Development of a Recombinant Antibody with Specificity for Chelated Uranyl Ions

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Abstract

The goal of our project is to continue the development of new techniques for rapid, automated identification of radionuclides, metals, and chelators that may contaminant surface and groundwater at DOE sites. One of the four specific aims of the present project is to develop new technologies in antibody engineering that will enhance our immunosensor program. Recombinant antibodies have potential advantages over monoclonal antibodies produced by standard hybridoma technology. The cloned genes represent a stable, recoverable source for antibody production. In addition, the recombinant format offers opportunities for protein engineering that enhances antibody performance and for studies that relate antibody sequence to binding activity. In this study, a hybridoma that synthesized an antibody (12F6) that recognized a 1:1 complex between 2,9-dicarboxyl-1,10-phenanthroline (DCP) and UO_2^{2+} was used as a source of RNA for the development of a recombinant (Fab)'$_2$ fragment. RNA was isolated from the 12F6 hybridoma and the cDNA encoding the entire κ light chain and the linked VH and C1 portions of the heavy chain were amplified from total RNA. cDNA sequences were verified by comparison with the N-terminal amino acid sequences of the light and heavy chains of the native 12F6 monoclonal antibody. A leader sequence and appropriate restriction sites were added to each chain, and the fragments were ligated into a commercial dicistronic vector (pBudCE4.1, Invitrogen, Inc.). COS-1 cells were transfected with this vector and the culture supernatant was assayed for activity and the (Fab)'$_2$ protein. Cells transfected with vector containing 12F6 cDNA synthesized and secreted recombinant (Fab)'$_2$ fragments that bound to the UO_2^{2+}-DCP complex with an affinity indistinguishable from that of a (Fab)'$_2$ fragment prepared from the native antibody. Molecular models of the heavy and light chain variable domains were constructed according to the canonical structures method detailed by Morea et al. (*J. Mol. Biol.* 275:269), and the participation of specific residues in antigen recognition was assessed using site-directed mutagenesis. Three amino acids in the light chain variable region, H39, Y54 and F103, were particularly important in antigen recognition.

In a separate series of experiments, a recombinant phage-displayed antibody library has been prepared using RNA isolated from the spleens of sheep and rabbits immunized with specific metal-chelate complexes. Phage-display libraries produced from an immunized source are inclined to include variable genes specific for the immunized antigen(s), many of which are already affinity matured. An antibody fragment specific for the UO_2^{2+}-DCP complex was isolated from this combined phage display library. While the binding affinity of this antibody fragment for UO_2^{2+}-DCP was not as high as that of the 12F6 monoclonal antibody, the beauty of antibody phage display technology is that it allows for the potential manipulation and maturation of the antibody's binding affinity, which may drastically improve and ultimately surpass that of monoclonal antibodies.

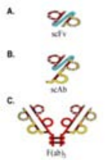

Figure 1. Diagram of the three most common formats for the expression of recombinant antibodies. *Panel A*, The single chain variable fragment format, scFV, consists of the heavy chain variable region (red) and the light chain variable region (yellow) joined by a flexible linker region (blue). *Panel B*, The scAb format consists of the heavy chain variable region (red) and the entire light chain (yellow) joined by a flexible linker region (blue). The F(ab)$_2$ format contains the heavy chain variable region and C1 constant region (red) and the entire light chain (yellow). The construct reported herein also contains the hinge region and cysteines required for disulfide bond formation.

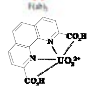

Figure 2. UO_2^{2+}-DCP complex recognized by the recombinant antibodies.

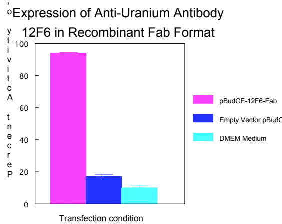

Figure 3. Activity of culture supernatants after transfection with pBudCE-12F6-Fab. The coding region of the light chain and the variable and first constant region of the heavy chain were amplified from RNA isolated from the 12F6 hybridoma. After an intermediate cloning step, leader sequences and N-terminal portion of the IgG1 murine hinge region were added using PCR with the appropriately designed primers (1). These heavy- and light-chain constructs were cloned into the CMV and EF1-1α promoter sites, respectively, of pBudCE 4.1, a dicistronic vector available from Invitrogen, to yield the plasmid pBudCE-12F6-Fab. Plasmid DNA (2 μg/100 mm culture flask) was mixed with Lipofectamine 2000 reagent (2.5 μL reagent per μg DNA) in serum-free DMEM. After incubation at room temperature for 20 minutes, the mixtures were added to newly confluent COS-1 cells and the cultures were incubated for 6 hours at 37°C, after which the complexes were removed and replaced with growth media. Expression proceeded for 36 hours, then the culture supernatants were removed and tested for their ability to bind to an ELISA plate coated with a UO_2^{2+}-DCP-bovine serum albumin conjugate (5 μg/ml, prepared as described in (2)). Bound recombinant antibody was detected using an HRP-labeled goat anti-mouse Fab antibody (Sigma Biosciences, St. Louis, MO). Data are expressed as percent of the activity displayed by a F(ab)'$_2$ fragment prepared by proteolysis of the native monoclonal antibody (n12F6-F(ab)'$_2$). Negative controls included culture supernatant from cells transfected with an empty vector (pBudCE4.1 only) and unused culture supernatant containing 10% fetal bovine serum (DMEM). Values are reported as mean ± SD (n=3).

