Identification of Residues in the Monoclonal Antitumor Antibody L6 Important for Binding to Its Tumor Antigen

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ABSTRACT: L6 is a monoclonal antitumor antibody which recognizes an epitope located in a 42-residue extracellular domain of a tumor-associated ~22 kDa glycoprotein antigen. The L6 mAb localizes to solid tumors *in vivo* and triggers complement activation and antibody-dependent cellular cytotoxicity. It has been the subject of phase I clinical trials. Previously, we had reported the derivation and analysis of a three-dimensional model of the L6 Fv. The model suggests that L6 displays a generally aromatic CDR surface. We aim at improving the affinity for tumor antigen of L6 by *in vitro* mutagenesis. As the first step toward this end, we have attempted to identify residues critical for the binding of L6 to tumor antigen. On the basis of the model, seven residues were selected which we thought may be critical for L6 antigen binding. Criteria for the selection of these residues were their accessibility and central position on the CDR surface and the residue character. Large polar or charged residues such as arginine, asparagine, and tyrosine were preferred. Nine site-specific single and double mutants were generated using oligonucleotide-directed mutagenesis in an M13 expression vector encoding the L6 Fab. The binding of these mutant Fabs to the L6 tumor antigen and a set of three anti-idiotypic antibodies was quantified in an ELISA. In eight out of nine mutants, binding to L6 tumor antigen was either abolished or substantially reduced. In contrast, the binding of the mutants to the anti-idiotypic antibodies was largely unaffected, suggesting that no significant structural perturbations were introduced as a consequence of these mutations.

The murine monoclonal antitumor antibody L6 (L6 mAb)[1] was raised against human lung carcinoma cells (Hellström et al., 1986) and recognizes a protein antigen (Fell et al., 1992) expressed on a variety of human carcinomas and, at much lower levels, on some endothelial cells. L6 has been cloned and a chimeric form, consisting of murine variable and human constant regions, has been generated (Liu et al., 1987; Fell et al., 1992). A cDNA encoding the human L6 tumor antigen has been isolated and shown to encode an integral membrane protein of ~22 kDa which belongs to a family of proteins which traverse the membrane four times (Marken et al., 1992). This family includes several leukocyte antigens such as TAPA-1 (Oren et al., 1990) and the tumor-associated antigen CO-029 (Szala et al., 1990). The L6 mAb recognizes an epitope in a 42-residue extracellular domain located between the third and fourth membrane-spanning region of the L6 antigen (Marken et al., 1994).

The L6 mAb is capable of mediating complement activation and antibody-dependent cytotoxicity (Hellström et al., 1986) and has been the subject of a phase I clinical trial in which 18 patients with recurrent breast, lung, colon, or ovary cancer were treated with murine L6 (Goodman et al., 1990). L6 mAb was well tolerated by patients and localized effectively to tumors. One breast cancer patient underwent complete, albeit temporary, remission after treatment with L6. In addition, ^{131}I-labeled chimeric L6 mAb caused partial remissions of metastatic breast carcinomas in one-third of the patients treated in the course of another *in vivo* study (DeNardo et al., 1991).

Previously, we have reported the construction and analysis of a three-dimensional model of the L6 mAb variable regions and have used this model in our attempts to characterize the L6 antigen binding site (Fell et al., 1992). It was possible to construct this L6 model entirely from known structural templates including the framework regions, the five canonical CDR loops (Chothia et al., 1989), and the noncanonical CDR loop H3. The L6 model did not suggest the presence of a pocket or cavity-shaped binding site as generally seen in antibodies which bind haptens, peptides, or carbohydrates. Instead, L6 was predicted to have a rather flat and irregular CDR surface as is frequently seen in antibodies which recognize protein surfaces (Webster et al., 1994). This led to the hypothesis that L6 mAb recognizes a protein antigen which was then confirmed (Fell et al., 1992). A prominent feature of the L6 antigen binding site is its largely aromatic character. Eleven aromatic residues were predicted to be at least partially solvent exposed and distributed over the L6 CDR surface.

Ultimately, we aim to improve the affinity of L6 to tumor antigen to enhance its therapeutic potential. The purpose of the present study was to determine whether it was possible to identify residues critical for antigen binding on the basis of inspection of the L6 model. We have initially focused our site-specific mutagenesis on residues of the three CDR

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[1] Abbreviations: 2×YT, 2× yeast tryptone medium; CDR, complementarity determining region; cfu, colony-forming unit; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Fab, antigen binding fragment; Fv, variable fragment; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria−Bertani medium; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); pfu, plaque-forming unit; PMSF, phenylmethanesulfonyl fluoride; RT, room temperature; SAS, saturated ammonium sulfate; SAS, sodium ammonium sulfate; ssDNA, single-stranded DNA; TES, 30 mM Tris, 2 mM EDTA, and 20% w/v sucrose; TMB, tetramethylbenzidine.

