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Humanization of murine monoclonal antibodies through variable domain resurfacing

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ABSTRACT **Two murine monoclonal antibodies, N901 (anti-CD56) and anti-B4 (anti-CD19), were humanized by a process we call "resurfacing." A systematic analysis of known antibody structures has been used to determine the relative solvent accessibility distributions of amino acid residues in murine and human antibody variable (Fv) regions and has shown that the sequence alignment positions of surface amino acids for human and murine variable region heavy (V_H) and light (V_L) chains are conserved with 98% fidelity across species. While the amino acid usage at these surface positions creates surface residue patterns that are conserved within species, there are no identical patterns across species. However, surprisingly few amino acid changes need to be made to convert a murine Fv surface pattern to that characteristic of a human surface. Resurfacing was used to change the patterns of surface accessible residues in the Fv regions of the N901 and anti-B4 antibodies to resemble those found on the Fv regions of human antibody sequences. Two different procedures for selecting a human sequence were compared. For anti-B4, a data base of clonally derived human V_L–V_H sequence pairs was used, while for N901, sequences for V_L and V_H were independently selected from the Kabat *et al.* data base [Kabat, E. A., Wu, T. T., Reid-Miller, M., Perry, H. M. & Gottesman, K. S. (1991) *Sequences of Proteins of Immunological Interest* (DHHS, Washington, DC), 5th Ed.]. Resurfaced N901 and anti-B4 antibodies had apparent affinities for their cell surface ligands that were identical to those of their respective parent murine antibodies. These data provide evidence that, despite the differences in the surfaces of mouse and human Fv regions, it is possible to substitute one for the other while retaining full antigen binding affinity.**

The generation of high-affinity murine monoclonal antibodies (1) against chosen human antigens is now routine. Therapy in humans, however, requires human antibodies to fully exploit immune effector functions and to prevent anti-globulin responses (2). "Humanization" or "reshaping" of murine antibodies is an attempt to transfer the full antigen specificity and binding avidity of murine monoclonal antibodies to a human antibody by grafting the murine complementarity-determining regions (CDRs) onto a human variable region framework (3). However, to preserve the binding affinity, the majority of CDR-grafted antibodies require additional amino acid changes in the framework region, because such amino acids either are conformationally important or are in direct contact with the antigen (3–5). Such necessary framework changes may introduce new antigenic epitopes and, if many changes are needed, the advantages of CDR grafting over chimeric antibody (6) constructions will be lost. A solution to this problem is to maintain the CDRs and the core of the murine variable region framework, but to replace the surface residues of the framework region with those from a human variable region by a process we call "resurfacing."

A premise of the resurfacing approach is that the immunogenicity of murine antibody variable regions originates with the surface residues. It is generally accepted that either mobility (7, 8) or accessibility (9, 10) is the major determinant of protein antigenicity, and hence that the surface will carry most if not all of the antigenic potential of a protein. Attempts to specify variable region surface residues have been described (11), but no systematic analysis of antibody structures has been previously carried out. We have done such an analysis of the relative solvent accessibility distributions of amino acid residues in 12 crystal structures of murine and human antibody variable (Fv) regions (12). This analysis has shown that the sequence alignment positions of 45 residues defined as surface-exposed were conserved with 98% fidelity, and it has allowed the development of a general algorithm for humanizing antibodies by resurfacing (12). Using this approach, we have designed and constructed humanized variable region genes for two monoclonal antibodies, N901 (IgG1, κ; anti-CD56; refs. 13 and 14) and anti-B4 (IgG1, κ; anti-CD19; ref. 15), spliced the genes to human constant regions, expressed the antibody protein products, and characterized the binding properties of the purified resurfaced antibodies.

MATERIALS AND METHODS

Sequence Alignments of Structurally Characterized Fv Regions and Calculation of Relative Surface Accessibility of Framework Amino Acid Residues. Light and heavy chain framework sequences and the coordinates of 11 crystal structures of antibody fragments were obtained from the Protein Data Bank at Brookhaven National Laboratory (16). The structure of a twelfth antibody, Gloop 2, was obtained from P. Jeffrey (17). Sequence numbering and alignments of framework regions are according to Kabat *et al.* (18), unless otherwise indicated, while CDRs are as defined in ref. 19. To determine the positions of the solvent-accessible residues on the surface of the Fv region, the residue accessibilities were calculated for the above 12 structures by using a modification of the DSSP (Dictionary of Secondary Structure in Proteins) routine (20) in which explicit atomic radii are used. A residue was defined as being accessible when its relative accessibility was greater than 30%. Further details of the procedure can be found in ref. 12.

