (LC). However, a minimal amount of radioactivity was associated with bands of unknown identity. The radioactivity profile of the Glu-C digest was complex, consisting of three major and two minor peaks. These peaks are labeled A-E in Figure 3. Very little radioactivity was found in the flow-through fractions, indicating that the radiolabel was stable under the conditions used for reduction, alkylation, digestion and separation. The uv absorbance profile at 214 nm was extremely complex, indicating that digestion of the antibody had proceeded well. Chromatographic analysis of radioactive fractions from peaks A-E in general yielded one or two major peaks of radioactivity (data not shown). These radioactive fractions were subjected to sequence analysis.

All sequenced fractions contained a mix of peptides, but in fractions containing appreciable amounts of radioactivity, one of two different forms of peptides derived from the COOH-terminal of the heavy chain were seen. A summary of the Glu-C peptide sequencing results is shown in Table 3. The COOH-terminal 20 residues of the 1209W95 heavy chain (residues 423-442) is: VMHEALHNHYTQKSLCLSLG. The tetrapeptide Leu-X-Leu-Ser (heavy chain residues 437-440) was recovered from two of the major peak fractions (peaks B and D, see Figure 3), while a longer peptide spanning residues 437-440 was identified in another major fraction (peak C). In both cases the X corresponds to the Cys that was the expected site of attachment of the chelator. The observed heterogeneity in the elution times of radioactive peptides with the same
apparent sequence could be due in part to the known instability of the chelator to high temperatures for several hours (unpublished result), as was required during the digest of the chelated protein (37°C for several hours). Both the tetrapeptide and the longer fourteen residue peptide result from cleavage of the antibody heavy chain after Ser residues, an unexpected cleavage point for Glu-C. However, similar post-Ser cleavages were also seen in unlabeled peptides from this digest.

The amounts of COOH-terminal peptides present in each fraction were estimated from the levels of Leu residues at the appropriate cycles, or from other residues beyond the first cycle when possible. The picomolar amounts of these COOH-terminally derived peptides could be correlated with the amount of radioactivity in the sequenced fraction (data not shown). This was not true for any other peptide sequences, which varied from fraction to fraction. Many of the other peptides found did not even contain Cys. In other cases, a PTH-amino acid derivative corresponding to carboxamidomethylated Cys was seen in the appropriate cycle. Fractions derived from peak A in Figure 3 contained a low-level, complex mix of sequences from which the COOH-terminal peptide was not identified. A very low level of a COOH-terminal sequence was found in one peak from the E fraction, although the identification is tentative as the sequence contained a mixture of species. This very low level is consistent with the lower levels of radioactivity present in the fraction (see Table 3). Based on the radioactivity present in the fractions, peaks B-D together account for approximately 83% of the radioactivity recovered post-flow through. Including radioactivity from peak E fractions, approximately 91% of recovered radioactivity can be associated with the COOH-terminus of the heavy chain at the expected site of chelation.
The results of the Glu-C digest indicated that the site of antibody labeling was at the COOH-terminus of the heavy chain, as expected. However, since the sequencing results were derived from peptide mixtures, the labeled antibody was digested with a second enzyme, Lys-C, in order to confirm that labeled peptides were only derived from the COOH-terminus of the heavy chain. The Lys-C digest radioactivity profile was less complex, and the results indicated that the heavy chain COOH-terminal sequence was associated with approximately 78% of the recovered radioactivity (data not shown).

