

## Reshaping a therapeutic CD4 antibody

(humanized antibody/chimeric antibody/tolerance/autoimmune disease/transplantation)

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**ABSTRACT** An immunosuppressive rat antibody (Campath-9) against human CD4 has been reshaped for use in the management of autoimmunity and the prevention of graft rejection. Two different forms of the reshaped antibody were produced that derive their heavy chain variable region framework sequences from the human myeloma proteins KOL or NEW. When compared to a chimeric form of the CD4 antibody, the avidity of the KOL-based reshaped antibody was only slightly reduced, whereas that of the NEW-based reshaped antibody was very poor. The successful reshaping to the KOL-based framework was by a procedure involving the grafting of human framework sequences onto the cloned rodent variable region by *in vitro* mutagenesis.

At present, unwanted immune responses in autoimmunity and graft rejection are managed by the long-term administration of immunosuppressants such as steroids, azathioprine, and cyclosporine. Patients receiving such long-term therapy are at continuous risk of infection and unwanted side effects of the drugs. An ideal alternative to sustained immunosuppression would be to establish a state of immunological tolerance to the inciting antigens. Here the intent is to provide short-term treatment to modulate the host immune system such that antigen-responsive T cells are either deleted or rendered anergic. In rodents, a short course of therapy with CD4 and CD8 monoclonal antibodies can tolerize to antigens as diverse as bone marrow, skin, and heart grafts (1–4) as well as preventing induction of a wide range of experimental autoimmune diseases (4–6). Remarkably, large doses of rat CD4 antibodies administered to mice can induce tolerance to themselves, thus avoiding an antiimmunoglobulin response that might neutralize their biological activity. However, lower doses fail to self-tolerize although they can still be tolerogenic for other antigens given under the CD4 umbrella (5, 6). In humans, rodent CD4 antibodies have thus far proven quite immunogenic despite their immunosuppressive properties (7, 8). This problem can be minimized by reshaping the rodent antibody to a human form. In this way a human antibody is created that contains only the six complementarity-determining regions (CDRs) from the heavy and light chain variable ( $V_H$  and  $V_L$ ) regions of the rodent antibody of interest (9). To maximize the opportunities to use CD4 antibodies for tolerance therapy, we have converted a known immunosuppressive CD4 monoclonal antibody of rat origin into a human form. The rat form of this antibody, Campath-9 (Wellcome Foundation), was demonstrated to be therapeutically useful in combination with Campath-1H (CDw52) antibody to achieve a long-lasting remission in a patient with autoimmune systemic vasculitis (10).

Reshaping antibodies is a relatively new procedure where success cannot necessarily be guaranteed for any individual antibody. Here, we describe a further approach of reshaping

that grafts onto a rodent variable (V) region the framework sequences from a human V region that is most homologous to that of the rodent. We compare the effectiveness of two reshaped versions of the rat Campath-9 antibody, where one has derived its human  $V_H$  region framework sequence from the myeloma protein KOL and the other from NEW.\*

### MATERIALS AND METHODS

**cDNA Cloning.** cDNA encoding the  $V_L$  and  $V_H$  regions were generated by a polymerase chain reaction (PCR)-based method (11) except that the primer 5'-TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC-3' was substituted for VH1FOR and 37°C and 50°C PCR annealing temperatures were used for  $V_L$  and  $V_H$  region cDNA amplifications, respectively.  $V_L$  and  $V_H$  cDNA regions were cloned into the M13-based vectors M13-VKPCR1 and M13-VHPCR1, respectively, as described (11).  $V_L$  region clones were screened by hybridization with a  $^{32}$ P-labeled probe (5'-GTT TCA TAA TAT TGG AGA CA-3') specific for CDR 3 of the Y3-Ag1.2.3  $V_L$  region cDNA (12); clones not hybridizing to this probe, and  $V_H$  region clones, were sequenced by the dideoxy method (13).