Sequence Data Bases. Human antibody sequences were obtained from the Kabat *et al.* data base of immunoglobulin

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Abbreviations: CDR, complementarity-determining region; Fv region, antibody variable region; V_L and V_H, light and heavy chain variable regions.

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sequences (18) and from the OWL nonredundant data base (21). Antibodies with known heavy chain–light chain pairing were identified from their common clone name.

Generation of Models of Resurfaced N901 and Anti-B4 Antibodies. Models were generated by using the methods of Martin *et al.* (22, 23) and recent modifications (19). The framework regions were modeled by using the structures of the most identical light and heavy chain variable region (V_L and V_H) sequences, KOL (24) and 4-4-20 (25), respectively for N901, and HyHEL5 (26) for both chains of anti-B4. The chosen frameworks were least-squares fitted onto the structurally conserved strands of the Fv β-barrel, as previously described (19). Framework side chains were substituted by using a maximum-overlap procedure. Where possible, CDR backbone conformations were generated by using canonical loops (27); otherwise they were built by using the CAMAL algorithm (19, 22, 23), which combines a C^{α} search of the Brookhaven data base with a conformational search using a modified version of CONGEN (19, 28) followed by screening with a solvent-modified potential and filtering based on torsion angles and sequence. Side chains for all CDR residues were constructed by using CONGEN. All models were subjected to 500 cycles of steepest descent energy minimization followed by 200 cycles of conjugate gradient minimization using the Discover force field (Biosym Technologies, San Diego).

Cloning of N901 and Anti-B4 cDNAs and Construction of Resurfaced V-Region Genes in Immunoglobulin Expression Vectors. The N901 and anti-B4 cDNAs were obtained by PCR amplification cloning (29) of mRNAs obtained from murine hybridomas. The resurfaced N901 V_H and V_L genes were constructed by extension and amplification of four overlapping oligonucleotides (120–130 nucleotides) making up alternate strands of the full-length resurfaced genes (30). For N901, the assembled V_H and V_L genes encoded the following sequences in order (5′ to 3′): a *Hin*dIII cloning site, the consensus Kozak sequence (5′-GCCGCCACC-3′) (31), an immunoglobulin signal sequence (32), an intron, the humanized V_H or V_L coding region, a 3′ noncoding sequence including a splice site, and a *Bam*HI cloning site. For anti-B4, the assembled V_H and V_L genes differ from the above by the elimination of an intron between the immunoglobulin signal sequence and the V_H or V_L coding region. The human immunoglobulin light and heavy chain expression vectors N901rLCEV and N901rHCEV were derived from HCMV-V_LLys-K_R (33) and contained either the resurfaced N901 V_L gene with the human constant κ exon or the resurfaced N901 V_H gene with the human γ-1 constant region gene (29), respectively. Expression vectors carrying the resurfaced anti-B4 V_L gene, α-B4rLCEVd, or the anti-B4 V_H gene, α-B4rHCEVd, differ from the resurfaced N901 expression vectors by the addition of the human *DHFR* cDNA positioned at the 5′ end of the cytomegalovirus (CMV) enhancer in reverse orientation with respect to the CMV promoter.

Expression, Purification, and Antigen-Binding Activity of Antibodies. Resurfaced N901 or anti-B4 antibodies were each expressed by transient transfection of COS cells cultured in serum-free medium (Hybridoma SFM, GIBCO), and then purified by staphylococcal protein A affinity chromatography. The antigen-binding activity of antibodies was assessed by two different methods, a competition binding assay and an indirect immunofluorescence binding assay. Competition binding assays of resurfaced N901 were performed with 3×10^5 SW-2 cells and fluorescein-labeled murine N901 (6 nM), while competition binding assays on resurfaced anti-B4 were performed with 3×10^5 Namalwa cells and fluorescein-labeled murine anti-B4 (2.9 nM) as described previously (34). Indirect immunofluorescence was performed on both N901 and anti-B4 antibodies by using fluorescein-labeled goat anti-mouse immunoglobulin or fluorescein-labeled goat anti-human immunoglobulin as secondary reagents (Sigma) and a FACScan flow cytometer (Becton Dickinson).