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loops of the variable heavy chain and one residue in CDR loop L2. Seven residues were selected and subjected to site-specific mutagenesis.

L6 mutagenesis was performed by cloning the L6 antibody genes into an M13 expression vector which allows the rapid isolation and characterization of mutant antibodies with changes in affinity or specificity (Huse et al., 1992; Glaser et al., 1992). Briefly, oligonucleotides incorporating the desired mutations were hybridized with a single-stranded, circular M13 DNA template encoding native L6 Fab, and after primer extension and ligation to produce functional vector encoding L6 Fab with the desired mutation, *Escherichia coli* were infected and Fab expression was induced. L6 Fab was isolated from the periplasmic space of infected bacteria and characterized in ELISAs.

The results of nine mutagenesis experiments are reported here. Although the majority of the residues were isosterically replaced, eight of nine mutations were found to abolish, or nearly abolish, the binding of L6 to its tumor antigen. In contrast, all of these mutations bound to at least two of three anti-idiotypic antibodies, indicating that the overall structure of the mutants remained intact. The results emphasize the critical role of single residue contacts for the integrity of antibody−protein interfaces and suggest that crucial antibody−antigen contact residues may be largely nonpermissive to changes.

MATERIALS AND METHODS

Selection of L6 Residues for Mutagenesis. Target residues for mutagenesis were selected on the basis of computer graphics inspection of the L6 Fv model (Figure 1). The CDR residues were identified that were predicted to be sufficiently accessible and most likely involved in antigen binding. Not all conceivable residues were selected, but preference was given to protruding asparagine, glutamine, or tyrosine residues because these residues are frequently found to mediate antibody−antigen interactions (Padlan, 1990; Mian et al., 1991) and because these residues are amenable to more conservative (such as isosteric) and less conservative changes (for example, Ala). No residues of known or putative importance for the conformation of the five canonical CDR loops (Chothia et al., 1989) were considered as mutagenesis sites. These structural determinant residues are important for conformational stabilization of single CDR loops, and their side chains are usually not solvent exposed.

Site-Directed Mutagenesis. L6 CDR mutations were introduced into the bacteriophage M13IXL604 (Huse et al., 1992) using the Muta-Gene M13 *In Vitro* Mutagenesis Kit, version 2 (Bio-Rad, Richmond, CA). Briefly, uracil-substituted M13IXL604 ssDNA was prepared as template for mutagenesis according to the manufacturer's instructions. Two hundred nanograms of template was mutagenized with 2−3 pmol of phosphorylated oligonucleotide in a total volume of 13 μL. For each mutagenesis experiment the template consists of the parental L6 sequence modified to include a deletion, stop codon, and restriction site to favor recovery of intact mutated L6 Fab (Figure 2). The reaction mixture was diluted to 100 μL with water, and 10 μL was transformed into *E. coli* strain XL-1 (Stratagene, San Diego, CA) and then titered onto a lawn of XL-1. Random plaques from each mutagenesis were picked for further analysis. Minipreparations of ssDNA from L604 mutant phage were prepared according to Sambrook et al. (1989) and sequenced to confirm construction of the correct mutants.

Antibodies and Reagents. L6 anti-idiotypic mAbs 1, 3, and 13 have been described previously (Hellström et al., 1990). Anti-id 1 recognizes an idiotypic determinant predominantly associated with the light chain V-region. Anti-id 13 recognizes an idiotypic determinant predominantly associated with the heavy chain V-region, and anti-id 3 requires the association of both the variable light and heavy chains. Mammalian chimeric L6 Fab fragments were generated by papain digestion using the kit available from Pierce (Rockford, IL). The following reagents were purchased from commercial sources: unconjugated goat anti-human κ light chain (Caltag, South San Francisco, CA), unconjugated goat anti-human Fab (Sigma, St. Louis, MO), HRPO-goat antihuman κ light chain (Caltag), biotinylated goat anti-human κ light chain (Fisher, Pittsburgh, PA), Vector ABC kit (Vector Labs, Burlingame, CA), and certain ELISA reagents (specimen diluent, conjugate diluent, TMB chromogen, and substrate buffer) (Genetic Systems, Redmond, WA).

