A Combinatorial Library Strategy for the Rapid Humanization of Anticarcinoma BR96 Fab*

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We have used a combinatorial mutagenesis strategy to humanize BR96, a monoclonal antibody that binds to the Lewis Y class of tumor antigens. This approach allows simultaneous assessment of hundreds of humanized variable regions to identify the molecules that best preserve affinity, thus overcoming the major drawback of current humanization procedures, the requirement to construct and analyze each humanized antibody separately. Murine residues of BR96 were mutated to human if they were solvent-exposed residues that did not participate in the formation of the antigen binding site and were not at the interface of the light and heavy chain. At positions that might be involved in binding to antigen, the choice between the murine and human residue was more difficult. Murine and human alternatives were incorporated into a combinatorial library at positions representing buried residues that might affect the structural integrity of the antigen binding site. By encoding this library of humanized BR96 Fabs in an M13 phage vector, we rapidly identified several candidates with nearly identical antigen binding, within 2-fold, of the chimeric Fab. Additional mutagenesis directed at sites suggested in the literature as potentially important for antigen binding in a similar anti-Lewis Y antibody yielded no further improvements.

The human immune response to antigenic sequences in rodent monoclonal antibodies (mAbs)1 has limited the therapeutic use of these proteins (1, 2). The creation of chimeric antibodies with the foreign variable regions (V regions) joined to human constant regions (3–5) has addressed this limitation, in part. Many chimeric antibodies, however, continue to induce an immune response directed to the foreign V regions (6, 7), and the immunogenicity of a given V region in humans is not predictable at present. The desire to further minimize the potential for immunogenicity of any xenogeneic V region has motivated the development of several methods for “humanizing” an antibody. “Humanization” involves changing foreign framework residues rare in human V region sequences to residues more commonly found. The involvement of complementarity determining regions (CDRs) in antigen binding generally requires preservation of these sequences.

Winter and colleagues (8–10) first described a method for transferring the specificity of a murine antibody by grafting its CDRs onto human framework regions. The simple grafting of CDRs onto human frameworks, however, often results in significantly reduced affinity for antigen (9, 11). Although humanization is straightforward in principle, in practice alterations at framework residues, particularly those interacting with CDRs, frequently affect antigen binding. Analysis of antibody-antigen complexes by x-ray crystallography has shown that some framework residues can interact directly with antigen (12), affect the conformation of the CDR loops (11, 13, 14), and influence packing interactions between the β-sheet strands (15). Molecular modeling and crystal data, when available, have helped to identify murine framework residues that most likely contribute to the integrity of the binding site. Preservation of these residues in humanized antibodies often maintains affinity for antigen comparable to the original antibody (9, 11, 14, 16). Detailed structural information is not available for many antibodies, which prompted development of other approaches for designing humanized V regions that maintain acceptable affinity. Such methods include computer assisted design (17, 18), variable domain resurfacing (19), framework exchange (20), a positional consensus method (21), and sequence homology comparisons (22).

These humanization methods generally require an iterative approach to create, characterize, and correct, if necessary, the sequence of a humanized V region. Even as our understanding of antibody structure increases and more information is available regarding positions that predominantly affect ligand binding, it remains difficult to generalize the approach. Designing a humanized antibody requires making choices of which residues to change and which to retain. Making these decisions is often subjective, but creating a set of humanized molecules that represents all of the alternatives at positions where the choice is difficult reduces the degree of subjectivity. Simultaneously determining the binding activity of the many different humanized forms contained in such a set or “library” reduces or eliminates the need to rely on a trial and error strategy.