RESULTS AND DISCUSSION

Analysis of Surface Accessible Residues in Structurally Characterized Antibody Fv Regions. The surface accessible residues of immunoglobulin Fv regions were defined as those in which greater than 30% of all residue atoms are solvent exposed (12). Comparison of the surface residues in the 12 Fv structures has shown that the residues in 45 positions in the aligned sequences were conserved with 98% fidelity. This has allowed the identification of a unique set of surface accessible framework amino acid positions for all structurally characterized human and murine V_H and V_L chains. The aligned framework sequences of the 12 crystal structures used in this analysis and the locations of the surface accessible residues are shown in Fig. 1 *a* and *d*.

Sequence Selection and Modeling of Resurfaced N901. Individual data bases of non-redundant human immunoglobulin V_L and V_H sequences were screened to identify patterns of surface exposed residues that most closely matched the patterns found on V_L and V_H of N901. The human sequences found with the most identical surfaces were KV4B for V_L (35) and PLO123 for V_H (36) (Fig. 1 *b* and *e*). To resurface the N901 variable domains, the amino acids constituting the set of surface residues were then replaced with the set of human light and heavy chain surface amino acids to produce the composite sequences N901L/KV4B and N901H/PLO123. Inspection of these sequences shows the unexpected result that the murine and human sequences differ only by three residues in the light chain and seven residues in the heavy chain. To visualize the proximities of surface residues to the CDRs, the structures of the resurfaced N901 Fv and the murine N901 Fv were modeled (Fig. 2 *Upper*) (19, 23). Substitutions were examined when the surface residue was within 5 Å of the CDRs in either of the Fv models. If there was a change in size, charge, hydrophobicity, or potential to form hydrogen bonds, which could significantly alter a CDR conformation, the murine residue was retained. In the framework of the resurfaced N901 Fv, the retention of only one murine surface amino acid was required, namely leucine at position 3 of V_L (Fig. 2 *Lower*).

Sequence Selection and Modeling of Resurfaced Anti-B4. For anti-B4, a different selection procedure was used. The closest V_L and V_H sequences, selected by scoring identity across the entire framework sequences, were identified from a data base in which only clonally derived heavy and light chain pairs were represented. The surface residues of the chosen V_L–V_H pair (LS5, ref. 37) were then substituted in the murine anti-B4 Fv (Fig. 1 *c* and *f*). There were a total of 6 amino acid differences in V_L and 10 in V_H between anti-B4 and LS5. Of these 16 differences only 3 were seen to be likely to influence CDR conformation, after modeling and inspection using the 5-Å proximity procedure previously described. These residues were at position 76 in V_L (aspartate in LS5, serine in anti-B4) and positions 186 and 195 in V_H (glutamine in LS5, lysine in anti-B4 and threonine in LS5, lysine in anti-B4, respectively). In all three cases, the murine residues were retained. Models of these sequences and of the residues involved in potential framework–CDR clashes are not shown, since the principle has already been illustrated with N901.

Construction, Expression, and Biochemical Analysis of Resurfaced N901 and Anti-B4 Antibodies. To evaluate whether the resurfaced N901 and anti-B4 antibodies would retain the binding characteristics of the original murine antibodies, genes encoding resurfaced N901 V_L or anti-B4 V_L were constructed and cloned in eukaryotic expression vectors N901rLCEV and αB4rLCEVd, respectively, each carrying the human κ constant region gene, while genes encoding