**Construction of Genes for Chimeric Antibodies.** The plasmid p $V_H$ rat/ $C_{G1}$  encoding the chimeric heavy chain consists of the following adjacently ligated fragments: the 6.6-kilobase (kb) vector p $\beta$ APr-1 (14) linearized at its cloning site with *Hind*III and *Bam*HI containing the  $\beta$ -actin promoter, xanthine-guanine phosphoribosyltransferase, and ampicillin-resistance genes; a 39-base-pair (bp) *Hind*III-*Nco* I synthetic linker fragment (5'-AAG CTT TAC AGT TAC TGA GCA CAC AGG ACC TCA CCA TGG-3'); a 698-bp *Nco* I-*Bam*HI fragment from an M13-VHPCR1 clone containing the Campath-9 antibody  $V_H$  region cDNA; a 2.3-kb *Bam*HI-*Sph* I fragment containing the human  $G1$  constant ( $C_{G1}$ ) region gene (15); and a 20-bp *Sph* I-*Bgl* II synthetic linker fragment (5'-GCA TGC GCG GCC GCA GAT CT-3').

The plasmid p $V_L$ rat/ $C_\kappa$  encoding the chimeric light chain consists of the following adjacently ligated DNA fragments: a 7.5-kb *Bam*HI-*Hind*III fragment from the plasmid pLD9 (16) containing the  $\beta$ -actin promoter, dihydrofolate reductase, and ampicillin-resistance genes; the 39-bp *Hind*III-*Nco* I synthetic linker; a 587-bp *Nco* I-*Bam*HI fragment from an M13-VKPCR1 clone containing the Campath-9 antibody  $V_L$  region cDNA; and a 4.9-kb *Bam*HI fragment containing the human  $\kappa$  constant ( $C_\kappa$ ) region gene (17).

**Construction of Genes for Reshaped Antibodies.** The plasmid p $V_H$ NEW/ $C_{G1}$  encoding the NEW-based reshaped heavy chain is identical to the plasmid pNH316 (16) except that the three CDRs of the Campath-9 antibody  $V_H$  region

Abbreviations: V, variable;  $V_H$ , heavy chain variable;  $V_L$ , light chain variable;  $C_{G1}$ , G1 constant;  $C_\kappa$ ,  $\kappa$  constant; CDR, complementarity-determining region; PCR, polymerase chain reaction; ADCC, antibody-dependent cell-mediated cytotoxicity.

\*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M61884 and M61885).

were CDR-grafted (9) into the 1.5-kb *Hind*III fragment from pNH316 encoding the Campath-1H antibody heavy chain by *in vitro* mutagenesis with three oligonucleotides.

The KOL-based reshaped V<sub>H</sub> region was created by *in vitro* mutagenesis of a Campath-9 antibody V<sub>H</sub> region cDNA clone in M13-VHPCR1 with five oligonucleotides that were designed to mutate the Campath-9 V<sub>H</sub> region framework residues into the corresponding residues of the KOL antibody (18). These five mutagenic oligonucleotides were simultaneously introduced in a single mutagenesis reaction. Twelve clones were sequenced and each clone had incorporated the five mutagenic oligonucleotides. The plasmid pV<sub>H</sub>KOL/C<sub>G1</sub> encoding the KOL-based reshaped heavy chain consists of the following adjacently ligated fragments: the 9.8-kb vector pHβAPr-1-gpt (14) linearized at its cloning site with *Hind*III and *Bam*HI containing the β-actin promoter, xanthine-guanine phosphoribosyltransferase, and ampicillin-resistance genes; the 39-bp *Hind*III-*Nco* I linker; a 698-bp *Nco* I-*Bam*HI fragment encoding the KOL-based reshaped V<sub>H</sub> region; a 2.3-kb *Bam*HI-*Sph* I fragment containing a human C<sub>G1</sub> region gene (15); and the 20-bp *Sph* I-*Bgl* II synthetic linker.