Preparation of Cell Membranes Expressing the L6 Antigen for ELISA. The human tumor cell line H3347 which expresses a high level of L6 antigen (Hellström et al., 1990) was grown to confluency in Iscove's media plus 10% v/v fetal calf serum. Cells were harvested by EDTA treatment followed by washing in PBS. Cell pellets of 0.5−3.0-mL volume were suspended in 10 mL of lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, pH 10.2, 10.5 μg/mL aprotinin, 0.5 mM PMSF, 5 μg/mL leupeptin) at 4 °C and incubated for 15 min. The suspension was homogenized in a chilled Dounce homogenizer for 30−40 strokes and then centrifuged at 1500*g* for 5 min at 4 °C. The pellet was resuspended in membrane buffer (containing 1× PBS, 5 mM EDTA, pH 10.2, 10.5 μg/mL aprotinin, 0.5 mM PMSF, 5 μg/mL leupeptin, and 25 mM iodoacetamide), centrifuged twice at 82000*g* for 45 min at 4 °C, resuspended in 10 mL of membrane buffer and sheared through 18- and 21-gauge needles. Protein concentration was determined using the BCA protein assay (Pierce).

Preparation of Fab Expressed in the Periplasmic Space. Fab fragments were obtained from the periplasmic space of M13-infected, IPTG-induced cultures of bacteria as described (Huse et al., 1992). The *supO* nonsuppressor strain of *E. coli* MK30-3 (Boehringer-Mannheim, Indianapolis, IN) was grown in 2×YT until the cultures reached a density of 1.0 at A_{600}. Mutant M13 was added to the bacteria (1 pfu of phage/1 cfu of bacteria) and incubated with shaking for 1 h at 37 °C. Fab expression was induced by adding IPTG to a final concentration of 1 mM followed by overnight incubation of the cultures at 25 °C. The bacteria were pelleted at 5000*g* and resuspended in ice-cold TES ($^1/_{100}$ the volume of the original culture). An equal volume of lysozyme at 2 mg/mL in TES was added to the resuspended pellet, with gentle agitation, for 10 min. The periplasmic fraction containing the Fab was separated from the bacterial cell pellet by spinning at 12000*g* for 10 min at 4 °C.

Quantifying the Fab Concentration in Periplasmic Fractions. The concentration of periplasmic Fab was determined using quantitative ELISAs with proteolytically derived chimeric L6 Fab as the reference standard. Plates were coated with 100 μL of goat anti-human κ light chain at 1 μg/mL or goat anti-human Fab at 0.3 μg/mL in 50 mM

sodium carbonate−bicarbonate buffer, pH 9.6, overnight at 4 °C, blocked with specimen diluent, and washed with saline containing 0.02% Tween 20. We chose quantitation assays employing two different capture specificities to increase accuracy and to ensure that the assays were not biased by serological differences between the phage expressed and reference Fab. A standard curve of the chimeric L6 Fab reference was plated at seven different concentrations ranging from 1 to 15 ng/mL (for the anti κ coat) and 1 to 7 ng/mL (for the anti-Fab coat). These concentration ranges were predetermined to give a linear standard curve. The standard curve and all samples were plated in quadruplicate at 100 μL/well. Periplasmic fractions, which generally contain 1−10 μg/mL Fab, were initially diluted 1:500 in specimen diluent, serially diluted 1:2, and incubated overnight at 4 °C. After being washed, for both assays, 100 μL of biotinylated goat anti-human κ light chain was added at 1 μg/mL in conjugate diluent for 1 h at RT, followed by washing and then a 30-min incubation with 100 μL of Vectastain ABC (avidin and biotinylated HRPO complex; Vector Labs, Burlingame, CA) using a slight modification of the manufacturer's instructions. After being washed, TMB in buffered substrate was added for 15 min at RT. Incubations were stopped with 100 μL of 1 N H_2SO_4, and absorbance values were determined at 450/630 nm in a Bio-Tek ELISA plate reader (Winooski, VT). A separate standard curve was run on each plate to eliminate between-plate variability, and the standard curve was required to have a correlation coefficient (R^2) greater than 0.99. The concentration of Fab in the periplasm was determined using values from all dilutions falling within the linear range of the assays, generally between 8 and 16 separate values. The two assays agree with one another on average to within 20%.