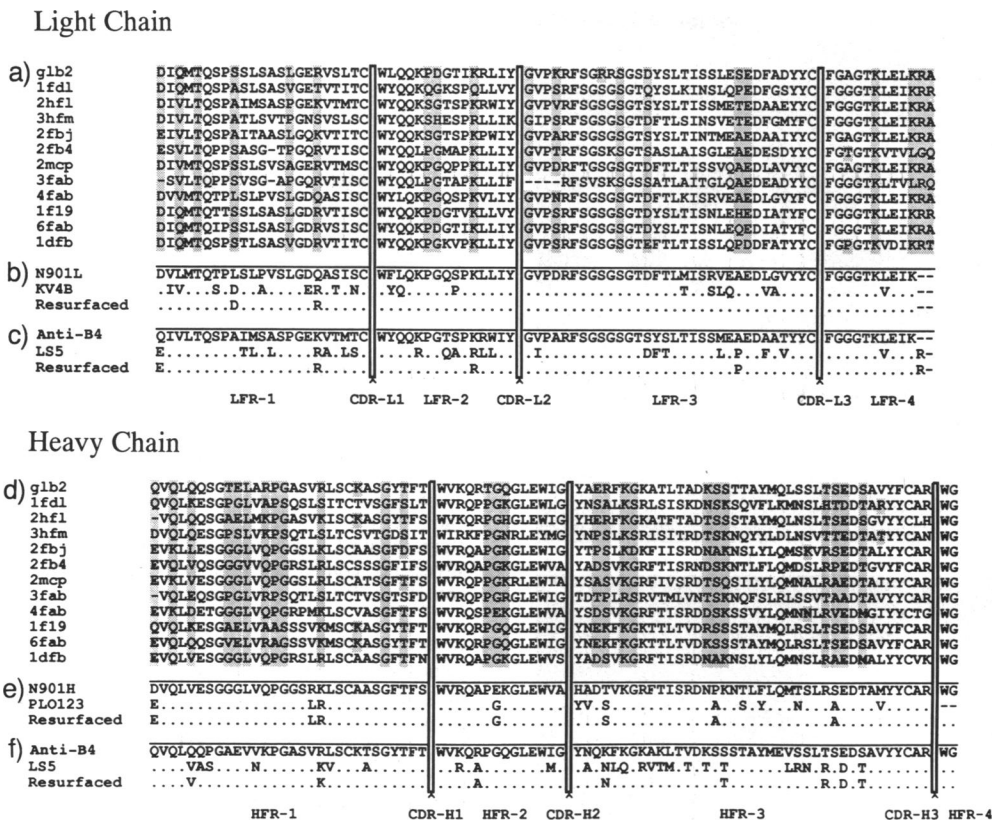

FIG. 1. Alignment of light and heavy chain framework (LFR and HFR) sequences for 12 Fv crystal structures (*a* and *d*). Residues whose side chains have a relative surface accessibility higher than 30% are shaded. CDRs have been eliminated from the alignment and their positions in the sequence are indicated by open boxes. Shown for N901 (*b* and *e*) are the sequences of the original murine antibody light and heavy chain V regions, the most identical human sequences (KV4B/PLO123) with respect to surface residues, and the resurfaced sequences of N901. Shown for anti-B4 (*c* and *f*) are the sequences of the original murine antibody, sequences of the most identical human antibody pair (LS5) with respect to surface residues, and the resurfaced sequences of anti-B4. Although residue 135 of the heavy chain is not normally defined as "surface," N901 is unusual in that it has an arginine at this position in the sequence GGSRKL (*e*), which is now surfaced exposed. In 98% of murine and 98.5% of human heavy chains this residue is Leu, Val, or Met. What is more, in human heavy chains arginine never occurs at this position. For this reason, the human residue (L) was substituted in the resurfaced N901 heavy chain. CDR amino acid sequences of murine and resurfaced N901 are as follows: CDR L1, RSSQIIIHSDGNTYLE; CDR L2, KVSNRFS; CDR L3, FQGSHVPHT; CDR H1, SFGMH; CDR H2, YISSGSFTIY; and CDR H3, MRKGYAMDY. CDR amino acid sequences of murine and resurfaced anti-B4 are as follows: CDR L1, SASSGVNYMH; CDR L2, DTSKLAS; CDR L3, HQRGSYT; CDR H1, SNWMH; CDR H2, EIDPSDSYTN; and CDR H3, GSNPYYYAMDY.

resurfaced N901 V_H or resurfaced anti-B4 V_H were constructed and cloned in the expression vectors N901rHCEV and αB4rHCEVd, respectively, each carrying the human IgG1 constant region genes. Resurfaced N901 antibody, purified from the supernatant of transiently transfected COS cells, was subjected to SDS/PAGE and found to be greater than 95% pure as shown by the presence of a single band (apparent M_r = 170,000) under nonreducing conditions and by two bands representing heavy (apparent M_r = 53,000) and light (apparent M_r = 27,500) chains under reducing conditions (Fig. 3*a*, lanes 1 and 2). Similar results were obtained for purified resurfaced anti-B4 antibody (Fig. 3*b*, lanes 1 and 2).