We have humanized the anticarcinoma mAb BR96 by a combinatorial library design strategy that created a set of humanized V regions from which sequences that best preserved affinity were rapidly selected. BR96 recognizes a tumor-associated antigen expressing a Lewis Y (Le+) related carbohydrate on the
surface of many human carcinomas (23, 24). An immunooconju-
gate of chimeric BR96 with doxorubicin induces complete re-
gressions and cures of human tumor xenografts growing in athymic mice (25). The chimeric BR96 immunooconjugate is cur-
cently in human clinical trials. We have constructed an M13 phage Fab expression library containing combinations of hu-
nan and murine residues at several positions among those selected for humanization of BR96. In this report we discuss the design of a humanized BR96 library and the selection and char-
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bird's eye view to sequence-defined hypervariable regions of Kabat and Chothia and Lesk (30) spatially delineate the binding site better than the sequence-defined hypervariable regions of Kabat et al. (31). Sequence numbering and framework region alignments were according to Kabat et al. (31). Differences in residues between BR96 and selected human germline templates were mapped on a three-dimen-
sional model of the BR96 variable regions (32). From the model we identified residues important (13) or potentially important to the inter-
face of the heavy chain and light chain V regions or to the integrity of the antigen binding site. The selected human framework sequences were then inspected for conservation of buried (“packing”) residues as defined by Padian (33). Considering the modeling information and identity on the known residues, we made decisions to retain a murine residue, change it to the human counterpart, or include both the human and murine alternatives in the combinatorial library.

Oligonucleotide Synthesis—Oligonucleotides were synthesized and purified as described elsewhere (34). Synthesis of oligonucleotides incor-
porated a 1.1 mixture of murine codons to human codons at the selected library positions (35). The oligonucleotide mixtures were dou-
ble-deprotected with concentrated NH$_4$OH, purified by polyacrylamide gel electrophoresis, and eluted from the excised gel slices (36).

Library Construction—The molecule chosen for humanization was a

mixture of murine codons to human codons at the selected library positions (35). The oligonucleotide mixtures were dou-
ble-deprotected with concentrated NH$_4$OH, purified by polyacrylamide gel electrophoresis, and eluted from the excised gel slices (36).
for site-directed experiments was uridinylated single-stranded DNA from humanized Fab clone (2-40) with two affinity mutations. This affinity mutant, designated M3, had mutations in CDR2 (Gly53 → Asp) and CDR3 (Asp97 → Ala) of the heavy chain, which improved the binding affinity of chimeric BR96 for tumor antigen (34). Two site-specific mutants of humanized BR96 M3 were prepared by introducing mutations at position 19 or 82b in the heavy chain. The oligonucleotide sequence to mutate Arg to Lys at position 19 and Ser to Arg at position 82b in the heavy chain of humanized BR96 M3 were 5’-GATGCAAGCAGAGGATAACTTCAGGAGCCCTCCAG-3’ and 5’-CGTC-CCTCAGTGGTATTGC-3’, respectively.

**RESULTS**

**Construction of a Combinatorial Humanized BR96 Fab Antibody Library**—To design the humanized BR96 framework V region we selected a set of human germline sequences from a homology search of available immunoglobulin V region sequences. Because framework residues can influence the conformation of the antigen binding site, we chose human frameworks closely resembling that of murine BR96 (mBR96) to maximize the probability they would provide similar structural
The murine and human amino acids at buried residue positions included in the humanized BR96 M1 combinatorial library are listed.

| Heavy chain positions | Mouse | Human |
|-----------------------|-------|-------|
| 24                    | Thr   | Ala   |
| 49                    | Ala   | Ser   |

| Light chain positions | Mouse | Human |
|-----------------------|-------|-------|
| 11                    | Leu   | Ser   |
| 36                    | Tyr   | Leu   |
| 37                    | Leu   | Gln   |
| 43                    | Ser   | Ala   |
| 104                   | Leu   | Val/Leu |

The antibody humanized in these studies was BR96 M1, a mutant of BR96 with improved affinity resulting from one amino acid substitution in CDR3 of the heavy chain (34). The heavy and light chain amino acid sequences of BR96 M1 and human template sequences were aligned and differences between the murine and human frameworks identified. We inspected a BR96 model (32) to map the predicted spatial position of each residue, and then decided whether to retain the murine residue, to substitute the human residue, or to include both the murine and human residues in the combinatorial library. We maintained the murine residues at structural determinant positions (marked with # in Fig. 1) that are responsible for the canonical loop conformations of an antibody (13). The only structural determinant position in the framework with a different residue in BR96 and the human template was position 2 in the light chain (Fig. 1), and we retained the murine residue valine. Examination of the model indicated that none of the solvent exposed residues different between BR96 M1 and the human template were likely to be important for maintaining the structure of the antigen binding site. We chose to mutate all solvent exposed residues, 11 residues in the light chain and 16 in the heavy chain, to the human amino acids (Fig. 1).