Characterizing L6 Mutants. To quantify the ability of mutant Fab to bind to tumor antigen, microtiter plates were coated with an H3347 cell membrane preparation at 10 μg/mL, 100 μL/well, overnight at 4 °C. After being washed and blocked as described above, the periplasmic Fab concentration was adjusted to 500 ng/mL in specimen diluent and 100 μL/well was incubated overnight at 4 °C. After being washed, biotinylated goat anti-human κ light chain was added at 1 μg/mL, 100 μL/well, for 1 h at RT, followed by Vectastain, TMB chromogen, and 1 N H_2SO_4 as described above. In this assay the substrate incubation time was 30 min.

To characterize mutant Fab in anti-idiotypic ELISAs, plates were coated with anti-id antibodies 1, 3, and 13 at 10 μg/mL, 100 μL/well, in carbonate−bicarbonate buffer overnight at 4 °C. After being blocked and washed, periplasmic Fab was diluted in specimen diluent to 55 ng/mL and 100 μL/well was incubated overnight at 4 °C. After being washed, 100 μL of HRPO-goat anti-human κ light chain in conjugate diluent at 1 μg/mL was added for 1 h at RT, followed by washing and 100 μL of TMB substrate. Substrate incubations were 5 min for the anti-id 1- and 3-coated plates and 20 min for the anti-id 13-coated plates. The plates were stopped and read as described above.

RESULTS

Mutagenesis Sites. Seven residues were selected as described above, and nine mutagenesis experiments were carried out (Table 1). The location of these residues in the L6 model is shown in Figure 1. The selected asparagine and glutamine residues were isosterically mutated to aspartic and glutamic acids, respectively. H56 Gln was also mutated to arginine. H53 Tyr was replaced by alanine. Also selected for mutagenesis were H94 Arg and H101 Asp of CDR loop H3 on the basis of a previous prediction that H94 Arg may form a molecular recognition site on the CDR surface of L6 (Fell et al., 1992). H101 Asp is predicted to be only partially accessible and likely to form an ionic interaction with H94 Arg (which is often a conserved residue in antibody variable heavy chains). This interaction, when present, is likely to stabilize the conformation of CDR loop H3. In the L6

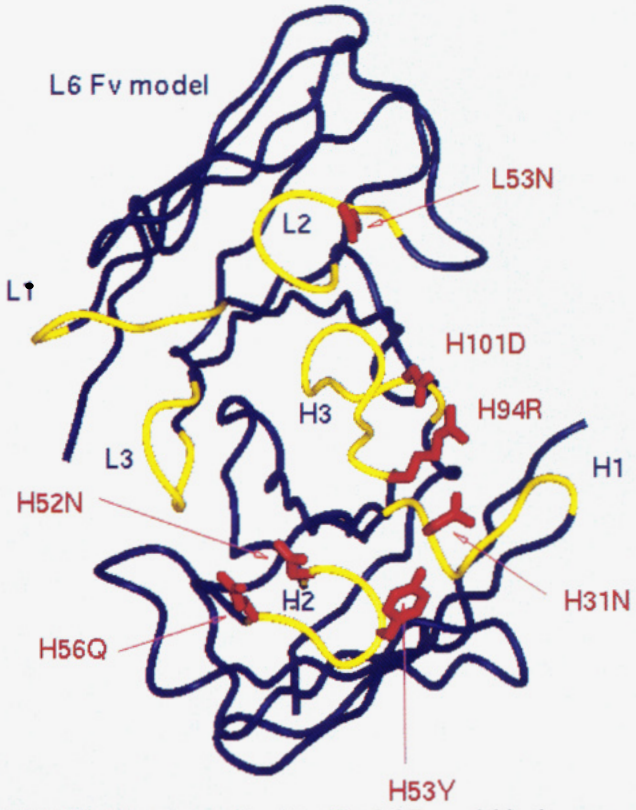

FIGURE 1: L6 mutagenesis sites. The L6 Fv model is shown as a blue solid ribbon in a front view facing the CDR loops (yellow, labeled H1−H3 and L1−L3). The side chains of CDR residues which were subjected to site-specific mutagenesis are labeled and colored in red. Asn31H is the only selected residue whose mutation did not affect binding of L6 mAb to the L6 antigen.