Analysis by isoelectric focusing gels (Fig. 3*a*, lanes 3 and 4) showed that resurfaced N901 is a more basic protein (pI 8.6–8.8) than murine N901 (pI 6.8–7.1) and, like murine N901, has a pattern of three to five evenly spaced bands that are characteristic of pure monoclonal antibodies (38, 39). Similar results were obtained for the purified resurfaced anti-B4 antibody (Fig. 3*b*, lanes 3 and 4), which has a pI of 8.6 (major band) compared with pI 6.5–6.9 for murine anti-B4. Since the constant regions of the two resurfaced antibodies are identical, it was not surprising that their pI values were similar. Interestingly, the pI values of murine IgG1 antibodies range from 6.2 to 7.1 (mean value of the three to five bands) (40), while the pI values of human IgG1 antibodies tend to be more basic, ranging from 7.2 to 8.4 in one study (41) and averaging 8.6 in another study (42). This increase in isoelectric point suggests that the resurfaced antibodies are more human than mouse-like in their charge distribution (42), which is probably largely due to the differences in charge between murine and human IgG1 constant regions.

Binding Analysis of Resurfaced Antibodies. In competitive binding assays, the resurfaced N901 and anti-B4 antibodies were equal to murine N901 or murine anti-B4 in their abilities to inhibit the binding of fluorescein-labeled murine N901 to SW-2 cells or labeled murine anti-B4 to Namalwa cells, respectively (Fig. 4 *a* and *b*). The binding of resurfaced N901 and murine N901 to SW-2 cells, and the binding of resurfaced anti-B4 and murine anti-B4 to Namalwa cells, were also assessed by indirect immunofluorescence assays (Fig. 4 *c* and *d*). The binding curves for the resurfaced N901 and the resurfaced anti-B4 are virtually identical to the binding curves for the murine N901 and the murine anti-B4, respectively, confirming that the affinity of the resurfaced antibodies for their respective antigen is identical to that of the relevant murine antibody. The midpoints of the binding curves are at 1.1×10^{-10} M and 2.3×10^{-10} M for N901 and anti-B4, respectively. The results of these binding studies allow us to conclude that the framework–CDR interactions in the resurfaced N901 and anti-B4 antibodies preserve the native conformations of the CDRs.

Resurfacing murine Fv regions is likely to minimize CDR–framework incompatibilities because a large number of mu-