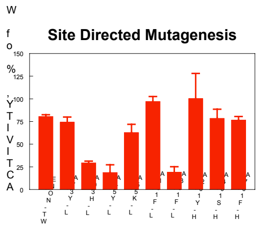

Figure 4. Binding of wild-type r12F6-Fab and mutants to immobilized UO_2^{2+}-DCP. Mutant clones were generated using the QuickChange Site-Directed Mutagenesis kit available from Strategene. COS-1 cells were transfected with plasmid DNA as described in the legend to Figure 2. Expression proceeded for 36 hours, then the culture supernatants were removed and tested for the expression of mouse Fab protein using sandwich ELISA (1). Culture supernatants were diluted with medium to the identical Fab concentration, then the ability of each sample to bind to immobilized UO_2^{2+}-DCP was assessed as described in the legend to Figure 2. Each bar represents the binding activity of a recombinant protein with a point mutation that resulted in the amino acid change shown on the X-axis (L, light chain, H, heavy chain). These data are also quantified in **Table 1** as % of wild-type activity. Values are reported as mean ± SD (n=3).

Name	Yield F_{ab} (ng/ml)	Activity (%)	IC_{50} (nM)	K_d (M)
none	8.8	80.45	1478±388	$9.1±1.2x10^{-9}$
L-Y37A	9.1	74.21	4491±2936	$2.0±0.4x10^{-6}$
L-H39A	4.7	29.35	NA[b]	ND[c]
L-Y54V	5.9	18.68	NA	ND
L-K55A	7.5	62.65	951±205	ND
L-F101A	6.7	96.89	1176±289	$7.8±1.4x10^{-9}$
L-F103A	2.3	19.01	NA	ND
H-Y102A	8.6	100.15	4435±2798	ND
H-S104A	8.7	78.44	1750±233	$5.2±0.5x10^{-8}$
H-F107A	7.3	76.46	2146±679	$2.7±0.4x10^{-7}$

TABLE 1. Biochemical and Immunological Characterization of the Different Mutants of the r12F6 Fab
[a]All plasmids were confirmed by sequencing to have only the mutation shown (primary data not presented). Transfection of the mutant plasmids into COS-1 cells using Lipofectamine was as described in the legend to Fig.2. Thirty-six hours after transfection the culture supernatants were removed and the yield of Fab protein from each transfection was determined by sandwich ELISA. Equivalent concentrations of Fab protein were tested for activity, as assessed by the ability to bind to an immobilized UO_2^{2+}-DCP complex (see Fig. 4 for details). The ability of the soluble UO_2^{2+}-DCP complex to inhibit binding was assessed by competitive ELISA (primary data not shown), and both the IC_{50} value from the competitive ELISA and the equilibrium dissociation constants (see Fig.5 for primary data) are presented herein.
[b]NA, binding to the immobilized UO_2^{2+}-DCP was too low for accurate IC_{50} determination.
[c]ND, not determination

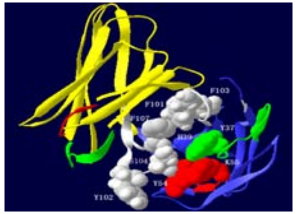

Figure 5. Ribbon representation of the r12F6-Fab homology model. Molecular models were constructed according to the canonical structures method detailed in Morea et al. (3) using the Swiss PDB Viewer program (version 3.7). Amino acid residues targeted for site-directed mutagenesis are shown in space-filling format. The light chain framework regions are indicated by *blue ribbons*, and the heavy chain framework regions by *yellow ribbons*. The CDR1, CDR2 and CDR3 are designated with *green, red and gray ribbons*, respectively. The amino acid residues chosen for mutagenesis based on this model are also listed in **Table 1**, below.

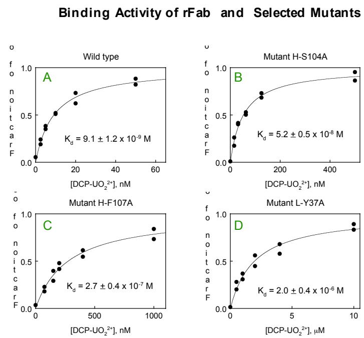

Figure 6. Determination of equilibrium dissociation constants for wild type r12F6-Fab and selected mutants. The concentration dependence of protein binding to soluble 2,9-dicarboxyl 1,10-phenanthroline (DCP) in the presence of 1000 nM UO_2^{2+}. Binding studies were performed as described in (1). Each experiment was performed in duplicate, and the curves drawn through the points were generated using the following equation:Fraction of occupied sites = [L]/(Kd +[L]), where [L] is the concentration of soluble UO_2^{2+}-DCP complex and Kd is the equilibrium dissociation constant. *Panel A,* wild type recombinant Fab; *Panel B*, Heavy chain serine 104 mutated to alanine; *Panel C*, Heavy chain phenylalanine 107 mutated to alanine; *Panel D*, Light chain tyrosine 37 mutated to alanine. Some mutants bound too poorly for an accurate Kd determination. Values for all Kd determinations are shown in Table 1.