The plasmid pV<sub>L</sub>REI/C<sub>κ</sub> encoding the reshaped light chain is identical to the plasmid pLD9 (16) except that the three CDRs of the Campath-9 antibody V<sub>L</sub> region were CDR-grafted (9) into the 748-bp *Hind*III fragment from pLD9 encoding the Campath-1H antibody light chain by *in vitro* mutagenesis with three oligonucleotides.

**Transfections and Antibody Purification.** The CD4-expressing cell line HCD4-NB2 is a clone of the rat T-cell line NB2-6TG stably transfected by electroporation with the expression vector pSFSVneo (19) containing cDNA encoding the human CD4 antigen (20).

Plasmids encoding antibody chains were cotransfected as described (21) into dihydrofolate reductase-deficient Chinese hamster ovary cells (10<sup>6</sup> cells per 75-cm<sup>2</sup> flask) using 9 μg and 1 μg of the appropriate heavy and light chain constructs, respectively. Transfectants were selected in medium containing 5% dialyzed fetal bovine serum for 2–3 weeks, and antibody-secreting clones were identified by ELISA of culture supernatants. Chimeric and reshaped antibodies were purified from culture supernatants using protein A-Sepharose CL-4B (Pharmacia) column chromatography as described (22). Antibody concentrations were determined by absorbance at 280 nm.

**Immunofluorescence and Flow Cytometry.** HCD4-NB2 cells were washed with staining medium (phosphate-buffered saline containing 0.1% bovine serum albumin, 1% heat-inactivated normal rabbit serum, and 0.1% sodium azide) and then incubated with either the chimeric or reshaped antibodies (10<sup>5</sup> cells per 0.1 ml) diluted in staining medium for 1 hr at 4°C. The cells were washed and then incubated with fluorescein isothiocyanate-conjugated anti-human IgG1 (γ-chain-specific) antibodies (The Binding Site, Birmingham, U.K.) diluted 1:30 in staining medium for 1 hr at 4°C. Propidium iodide (100 μg/ml final concentration) was added during the last 10 min of incubation. Cells were thoroughly washed and resuspended in 0.5 ml of staining medium. Mean cellular fluorescence (3000 live cells per sample) was determined with a Cytofluorograph (model 50-H Ortho Instruments). Propidium iodide-stained dead cells were gated-out. Fifty percent antigen binding titers were determined by fitting the data to a sigmoid curve by a least squares iterative procedure (23).

**Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC).** Antibodies were assayed by ADCC with activated human peripheral blood mononuclear cells (24). Briefly, 5 × 10<sup>4</sup> HCD4-NB2 cells were labeled with <sup>51</sup>Cr and incubated for 1 hr at room temperature with different concentrations of antibodies. A 75-fold excess of activated cells was added as

effectors. After 4 hr at 37°C, cell death was determined by measuring <sup>51</sup>Cr release.

## RESULTS

**Cloning of V<sub>L</sub> and V<sub>H</sub> Region cDNA.** cDNAs encoding the V<sub>L</sub> and V<sub>H</sub> regions from the Campath-9 antibody-secreting clone YNB46.1.8SG2B1.19 (10) were isolated by PCR using primers that amplify the segment of cDNA encoding the amino-terminal region through the joining region (11). V<sub>L</sub> region clones were first screened by hybridization with a <sup>32</sup>P-labeled oligonucleotide probe complementary to CDR 2 of the light chain expressed by the rat Y3-Ag1.2.3 myeloma cell line (12) that was used as the fusion partner to generate the Campath-9 antibody-secreting hybridoma. Subsequent nucleotide sequence analysis was restricted to clones that did not contain sequence complementary to this probe (about 5% of clones). In this manner, two cDNA clones from independent PCR amplifications were identified that encoded identical V<sub>L</sub> regions. Nucleotide sequence analysis of random V<sub>H</sub> region clones from two independent PCR amplifications revealed a single species of V<sub>H</sub> region cDNA. These cDNA sequences have been submitted to the GenBank data base, and their predicted amino acid sequences are shown (Fig. 1). As no additional V<sub>L</sub> or V<sub>H</sub> region-encoding clones were identified, it was assumed that these sequences were derived from the Campath-9 antibody genes.