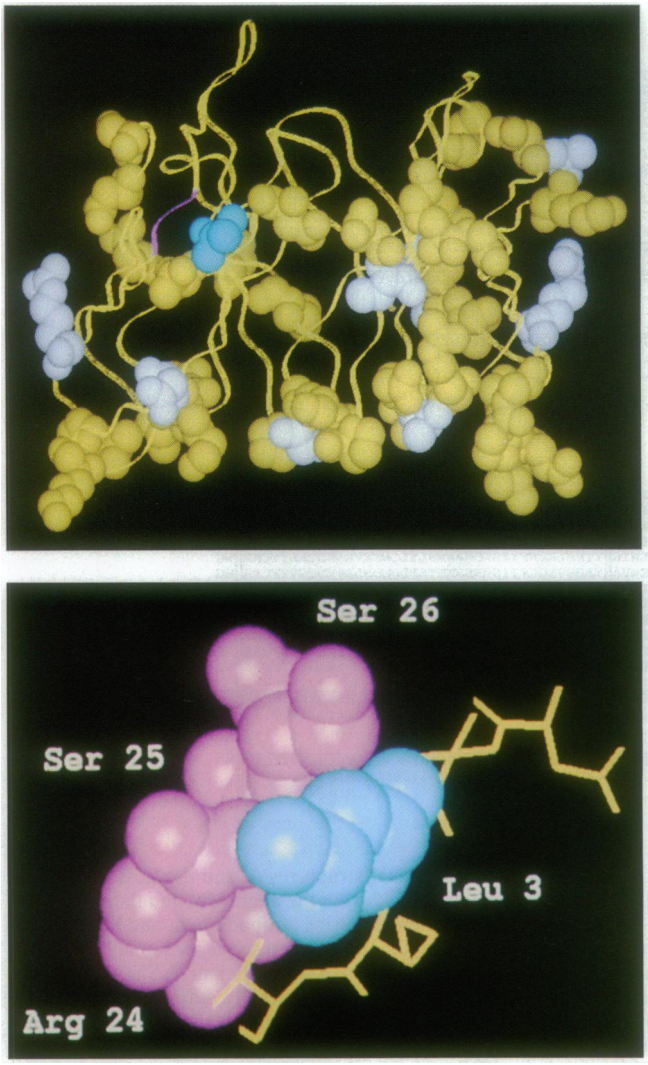

FIG. 2. Models of resurfaced N901 Fv generated by using methods previously described (19, 22, 23). (*Upper*) Backbone for the whole resurfaced Fv. Residues with more than 30% relative accessibility (all atoms) are space-filled. (*Lower*) Interaction of the light chain surface framework residue Leu-3 with residues Arg-24, Ser-25, and Ser-26. The amino acid residue at position 3 is leucine in murine N901 and valine in the human sequence KV4B. A large difference in the conformation of Arg-24 was seen between the models for murine and resurfaced N901; therefore, the murine residue, leucine, was retained in position 3 of the light chain. Coloring of resurfaced N901 model (*Upper*): yellow ribbon for the core framework and CDRs; yellow space-filled atoms where the framework surface residues in the murine and human sequence are the same; white space-filled atoms where the framework surface residues were changed from murine to human; blue space-filled atoms where the framework residue from the murine sequence was retained due to interactions with CDR residues; and magenta ribbon for residues 24, 25, and 26 of CDR1 of V_L.

rine surface residues can be retained. The total number of differences between the surface residue patterns of the murine N901 and the most identical human V region was remarkably low, so that only a small number of amino acid changes needed to be made to humanize the antibody. Again, few amino acid changes from the murine sequences were needed to resurface anti-B4, where the most identical human V_L–V_H pair was selected. The fact that resurfaced N901 retained its full affinity for its antigen suggests that humanization by resurfacing is not absolutely dependent on selection of V_L and V_H sequences that are clonally derived, as used for anti-B4. Nevertheless, to avoid the possibility of

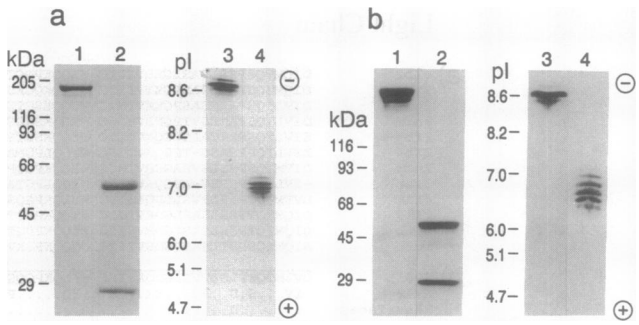

FIG. 3. Analysis of purified resurfaced N901 and anti-B4 antibodies. (*a*) Analysis of purified resurfaced N901 antibody by SDS/PAGE under nonreducing conditions (lane 1; 4–12% gradient gel) or reducing conditions (lane 2; 11% gel) and by isoelectric focusing (IEF) gel (38, 39), pH range 3.0–9.0 (lane 3). (*b*) Analysis of purified resurfaced anti-B4 antibody by SDS/PAGE (4–12% gradient gel) under nonreducing conditions (lane 1) or reducing conditions (lane 2), and by IEF gel, pH range 3.0–9.0 (lane 3). The SDS/PAGE gels were stained with Coomassie blue, and the IEF gels were stained with silver. For comparison, purified murine N901 (*a*, lane 4) or murine anti-B4 (*b*, lane 4) are shown on the IEF gel.

creating neoepitopes due to combinations of V_L and V_H regions that may not naturally occur, the resurfacing procedure in which selection from the paired sequence data base is used may be preferred.