The results from both digests support the assignment of Cys-438 as the site of chelator attachment to the antibody. No other peptide sequence was consistently present in radioactive fractions. In addition, the level of radioactivity in a sequenced fraction was consistent with the estimates of the amount of the COOH-terminal peptide in that fraction. Prior studies on a Campath-1H antibody engineered to contain a similar COOH-terminal Cys produced the same finding, namely, that radiolabeled Glu-C peptides were derived from the COOH-terminus of the heavy chain. The same unusual cleavages after Ser were also observed for this antibody.
DISCUSSION

The power of monoclonal antibodies is their therapeutic versatility. They can be used in immunotherapeutic approaches either as unmodified antibodies with inherent effector functions or as a delivery vehicle. Our ultimate goal was to design the optimal antibody delivery vehicle for radioimmunotherapeutic applications. Previous work had demonstrated that an antibody devoid of immune effector function resulted in reduced binding to normal organs and reduced immunogenicity in vivo (2). Once these improvements had been engineered, it became important to design a site for conjugation of a bifunctional chelator. Introduction of the modification in the final product should attempt to meet the following set of criteria: 1) the modification should not affect antigen recognition or effector function, 2) the conjugation reaction should produce a homogeneous product with all IgG antibody isotypes, 3) the engineered site should be easily accessible for conjugation; generic protocols can be developed to accomplish conjugation, and 4) conjugation chemistry should not be easily hydrolyzed in human serum. The modification that appeared to meet all these criteria was the introduction of a cysteine residue in the C_{H3} domain of an IgG antibody.

We have described a process in which Ser-442 in the C_{H3} of the heavy chain has been modified to a cysteine residue, and following controlled reduction, can be conjugated with a chelator such that the antibody was only modified on the heavy chain. The selection of this position was based on molecular modeling that indicated unhindered solvent accessibility, and the potential for good conjugation reactivity. Based on Shope’s experience with introduction of Cys-444 into the C_{H3} region of antibodies, we were concerned about the potential for formation of a heterogeneous product, monomeric and
dimeric IgG. In crystal structures of both IgG1 and IgG4 (35), the carboxy-terminal residues 444-446 were undefined and presumably disordered and extended into solvent. This apparent flexibility and accessibility of Cys-444 in Shope’s modified antibody may explain the corresponding propensity for dimer formation. In contrast, our experience with the Ser→Cys 442 modification was similar to that described by Lyons and colleagues (25); production of the engineered antibody resulted in minimal formation of aggregates, and the introduced cysteines were blocked reversibly. Although, Lyons and colleagues were not successful in reducing the blocked cysteines without cleaving hinge disulfides and dissociating inter-heavy chain disulfides, we found that controlled reducing conditions employing MEA or a solid-phase reductant were capable of selectively reducing the engineered cysteines. Thus, we were able to subsequently perform conjugation chemistry on the reactive cysteines. We can speculate that our success in accomplishing the reduction was dependent on two factors: proximity of the modification to hinge disulfides and choice of reductant. Modification in the CH3 region is far removed from any intrinsic disulfides, and therefore, lowers the probability of exchange with intrinsic disulfide bonds. In addition, MEA is an extremely mild reducing agent, and it was easy to control the extent of reduction.

Thioether linkages have shown superior serum stability (3). Numerous reagents claim to be able to alkylate cysteines specifically. In practice, however, we found that bromoacetyl reagents were optimal for selective alkylation of cysteine 442. Most surprising were the results with maleimide-TMT; at both room temperature and 37°C approximately 20% of the reagent was incorporated into antibody light chain polypeptides. According to Partis et al. (37), the selectivity of maleimides for reaction
with cysteines increases at lower pH values. However, conjugation reactions attempted at pH values less than our standard of pH 8.5 led to greater than 10% incorporation into the light chain polypeptides. The results of the enzymatic digestion of radiolabeled-1209W95 provided evidence to support our conclusion that the conjugation was in fact site-specific.