**Chimeric Antibody Constructs.** Plasmids were constructed that encoded a rat/human chimeric version of the Campath-9 antibody. The plasmid pV<sub>H</sub>rat/C<sub>G1</sub> encodes a chimeric heavy chain consisting of the Campath-9 V<sub>H</sub> region (Fig. 1A) and a human C<sub>G1</sub> region. The plasmid pV<sub>L</sub>rat/C<sub>κ</sub> encodes a chimeric light chain consisting of the Campath-9 V<sub>L</sub> region (Fig. 1B) and a human C<sub>κ</sub>. These chimeric heavy and light chains were coexpressed in Chinese hamster ovary cells to produce a chimeric antibody.

**Reshaped Antibody Heavy Chain Constructs.** Possibly the largest unknown variable when reshaping an antibody is the selection of the human immunoglobulin V region from which the framework sequences are derived. Because the framework regions hold the CDRs in their correct spatial orientation and can sometimes even participate in antigen binding (29), this selection could be important. At present, there are insufficient published reshaping results to generalize a "best framework" selection strategy. Reshaping experiments to date (9, 30–32) have not compared the effectiveness of different human frameworks incorporating the same rodent CDRs.

To investigate the importance of framework selection and to maximize our chances of producing a functional reshaped CD4 antibody, we have designed two different versions of reshaped V<sub>H</sub> regions. In the first case, we designed a reshaped V<sub>H</sub> region that derives its CDRs from the Campath-9 V<sub>H</sub> region and its framework sequences from the NEW-based framework that had been used previously for the reshaped antibody Campath-1H (9) and others (30, 31). Given the demonstrable antigen binding of these antibodies, it was reasonable to try the same framework sequences as well. A plasmid was thus constructed, pV<sub>H</sub>NEW/C<sub>G1</sub>, that encodes a reshaped heavy chain consisting of an NEW-based V<sub>H</sub> region with Campath-9 V<sub>H</sub> region CDRs (Fig. 1A) and a human C<sub>G1</sub> region.

In the second case, we designed a reshaped V<sub>H</sub> region that derives its CDRs from the Campath-9 V<sub>H</sub> region and its framework sequences from the V<sub>H</sub> region of the human myeloma protein KOL (18). The V<sub>H</sub> region of KOL was chosen because of all known human heavy chain V regions its overall amino acid sequence is very homologous to the Campath-9 V<sub>H</sub> region (Fig. 1A) containing 72% identical residues (excluding gaps introduced for alignment purposes).