Table 1: Positions of Site-Specific Mutations in L6

Light Chain											
CDR 2 Parent Sequence	A	T	S	N	L	A	S				
L53D				D							
Heavy Chain											
CDR 1 Parent Sequence	N	Y	G	M	N						
H31D	D										
CDR 2 Parent Sequence	W	I	N	T	Y	T	G	Q	P	T	
H52D			D								
H53A					A						
H56E								E			
H56R								R			
H52D/H56E			D					E			
CDR 3 Parent Sequence	R	F	S	Y	G	N	S	R	Y	A	D	Y
H101N											N	
H94D/H101R	D										R	

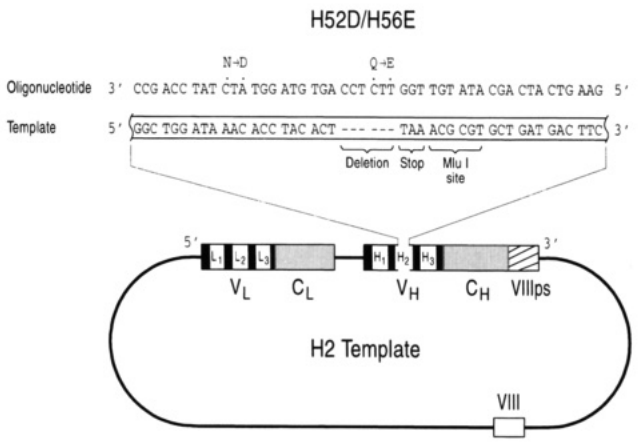

FIGURE 2: M13IXL604, the filamentous phage vector for parent L604 and mutant Fabs. The vector encodes the genes for expression of L6 Fab either as free antibody or as a fusion protein with pVIII on the phage surface. The lac operon controls expression of both the heavy and light chains. Mutations can be easily introduced into the vector by oligonucleotide-directed mutagenesis. The expanded insert illustrates the wild-type sequence of the vector compared to modifications in the template DNA (specific to the CDR to be mutated) including a 6-base-pair deletion, stop codon, and cloning site. Oligonucleotides used in site-directed mutagenesis correct the changes in the template, restoring the sequence of the full Fab molecule incorporating the desired mutations.

model, these two residues are surrounded by a cluster of aromatic residues. H94 Arg was predicted to be one of only two charged residues exposed on the largely hydrophobic CDR surface of L6.

Expression of L6 Mutants in M13. Figure 2 is a map of the M13 vector into which the L6 Fab genes were inserted. The vector, M13IXL604, was developed as described (Huse et al., 1992; Glaser et al., 1992). This vector encodes the L6 Fab under the control of the lac operon with bacterial signal sequences that direct expression to the periplasmic space. The Fab can be expressed either free in the periplasmic space or on the surface of the phage as a fusion protein with pVIII, depending on the bacterial strain used for expression. In these experiments, a nonsuppressor strain of *E. coli*, MK30-3, was used which produces only free Fab. Earlier results demonstrated that the periplasmic Fab binds to tumor antigen and anti-idiotypic antibodies with activity identical to that of Fab derived from IgG produced by mammalian cells. Thus, the serologically detected Fab appears to be properly folded, assembled, and fully active. Each mutation was constructed by oligonucleotide-mediated mutagenesis. Individual plaques containing each mutant were isolated, sequenced, and used to infect *E. coli* to generate periplasmic fractions containing Fab. The concentration of Fab in each preparation was carefully quantified in two ELISAs.

Characterization of Mutants by ELISA. Each mutant was characterized for its ability to bind to membrane preparations of a cell line which expresses approximately $(1-5) \times 10^5$ molecules per cell of the L6 tumor antigen (Hellström et al., 1986). Figure 3 shows that the binding of six (H52D, H53A, H56E, H52D/H56E, H101N, and H94D/H101R) of the nine mutants was essentially abolished, whereas two mutations (L53D and H31D) showed substantially reduced binding, and the binding of one mutant (H56R) was comparable to that of wild type.

To show that the conformational integrity of the CDR loops was not altered substantially by any of these mutations,

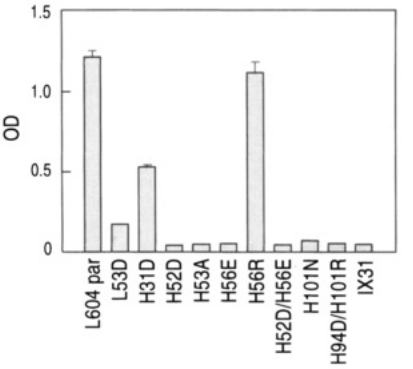

FIGURE 3: ELISA showing binding of wild-type L6 and mutants to H3347 tumor cell antigen. IX31 represents the negative control comprising the M13 vector lacking genes encoding Fab. Periplasmic Fab concentrations have been adjusted to 500 ng/mL, a concentration of Fab falling within the dynamic range of this assay.