The cysteine modification at Ser-442 was successfully introduced into antibodies of differing specificities and subtypes without compromising antigen binding affinity or effector function. In addition, all of these cysteine-modified monoclonal antibodies were successfully conjugated on small (< 20 mg) and large scale (>20 mg) using the reduction and conjugation protocols described. Therefore, we conclude that introduction of a cysteine residue at position 442 of the C重金属3 domain of IgG2 and IgG4 antibodies produces a homogeneous product that can be conjugated in a site-specific manner without compromising immunoreactivity. These reagents have been used to perform tumor targeting studies in mice that will be the subject of future publications. The major focus of our work has been the design of the optimum radioimmunotherapeutic, but clearly these antibodies containing engineered cysteines can be used for other site-directed applications.
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**ACKNOWLEDGEMENT**

The authors would like to acknowledge the synthetic expertise of W. J. Bock of the Organic Chemistry Division of Wellcome Research Laboratories for the preparation of bifunctional reagents. We would also like to acknowledge the tissue culture and antibody purification expertise of Jane M. Bynum, Jane Rosemond, and Marie E. Stockstill. In addition, we would like to acknowledge Biotechnology Development
Laboratories in Beckenham, England for the production and quality analysis of 1209W95. Finally, we would like to acknowledge the help of Aaron Miller of Structural Chemistry for the production of Figure 1.
Figure 1. **Molecular Environment of Ser-442 in Human IgG4.** The structure of IgG4 Fc (PDB accession code 1ADQ) in the region of Ser-442 is illustrated (36). Protein backbone is represented by the green tube. Atoms of Asn-421, Val-422, Ser-440, Leu-441, and Ser-442 are represented as spheres that are color-coded by atom type: carbon, white; oxygen, red; and nitrogen, blue.

Figure 2. **Gel Electrophoresis of Reduced/Alkylated $^{177}$Lu-1209W95.** 1209W95 was trace-labeled with lutetium-177 and reduced and alkylated as described in “Experimental Procedures”. A sample was fractionated by electrophoresis on a SDS, 4-16% polyacrylamide gel under non-reducing conditions. The relative migration of stained non-radioactive heavy chain (HC) polypeptides and light chain (LC) polypeptides are indicated by arrows on the left, and the relative migration distance for pre-stained molecular mass markers is shown in kilodaltons on the right ($\beta$-galactosidase, 139; carbonic anhydrase, 42; soybean trypsin inhibitor, 32).

Figure 3. **Radioactivity profile of a Glu-C digest of 1209W95.** Proteolytic digestion was performed according to protocols in “Experimental Procedures”. Peptides were eluted from a YMC analytical C-18 column (4.6 x 250 mm) at a flowrate of 0.5 mL/min with a 90-minute linear gradient of 20 mM potassium phosphate pH 6.5 to 70% acetonitrile, 30% 20 mM potassium phosphate, pH 6.5. Fractions were collected in one-minute increments and monitored off-line for radioactivity using an LKB gamma counter. Radioactive fractions were treated as described in “Experimental Procedures” prior to rechromatography on an Aquapore C-8 2.1x150 mm column at a flowrate of 0.5 mL/min using the same HPLC and buffer system. Radioactive fractions that had undergone two
rounds of chromatography were sequenced on a Hewlett-Packard model 1005 amino acid sequencer.
Table 1. **Optimal Conditions for MEA Reduction.** MEA (Pierce) was dissolved in 0.1 M sodium phosphate pH 6.0 and 5 mM DTPA at a concentration of 50 mM. MEA was added to the solution in excess over the protein concentration (300 µM) as indicated in the table. The reduction proceeded at room temperature for 60 minutes. Following reduction, the protein solution was passed through Bio-Spin 30 columns that had been pre-equilibrated in 0.1 M TMAP pH 8.2, 25 µM DTPA for 2 minutes at 150 x g. The protein concentration was determined by absorbance at 280 nm. Free thiol content was determined by addition of 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) and correlated to a standard curve constructed using MEA.