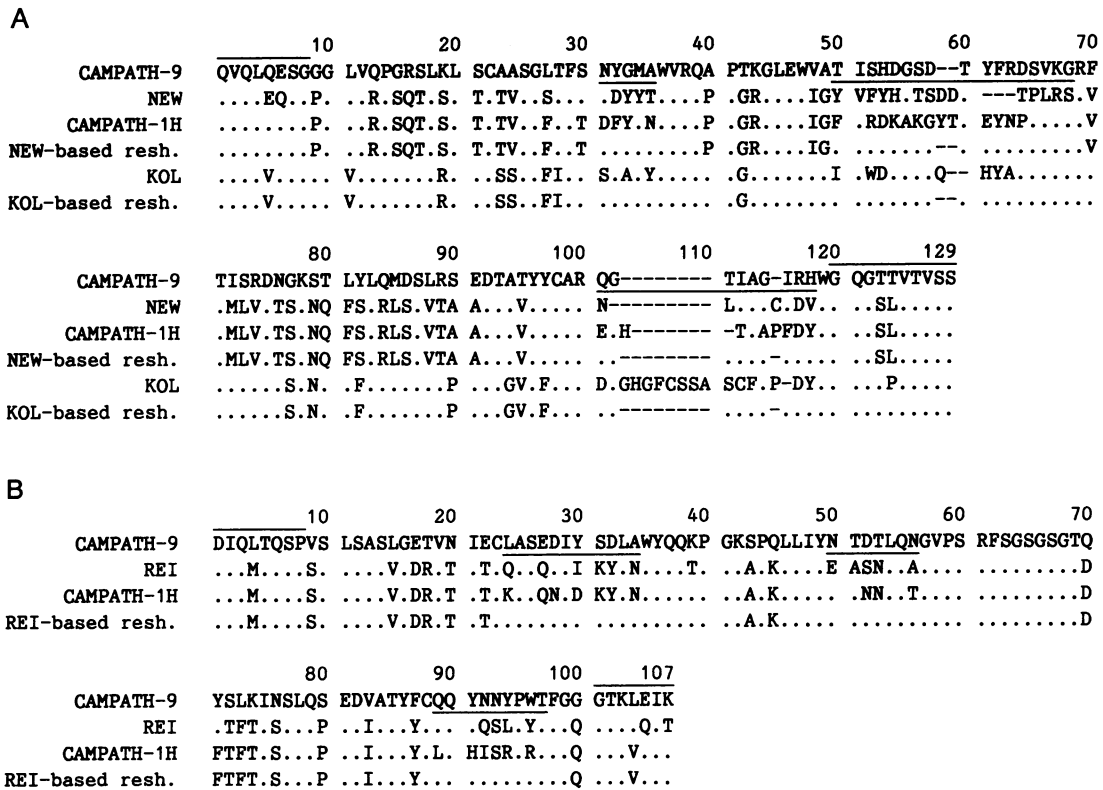


FIG. 1. Comparison of the amino acid sequences of the heavy (A) and light (B) chain V regions described in the text. Dots indicate residues that are identical to the corresponding residue in Campath-9. Hyphens represent spaces introduced in the sequences by GAP (25) to aid the alignment. CDRs of Campath-9 are underlined and residues encoded within the amplification primers and cloning vectors are overlined. resh., Reshaped. Sequences of NEW, KOL, and REI are from the Swiss-Prot protein sequence data base, release 14. It should be noted that there are some minor sequence differences of NEW and KOL as recorded in the various data bases—for example, Swiss-Prot and Brookhaven (26). The actual framework sequences of the NEW- and REI-based reshaped V regions described here are identical to those of the Campath-1H antibody (9), which differ only slightly from the reported framework sequences of NEW (27) and REI (28). For consistency, and given the demonstrable antigen binding of this reshaped antibody, identical framework sequences were used here.

This was determined by a computer search of several data bases. By contrast, the NEW V<sub>H</sub> region sequence has only 47% identical residues. We reasoned that since the primary function of the framework sequence is to hold the CDRs in their correct spatial orientation, we could maximize the chances of retaining correct CDR structure (and hence antigen affinity) by deriving framework sequences from a human V<sub>H</sub> region that is most homologous to that of the rodent. Of the several homologous human V<sub>H</sub> regions available, the choice of KOL was made because its three-dimensional structure is well characterized. A plasmid was thus constructed, pV<sub>H</sub>KOL/C<sub>G1</sub>, that encodes a reshaped heavy chain consisting of a KOL-based V<sub>H</sub> region with Campath-9 V<sub>H</sub> region CDRs (Fig. 1A) and a human C<sub>G1</sub> region.