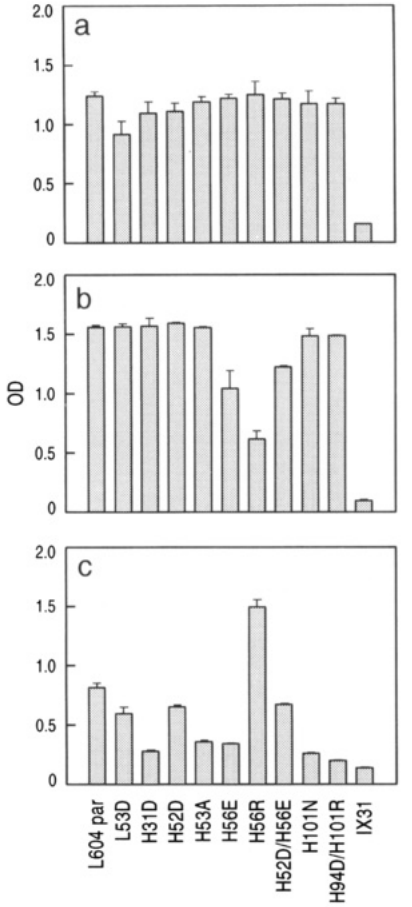

FIGURE 4: ELISA showing binding of wild-type L6 and mutants to anti-idiotypic antibodies. As in Figure 3, ELISA values are shown for all samples falling within the dynamic range of each assay. For all assays on anti-idiotypic antibodies, concentrations of Fab are 55 ng/mL. Panels a, b, and c refer to assays performed with anti-ids 1, 3, and 13, respectively.

each mutant was assayed against a panel of anti-idiotypic antibodies that recognize three distinct determinants on both the heavy and light chains of L6. Figure 4a shows the reactivity of the mutants on anti-idiotype 1, whose idiotope is contained largely, if not completely, within the L6 light chain (Hellström et al., 1990). None of the mutations in the L6 heavy chain affected the binding to this anti-idiotype. The single mutation in the light chain, L53D, diminished binding minimally. Figure 4b shows the reactivity of the mutants on anti-idiotype 3, which requires the association

Table 2[a]

mutations	antigen	1 (L chain)	3 (H + L)	13 (H chain)
L604 parent	+++	+++	+++	+++
L53D	+	++	+++	++
H31D	++	+++	+++	+
H52D	0	+++	+++	++
H54A	0	+++	+++	+
H56E	0	+++	++	+
H56R	+++	+++	+	++++
H52D/H56E	0	+++	++	++
H101N	0	+++	+++	+
H94D/H101R	0	+++	+++	+
IX31	0	0	0	0

[a] Symbols: 0, abolished binding; + greatly reduced binding; ++, moderately reduced binding; +++, binding comparable to wild type; ++++, binding greater than wild type.

of the L6 heavy and light chains for recognition. Only three mutations reduced binding: H56E and H52D/H56E moderately and H56R somewhat more. Interestingly, all three mutations involved residue 56 in H2. Figure 4c shows the reactivity of the mutants on anti-idiotype 13, which binds predominantly to the L6 heavy chain. This anti-idiotype was the most frequently affected by the mutations in that no mutation appeared identical to parent L6 and one mutation appeared to improve binding to the anti-idiotype. Mutants L53D, H52D, and H52D/H56E are moderately reduced compared to parent. Mutants substantially reduced in their anti-id 13 binding, but not negative in repeated assays, are H31D, H53A, H56E, H101N, and H94D/H101R. It is interesting to note that the glutamic acid substitution at H57 and the substitution of arginine at the same residue had dramatically opposite effects on anti-id binding. Taken together, these data support the contention that none of these mutations introduce global structural distortions in the antibody combining site.