The residues considered for library positions were buried amino acids as defined by Padlan (33). Humanization of the anti-lysozyme antibody, D1.3 (11) and of an anti-CD18 mAb, mIB4 (22) demonstrated the critical nature of some buried residues. CDR grafting of D1.3 caused a 45-fold decrease in its binding affinity. Changing four buried framework residues back to the murine residues restored binding activity to within 4-fold of the murine antibody. For humanized 1B4, recovery of a 3-fold loss of activity measured in an in vitro adhesion assay occurred by changing three buried residues back to the murine residues (22).

The BR96 library included buried residues at heavy chain and light chain positions where the murine and human choices were between nonconservative residues. In the heavy chain of BR96 four buried positions differed between the murine (m) and human (h) template sequences, position 20 (m = Val, h = Leu), 24 (m = Thr, h = Ala), 49 (m = Ala, h = Ser), and 77 (m = Thr, h = Ser). We included positions 24 and 49 (Table I) in the library, since they are less conservative choices. The amino acid choices at positions 20 and 77 were conservative and we chose to humanize both. In addition, at position 20, the murine residue Val rarely occurs in human VHIII sequences (less than 5%) (31). The fourth potential library position, 77, is not proximal to a CDR loop and the computer model suggested that neither the Thr (murine) nor Ser (human) would affect CDR conformation.

Inspection of buried residues in the light chain V regions revealed different residues at six positions, position 2 (m = Thr, h = Ser), 11 (m = Leu, h = Ser), 36 (m = Tyr, h = Leu), 37 (m = Leu, h = Gln), 43 (m = Ser, h = Pro), and 104 (m = Leu, h = Val). As noted above we maintained the murine residue at position 2 of BR96 because this position is a canonical structural determinant for the L1 loop (13). Even though the two amino acid choices are conservative at position 104, we included this position in the combinatorial library since representation of Leu and Val in the human Jk2 segment is approximately equivalent in human J region sequences homologous to BR96. Therefore, the humanized antibody library incorporated the murine and human alternative amino acids at light chain positions 11, 36, 37, 43, and 104 and at heavy chain positions 24 and 49 (Table I).

We constructed the combinatorial library of humanized BR96 M1 in an M13 Fab expression vector. De novo synthesis of the humanized V region genes by PCR amplification of long overlapping oligonucleotides was used so that all combinations of the alternatives at library positions were introduced in a single reaction. The single-stranded DNAs, produced by a second, asymmetric PCR, were incorporated into the uridinylated M13IX104 phage vector by hybridization mutagenesis (37, 39). Inclusion of codons for the murine and human alternatives at library positions into the oligonucleotides yielded PCR products representing all permutations. The resulting library contained 2² different heavy chains and 2² different light chains for a total of 128 variant humanized BR96 M1 Fab molecules. If desired, substantially larger libraries can be created by this method to examine alternate residues at many more positions.

Selection and Characterization of Humanized BR96 Fabs—Screening of the combinatorial library by a nitrocellulose filter
Humanization of Anticarcinoma BR96 Fab

The heavy chain and light chain V regions of five humanized BR96 M1 clones were sequenced as described under "Experimental Procedures." The amino acids at the combinatorial library positions in the murine BR96 V region, in the human template sequences, and in the five humanized clones are presented.

Table III

Amino acid sequence of library positions in humanized BR96 M1 clones

| Construct | H24 | H49 | L11 | L36 | L37 | L43 | L104 |
|-----------|-----|-----|-----|-----|-----|-----|------|
| mBR96 M1  | Thr | Ala | Leu | Tyr | Leu | Ser | Leu  |
| Human template | Ala | Ser | Ser | Ser | Leu | Gla | Pro  |
| 1–29      | Ala | Ser | Pro | Ser | Tyr | Gla | Pro  |
| 1–76      | Ala | Ser | Ser | Tyr | Gla | Ser | Val  |
| 1–88      | Ala | Ser | Ser | Tyr | Gla | Ser | Val  |
| 2–31      | Ala | Ser | Ser | Tyr | Gla | Ser | Val  |
| 2–40      | Ala | Ser | Ser | Tyr | Gla | Ser | Leu  |