A majority of antibodies that are humanized by CDR grafting contain a number of internal and external murine framework amino acids because many original murine residues are used to replace human residues to restore the high

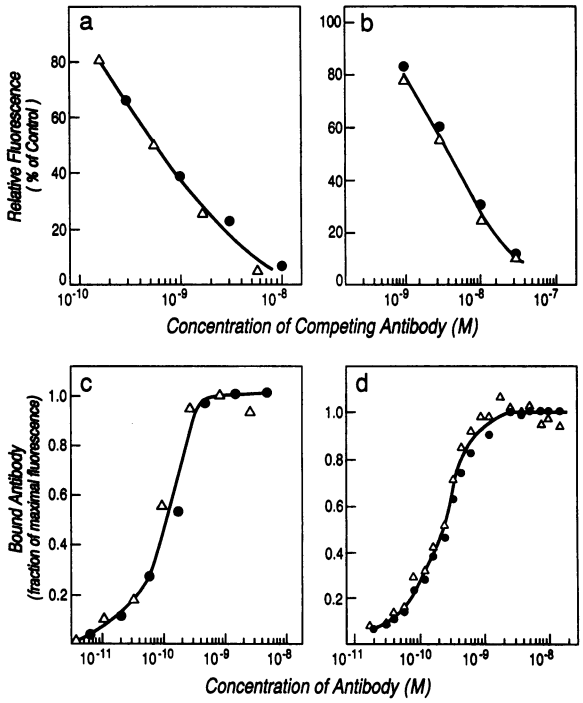

FIG. 4. Comparison of binding activities for resurfaced and murine N901 and anti-B4 antibodies. Resurfaced antibodies (△) and murine antibodies (●) were compared as follows: (*a*) Competition binding assay measuring the ability of resurfaced N901 and murine N901 to compete with fluorescein-labeled murine N901 for binding to SW-2 cells. (*b*) Competition binding assay measuring the ability of resurfaced anti-B4 and murine anti-B4 to compete with fluorescein-labeled murine anti-B4 for binding to Namalwa cells. (*c*) Binding of resurfaced N901 and murine N901 to SW-2 cells as measured by indirect immunofluorescence. (*d*) Binding of resurfaced anti-B4 and murine anti-B4 to Namalwa cells as measured by indirect immunofluorescence.

affinity of the original murine antibody and, even so, in many cases the full affinity of the parent murine antibody is not restored. The resurfacing approach was tested to examine the feasibility of constructing humanized antibodies that would retain the full antigen-binding specificity and avidity of the original murine monoclonal antibody by not interfering with the crucial framework interactions that support the CDRs. Foreign sequences, whether internal or external, in both CDR-grafted and resurfaced antibodies may be presented to T cells, and whether humanized antibodies will evade the immune system is a question that awaits clinical evaluation. However, the fact remains that there is a strong conservation and localization of surface accessible amino acid residues in the Fv regions of murine and human antibodies (12). This, together with the fact that the side chains of surface accessible residues are in general not critical to the structural integrity of Fv regions, may hint at a biological significance for the selective conservation of surface patterns in antibodies.

Note. When the model of the resurfaced anti-B4 antibody was being prepared, an error in the numbering of the amino acid positions of the heavy chain framework region 2 occurred, which led to the assignment of a surface position to arginine in the sequence WVKQRPGQ [position 159 as used in the antibody modeling program AbM (Oxford Molecular Ltd., Oxford, U.K.; refs. 12 and 19)]. Consequently, we replaced that amino acid with the corresponding human residue, alanine, of the LS5 antibody. We have now restored the original murine residue at amino acid position 159 by constructing expression vector α-B4rHCEVd-R159. Coexpression in COS cells of this resurfaced anti-B4 immunoglobulin heavy chain with the resurfaced anti-B4 light chain resulted in the production and purification of an antibody that was subjected to all the analytical procedures described in this report. A pI of 8.8 was determined by isoelectric focusing, and a half-maximal binding equal to that of the murine anti-B4 and the resurfaced anti-B4 antibody in this report was obtained. Therefore, this resurfaced anti-B4 antibody retains all the binding characteristics of the murine parent monoclonal antibody as well as the resurfaced anti-B4 antibody containing alanine at position 159 as described in this report. The side chain of arginine at position 159 lies along the bottom of the Fv region, almost 20 Å from the closest CDR residue.

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