Table 2 summarizes the ELISA data to illustrate the different effects these mutations had on antigen and anti-idiotype binding. L53D binding to antigen is minimal but not abolished. L53D, which shows moderately reduced binding to anti-id 1 (L specific), is comparable to wild-type binding on anti-id 3 (H + L chain). This mutation also moderately reduced the binding to anti-id 13, whose epitope was mapped to the heavy chain because it binds the L6 heavy chain in combination with irrelevant light chains (Hellström et al., 1990). The L53D mutation provides the first evidence that anti-id 13 may also recognize a part of the light chain. H31D shows moderately reduced binding to antigen and poor binding to anti-id 13, but it is comparable to wild type on anti-ids 1 and 3. Mutants H52D and H53A are similar in that binding of both Fabs to antigen is abolished, binding of both Fabs to anti-ids 1 and 3 is comparable to that of wild type, but binding to anti-id 13 is reduced (moderately for H52D, substantially for H53A). All mutations modifying H56 (H56E, H56R, and H52D/H56E) are comparable to wild type on anti-id 1. H56E does not bind to antigen and shows moderately reduced binding to anti-id 3 and substantially reduced binding to anti-id 13. H56R is the only mutant in which binding to antigen is comparable to that of wild type. It shows substantially reducing binding to anti-id 3 but is comparable to, and perhaps slightly better than, wild type on anti-id 13. The double mutant H52D/H56E does not bind antigen, shows moderately reduced binding to anti-id 3, and is comparable to wild type on anti-id 13. The H3 mutants, 108N and H94D/H101R, are similar to some H2 mutants, H53A and H52D, in their abolished binding to antigen, high binding to anti-ids 1 and 3, and reduced binding to anti-id 13.

DISCUSSION

Engineering monoclonal antibodies for therapeutic intervention is currently of significant scientific interest, particularly in efforts to increase affinity (Deng et al., 1994; Schlom et al., 1992). Several strategies can be pursued to identify mutations that improve antibody affinity and, conceivably, specificity. These approaches range from completely random mutagenesis over the entire variable regions (Hawkins et al., 1993) or restricted to the CDRs (Sharon et al., 1993) to mutagenesis based on structural models obtained from crystallography (Reichmann & Weill, 1993) or computer modeling (Kelly & O'Connell, 1993). Random approaches often suffer from the extremely large number of mutant sequences that need to be evaluated, whereas site-specific changes are limited by the lack of a fundamental understanding of the mechanism of antibody−antigen binding in most cases. Even when crystallographic data exist for an antibody−antigen complex, mutations that might improve an interaction are difficult to predict from these structures.

We have attempted to identify residues in the L6 mAb which are important for binding to the L6 tumor antigen. The architecture of L6 was predicted to be typical for an antibody that binds a protein antigen in that it presents a rather flat, largely hydrophobic surface punctuated by clusters of polar and a few charged residues. We felt that these polar residues were likely candidates to be involved in the L6 antibody−antigen recognition. Residues involved in binding and/or function are often identified by alanine mutagenesis (Cunningham & Wells, 1989; Kelly & O'Connell, 1993). We have introduced other mutations to probe the role of the selected Asn and Gln residues. The mutations of Asn and Gln residues to Asp and Glu, respectively, are isosteric. Therefore, these changes are, in contrast to alanine mutation, not likely to disturb the surface complementarity of an antibody−antigen interface. However, these changes introduce single charges in the interface. All but one of the Asn or Gln residues selected in this study showed greatly reduced binding to tumor antigen when mutated to their charged analogs. We have subsequently changed H56 Gln to arginine. Whereas the H56 Gln to glutamic acid mutation resulted in dramatically reduced binding to L6 to antigen, the change to arginine, albeit not isosteric and introducing a positive charge, resulted in binding at least comparable to that of wild type. This may be due to an enthalpy−entropy compensatory effect if H56 Gln would participate in a polar to ionic interaction involving a negatively charged antigen residue. Since changes of all three residues selected in CDR loop H2 reveal dramatic effects on binding, we conclude that H2 must substantially participate in the formation of the L6 antibody−antigen complex.

Residue H101D Asp is predicted to form a salt bridge with H94 Arg at the base of CDR loop H3. In the L6 model, this salt bridge is surrounded by a cluster of aromatic residues, and this aromatic cluster was previously proposed to be a molecular recognition site of the L6 mAb (Fell et al., 1992). We have addressed this prediction in two mutagenesis experiments. H101D Asp was first isosterically

replaced to asparagine which may retain hydrogen bonding to H94 Arg but introduces a positive net charge in this region of L6 mAb. Secondly, a double mutant (H94 Arg/Asp, H101D Asp/Arg) was prepared which does not introduce a net charge in this region and which may still permit an ionic interaction between these residues. This double mutation is, however, expected to distort or destroy the geometry of the putative L6 recognition site. Accordingly, we find nearly abolished binding of L6 to tumor antigen as a consequence of these mutations.