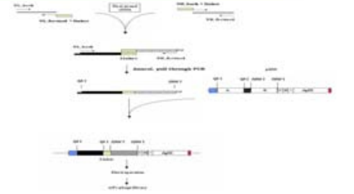

Figure 7. Pull-through strategyfor construction of a recombinant anti-metal chelate antibody library. Four rabbits and two sheep were immunized with mixtures of metal-chelate-protein conjugates. After the animals had demonstrated immune reactivity in a serum sample, they were sacrificed and their spleens were harvested. Spleenic RNA was used as a template for cDNA, then light- and heavy-chain variable coding regions were amplified by PCR using primers as described in (4). These inserts were subsequently used in the one-step cloning strategy used for construction of a pSD3:scFv libraries. A , B are VL and VH DNA fragments. C and H are c-myc and (His)$_6$ tags, respectively. The * indicates an amber codon. ΔgIII is the gene encoding the C-terminal domain of the M13 gene III protein (4).

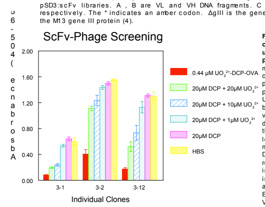

Figure 8. Results from competitive ELISA screening using soluble metal-chelate-protein conjugate or soluble metal-chelate complexes. Three clones were isolated from the pull-through libraries by panning against immobilized UO22+-DCP-conjugate followed by a low pH elution. A specific volume of scFv-phage was diluted with a equal volume of the inhibitor shown in the legend, then added to a microwell coated with UO22+-DCP-ovalbumin. Phage particles remaining in the microwell after incubation with soluble inhibitors were detected with anti-fd biotin conjugate and ExtrAvidin AP substrate (sigma). Values reported are mean ± SD, (n=4).

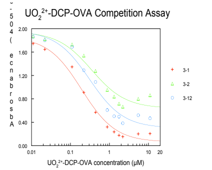

Figure 9. Competition ELISA with soluble UO_2^{2+}-DCP-OVA. scFv-bound to phage particles were used for these experiments. IC_{50} values range from 0.19μM to 0.31μM for clones 3-1,3-2,and 3-12.

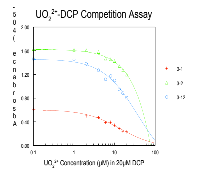

Figure 10. Competition ELISA with soluble UO_2^{2+}-DCP. scFv-bound to phage particles were used for these experiments. IC_{50} values ranged from 13.7μM to 37.8μM for clones 3-1,3-2, and 3-12.

Conclusions

- A monoclonal antibody (12F6) that binds to chelated uranyl ions (see ligand structure in Figure 2) with nanomolar affinity has been expressed as a recombinant Fab fragment.
- Molecular modeling and subsequent site-directed mutagenesis have identified several amino acid sidechains (histidine 39, tyrosine 54 and phenylalanine 103 in the light chain variable region) that are very important in the interaction between the 12F6 antibody and the UO_2^{2+}-DCP complex.
- Unexpectedly, another mutation in the light chain (transformation of phenylalanine 103 to an alanine residue) slightly increased the affinity of the recombinant antibody for the UO_2^{2+}-DCP complex.
- A recombinant library of antibodies to metal-chelate complexes has been prepared from animals immunized with a mixture of metal-chelate complexes.
- Preliminary panning of this library has yielded an antibody fragment that binds to the UO_2^{2+}-DCP complex with micromolar affinity.
- These recombinant protein constructs will provide the DOE with a source of antibody reagents that could be genetically manipulated to suit the assessment requirements of specific DOE facilities.

References
1. J.B. Delehanty, R.M. Jones, T.C. Bishop and D.A. Blake (2003) Identification of important residues in metal-chelate recognition by monoclonal antibodies, *Biochemistry*, **42**:14173-14183
2. R.C. Blake II, A.R. Pavlov, M. Khosraviani, H.E. Ensley, G.E. Kiefer, H. Yu, X. Li, and D.A. Blake (2004) Novel monoclonal antibodies with specificity for chelated uranium(VI): Isolation and binding properties, *Bioconjugate Chemistry* **15**:1125-1136
3. Morea, V., Tramontano, A., Rustici, M., Chothia, C. & Lesk, A. M. (1997) Antibody structure, prediction and redesign., *Biophysical Chemistry*. **68**: 9-16.
4. Li, Y., Cockburn, W., Kilpatrick, J. B. & Whitelam, G. C. (2000) High affinity ScFvs from a single rabbit immunized with multiple haptens. *Biochem Biophys Res Commun.* 268:398-404.

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