a H designates heavy chain, and L designates light chain.
b Proline was not included as a library residue and resulted from a PCR error.
We compared the residues humanized in each antibody and particularly noted the positions in B3 identified as important to antigen binding. The humanized B3(Fv)-PE38 immunotoxin, as originally designed, showed a 20-fold loss in activity, but two site-specific changes resulted in substantial recovery of ligand binding and cytotoxicity (20). Mutation of Leu104 (murine) to Val104 (human) in the B3 light chain restored binding activity 8-fold. The authors suggested that humanization of light chain residues 15 and 18, both to Pro, required that 104 also be humanized. For the humanization of BR96, the combinatorial library included light chain position 104 with the same amino acid alternatives as for B3. We found that substitution of either amino acid at that position in humanized BR96 M1 did not affect antigen binding. BR96 has Leu at position 15 not Pro, which may account for greater flexibility to substitute residues at 104.

An additional 2-fold recovery in binding of humanized B3 occurred by replacing human residue Ser with the murine residue Arg at position 82b in the heavy chain. In humanized BR96 we had also substituted the human Ser for the murine Arg so we questioned whether changing the residue at 82b in BR96 might affect its binding activity. We changed the human residue Ser back to the murine amino acid, Arg, at 82b by site-directed mutagenesis. The clone used for this experiment was humanized BR96 M3, a derivative of clone 2-40 with an additional affinity mutation, Gly to Asp at 53 in the heavy chain (34). The return to the murine residue did not alter binding to tumor (Fig. 4). The $k_{on}$ measured by SPR was also unchanged (data not shown).

Residue 19 of humanized B3 heavy chain fits the criteria that Benhar et al. (20) had established for humanization of B3. However, based upon their unpublished observations they suggested that mutations introduced at residue 19 resulted in substantial activity loss, and despite the conservative nature of the change (Lys to Arg), they retained the murine residue. We had chosen to humanize position 19 in BR96. To test whether this position affected antigen binding of humanized BR96, we changed the Arg back to the murine residue Lys. As shown in Fig. 4, changing the residue had no effect on binding activity. The $k_{off}$ of this molecule measured by SPR was similar to that of humanized BR96 M3 and the 82b site-directed mutant (data not shown). Thus, none of the positions that had or were suggested to have an impact on the humanization of B3 had a significant effect on humanized BR96 binding, despite the great sequence homology and similar antigen specificity of the two antibodies.

**DISCUSSION**

Many “humanized” forms of a monoclonal antibody are possible. A compromise between humanizing a murine framework residue to reduce its potential immunogenicity and retaining a residue critical to antigen binding and specificity is often nec-

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**FIG. 3. Alignment of murine and humanized BR96 sequences with murine and humanized B3 sequences and human templates for each. A, alignment of mBR96 light chain, hBR96 light chain, and human template HSIGVA23 with murine B3 (mB3) and humanized B3 (hB3) light chains, and human template, GM607 (20). B, alignment of mBR96 and hBR96 heavy chains and human template HSIGDP51 with mB3 and hB3 heavy chains and human template, 56P1 CL (20). Amino acids that are identical to the murine amino acid are designated by a dash.**
body-specific modeling information can be useful when deciding identification of positions as high, medium, or low risk for available databases are options (for a review, see Routledge) regarding which residues to humanize. Choosing homologous sequences identified in our data base search did not include the template for B3, GM607. If we had chosen GM607 as a template, our choice of buried residues for the library would have the template hessary. We have presented a combinatorial library strategy to examine murine and human alternative residues at positions potentially important for retaining antigen binding. Simultaneous evaluation of antigen binding of Fabs representing all combinations of selected residues eliminates repeated rounds of design and analysis frequently required to identify the “most” human sequence that maintains the best binding.

Selection of the human frameworks to use as templates for humanization of foreign V regions defines subsequent decisions regarding which residues to humanize. Choosing homologous templates from antibodies with known crystal structure, from germline, non-germline, or consensus sequences derived from available data bases are options (for a review, see Routledge et al. (43)). As discussed in more detail below, the choice of templates for the light chains of BR96 and B3, germline template for BR96, and a non-germline sequence for B3, dictated that different residues be humanized in the two antibodies. Even though we chose germline sequences to eliminate potential immunogenicity of somatic mutations, no particular template selection method has proven superior to another regarding immunogenicity. Too few humanized antibodies have been administered clinically to provide sufficient data about the immunogenicity of humanized mAbs.