The importance of residue L53 in CDR loop L2 is noteworthy. L2 is the CDR loop least frequently involved in antigen binding and has not yet been found to contribute to the binding of haptens to antibodies. CDR L2 has, however, been shown to contribute to the formation of crystallographic antibody−protein complexes (Wilson & Stanfield, 1993). Previously we have shown that a mutation in CDR loop L2 of the anti-CD18 antibody 60.3 abolished antigen binding (Hsiao et al., 1994). Here we show that a single isosteric change in CDR loop L2 is sufficient to dramatically reduce binding of L6 mAb to its tumor antigen.

The binding of L6 anti-idiotypic antibodies to L6 mutants was used to assess the global structural integrity of the mutated forms of L6. The binding of the mutants to the L6 anti-idiotypic antibodies that recognize different epitopes in the L6 variable regions suggests that no large conformational changes have been introduced as the consequence of the mutations. It follows that the observed reductions in tumor antigen binding are likely due to a change in contact residues rather than to secondary conformational effects. It is worth noting that these results are consistent with the canonical structure model of Chothia and colleagues (Chothia & Lesk, 1987; Chothia et al., 1989); i.e., changes in residues different from the so-called structural determinants (Chothia et al., 1989) in single canonical CDR loops do not affect the overall conformation of the loop. None of the residues targeted in this study was a known canonical determinant for the five canonical CDR loops.

We have found that six of the seven polar residues selected in L6 CDRs are important in binding to tumor antigen. Not all residues which contact antigen, however, determine the energetics of the antibody−antigen interaction. The analysis of crystallographic structures of uncomplexed Fab fragments or of modeled Fv structures is at best able to correctly identify residues which contact antigen. Only a fraction of antigen-contacting residues in antibodies may actively contribute to the free energy of binding whereas some contacts are energetically neutral or even opposing (Novotny et al., 1989). Recently, this concept has experienced experimental support in several instances, the most prominent perhaps being a study by Wells and co-workers (Jin et al., 1992), who analyzed the binding of 21 different monoclonal antibodies to human growth hormone. It follows that the identification of antigen-contacting residues by mutagenesis may often be problematic. The presence of enthalpy−entropy compensation of antibody mutants has frequently been observed (Ito et al., 1993; Kelly & O'Connell, 1993), but also the absence of these effects is known (Kelly & O'Connell, 1993). The mutation of residue H56 Gln to Arg did not exhibit an effect, but the mutation of this residue to Glu significantly reduced binding. These findings suggest that the L6 antibody−antigen interface is particularly sensitive to the introduction of negative charge. We classify residue H56 in L6 as important for antigen binding, but our results show that this remains dependent on the particular mutagenesis experiment.

The results of the mutagenesis study presented here have some implications for future attempts to increase the tumor affinity of L6 by M13 saturation mutagenesis. We have generated large libraries of mutations in the L6 CDRs (Glaser et al., 1992), which are difficult to screen because of their size. It has been possible to identify, on the basis of the L6 model, residues in L6 which are critical for antigen binding. The identification of residues important for antigen binding to L6 allows us to assign priorities and limit the number of residues for saturation mutagenesis. In addition, some of the identified residues may not tolerate any change without abolished, or nearly abolished, antigen binding (as indicated by the results of isosteric mutagenesis). The inclusion of such residues, which may not be permissive to any changes, in libraries of antibody mutants would increase the number of mutants which do not bind antigen and would hinder the screening for higher affinity mutants. Recently, Hawkins et al. (1993) have suggested that noncontact residues may be important in the natural mechanism of affinity maturation as well as in attempts to generate higher affinity antibodies *in vitro*. It may be advantageous to initially restrict mutations to residues proximal to contact residues while retaining the contacting residues as wild type in order to minimize both library complexity and the likelihood of introducing single mutations that destroy antigen binding.

CONCLUSIONS

We have presented an analysis of L6 mAb residues critical for binding to the L6 tumor antigen. Six of seven residues selected from a three-dimensional model of the L6 binding site were found to be critical for the binding to tumor antigen. In contrast, mutation of these residues did not abolish binding to L6 anti-idiotypic antibodies whose epitopes did not include the site of the mutation. This latter result supports the notion that solvent-accessible CDR residues in antibodies can be mutated without introducing significant structural perturbations of the antigen binding site. We found that the integrity of the L6 antibody−antigen interface was very sensitive to the introduction of negative charge. In one case, the subsequent and nonisosteric introduction of an opposite charge was readily tolerated.

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