| MEA  | 1 hr | 2 hr | 3 hr |
|------|------|------|------|
| 0    | 0.2  | 0.3  | 0.3  |
| 10x  | 2.0  | 2.3  | 2.5  |
| 30x  | 2.4  | 3.0  | 3.0  |
| 50x  | 3.0  | 3.6  | 4.4  |
| 70x  | 3.5  | 3.8  | 4.9  |
| 100x | 4.3  | 4.3  | 5.1  |
Table 2. **Free thiol content following exposure to Reduce-Imm™ gel (Pierce).** Antibody solutions were reduced with Reduce-Imm™ gel as described under “Experimental Procedures.” Thiols were quantitated by conventional means using Ellman’s reagent. Free thiol content is expressed relative to moles antibody. It is assumed that 2 moles of thiol were reduced per antibody thiol. The sem for triplicate measurements was ± 0.1 SH/antibody.

| Molar Ex Gel | Moles -SH/Antibody |
|--------------|--------------------|
|              | GW2838W94 | G4 control |
| Expt. 1      |           |            |
| 100          | 4.6       | .6         |
| 30           | 3.0       | .2         |
| 10           | 1.2       | .1         |
| 3            | .6        | 0          |
| 0            | .1        | 0          |
| Expt. 2      |           |            |
| 50           | 3.3       | .3         |
| 30           | 2.3       | .2         |
| 20           | 1.7       | .2         |
| 10           | 1.0       | .1         |
| 3            | .5        | .1         |
| 0            | .1        | .1         |
Table 3. **Sequence summary of radioactive Glu-C peptides of 1209W95.** The peak ID refers to Figure 3. More than one fraction across each peak was sequenced after rechromatography. In some cases equivalent fractions from two runs were sequenced. An X refers to an unidentified amino acid. B refers to carboxamidomethyl-Cys. This was found in one of the radioactive peptides at the site of Cys-438. The pmol amount for heavy chain COOH-terminal peptides was estimated from sequencer yields. The residue numbers are based on the actual heavy chain sequence. Residue 438, expected to be Cys in this sequence, would correspond to residue 442 by EU/OU number and to Kabat residue number 473.

| Peak ID | CPM   | Sequence(s) observed                        | approx. pmol |
|---------|-------|---------------------------------------------|--------------|
| A       | 76800 | Low level complex mix                       |              |
| B       | 371500| 1: LXLS, h. chain res. 437-440              | 60           |
|         |       | 2: ALHNHYTQKS res. 427-436                  |              |
| B       | 236600| 1: LXLS                                     | 30           |
|         |       | 2: l. chain res. 88-98                      |              |
| C       | 136000| h. chain residues 405-415                   |              |
|         |       | h. chain residues 344-353                   |              |
|         |       | Minor trace: ALHNHY                        | 10           |
| C       | 370200| complex mix                                 |              |
|         |       | ALHNHYTQKS(L)XLS                            | 50           |
| C       | 190100| ALHNHYTQ(K)XLBL(S)                           | 25           |
| C       | 184400| complex mix, no C-term identified           |              |
|         |       | ALHNHYTQKS(end)                              |              |
| C       | 276000| ALHNHYTQ(L)XL                               | 45           |
| D       | 325900| 1: h. chain res. 165-175                    |              |
|         |       | 2: LXLS                                     | 55           |
|         |       | 3: h. chain res. 269-279                    |              |
| D       | 208900| 1: h. chain res. 165-175                    |              |
|         |       | 2: LXL(S)                                   | 35           |
| D       | 148500| 1: ALHNHYTQKSLXL                            | 25           |
|         |       | 2: light chain res. 1 on                    |              |
| D       | 98200 | 1: Glu-C peptide                            |              |
|         |       | 2: LXL(S)                                   | 10           |
|         |       | 3: h. chain 117-131                         |              |
| D       | 315200| Complex mix                                 |              |
|       | LXLS |     |
|-------|------|-----|
| E     | 58100| 50  |
|       | 1: h. chain res. 361-376 |     |
|       | 2: h. chain res. 365-372 |     |
|       | Possible low-level LXLS | 3   |
Figure 1.
Figure 2.
Figure 3

Glu-C digest of $^{177}$Lutetium-labeled 1209W95