A patient’s immune response to solvent exposed residues of a mAb results in more rapid clearance of the mAb upon subsequent administrations (43). Therefore, we preferred to humanize all the solvent exposed residues to minimize immunogenicity. Although we wished to humanize as many residues as possible, we were hesitant to change residues at the light and heavy chain V region interface or adjacent to CDR loops, since they may affect antigen binding. Many of these amino acids are buried or “packing” residues (35) and are important to antigen binding (11, 22). Positions selected for the BR96 library included the buried positions for which the murine and human alternative amino acids represented nonconservative changes.

A library strategy is advantageous because it can incorporate any position for which the choice of human or murine amino acid is ambiguous. General structural information compiled from crystallized antibodies, e.g. CDR loop conformation (13), identification of positions as high, medium, or low risk for humanization (44), degree of solvent exposure (33), or antibody-specific modeling information can be useful when deciding to retain the murine residue, change it to human, or incorporate it as a library position. Furthermore, rodent and human V regions share homology in CDR sequences, and future attempts at humanization could include selected CDR residues as well as framework residues.

Humanization of buried residues did not adversely affect binding of BR96 to antigen. Several humanized BR96 M1 Fabs demonstrated binding activity comparable to the murine V region parent. In five clones sequenced, we found only the human residue at three positions, both human and murine residues at three positions, and only the murine residue at one position. The murine residue Tyr predominated at position 36 in the light chain and it also dominates in the human sequences homologous to BR96. This position is involved in the interface between the V regions of the heavy and light chains, and so it may not be permissive to change. The predominance of human residues at three positions was unexpected, but because we sequenced only five clones, we do not think that the data statistically prove an advantage of human over murine residues at these positions. While the binding activity of all selected clones was similar to mBR96 Fab, the expression of the humanized clones was higher in the bacteria. The humanized Fabs expressed in the bacterial periplasm all yielded 4–8-fold more Fab than typically found for the murine V region. The plaque lift assay used initially to select clones from the library is sensitive to expression level. The human residues that predominated in some clones could have affected their expression in bacteria, but we cannot distinguish whether library positions or the other humanized residues not included in the library caused this improvement. Application of a combinatorial strategy might also be useful solely to maximize protein expression, often an important issue in bacterial expression of mammalian proteins.

The different approaches taken to humanize BR96 and B3 illustrate how template choice and form of an antibody can influence the outcome of the process. We chose germline sequences for humanization, whereas Benhar et al. (20) selected the most highly homologous templates without the same constraint from their data base search. The BR96 heavy chain framework has sufficient dissimilarity to B3 that the 46 human sequences most homologous to BR96 did not include the template used for B3. The light chains, on the other hand, have only three amino acid differences in the frameworks, and homologous sequences identified in our data base search did include the template for B3, GM607. If we had chosen GM607 as template, our choice of buried residues for the library would have been reduced to one, position 104. The different choice of templates not only determined library composition, but also led us to mutate a somewhat different set of solvent-exposed residues. Ultimately, the two humanized mAbs had greater divergence in sequence than the original murine antibodies.

Humanization of B3(Fv)-PE38 as originally designed resulted in a molecule with a 20-fold loss in binding activity. By iterative design and analysis they identified the framework positions critical to recovery of binding activity. Despite the sequence similarity of B3 and BR96, humanization of positions or preservation of particular murine residues in BR96 was not critical to its activity. Because B3(Fv)-PE38 immunotoxin is a single-chain fusion protein isolated from inclusion bodies in E. coli and requires refolding, alterations to the amino acid sequence of the Fv could affect the refolding process. These amino acid substitutions might not affect BR96 similarly, since it is expressed as a soluble functional Fab.

Humanization is an empirical process and many modifications of xenogeneic antibodies are conceivable that might fit a definition of humanized. In this report many humanized BR96
Fabs with varied humanized sequences that bound comparably to tumor antigen and without significant loss in affinity were rapidly identified. Combinatorial library strategy offers a flexible approach to the humanization of antibodies and to other protein engineering projects.

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