**Reshaped Antibody Light Chain Construct.** We have designed a reshaped V<sub>L</sub> region that derives its CDRs from the Campath-9 V<sub>L</sub> region and its framework sequences from the REI-based framework that has been used previously for the reshaped antibody Campath-1H (9). Again, given the demonstrable antigen binding of this antibody, it was reasonable to try the same framework as well. A plasmid was constructed, pV<sub>L</sub>REI/C<sub>K</sub>, that encodes a reshaped light chain consisting of an REI-based V<sub>L</sub> region with Campath-9 V<sub>L</sub> region CDRs (Fig. 1B) and a human C<sub>K</sub> region. A second reshaped V<sub>L</sub> version was not created as with the reshaped V<sub>H</sub> region because REI is already highly homologous (67% identical residues) to the rat V<sub>L</sub> region of Campath-9. Thus this reshaped light chain was coexpressed with the reshaped heavy chains in Chinese hamster ovary cells to produce two reshaped antibodies (KOL- and NEW-based) differing only in their human-derived V<sub>H</sub> region framework sequences.

**Properties of Chimeric and Reshaped Antibodies.** The abilities of the chimeric and reshaped antibodies to bind the CD4<sup>+</sup> cell line HCD4-NB2 were compared by immunofluorescence staining (Fig. 2). The chimeric and KOL-based reshaped antibodies stained CD4<sup>+</sup> cells well. The titration curves of these two antibodies were fitted to a sigmoid curve, and the concentrations (mean ± SEM) of chimeric and KOL-based reshaped antibodies needed to achieve 50% antigen saturation were determined to be 2.21 ± 0.16 and 7.16 ± 0.45 μg/ml, respectively. Thus the avidity of the KOL-based reshaped antibody is only slightly reduced as it only

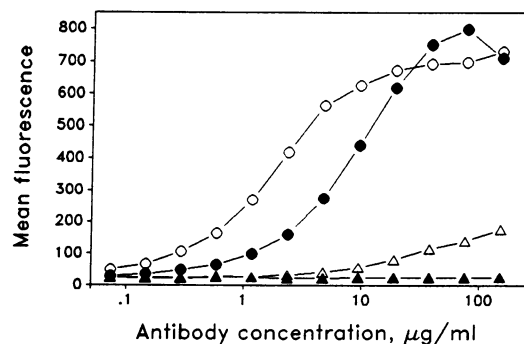


FIG. 2. Fluorescence of CD4<sup>+</sup> cells stained with chimeric and reshaped antibodies. ○, Campath-9 chimeric antibody; ●, KOL-based reshaped antibody; △, NEW-based reshaped antibody; ▲, Campath-1H antibody (isotype-matched negative control). The KOL- and NEW-based reshaped antibodies have the same REI-based reshaped light chain.

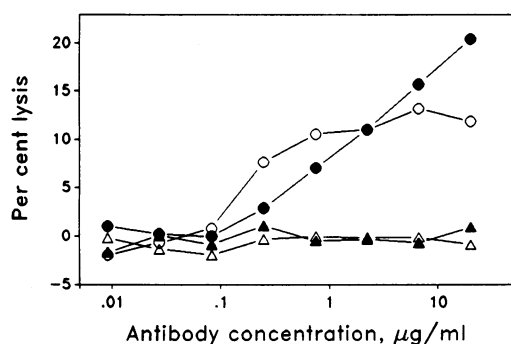


FIG. 3. ADCC with chimeric and reshaped antibodies. Symbols are the same as in Fig. 2.

takes three times the amount of this antibody to give the same 50% binding as the chimeric antibody. All KOL  $V_H$  region framework residues were incorporated into this reshaped  $V_H$  region except for the proline at position 124 (Fig. 1A), which was kept as threonine. A second version that incorporates this proline residue gave similar results (not shown). By contrast, the NEW-based reshaped antibody stained  $CD4^+$  cells only poorly even at the higher concentrations. The control Campath-1H antibody did not stain cells at any concentration. Also, the chimeric and KOL-based reshaped antibodies were effective in cell-mediated lysis, whereas the NEW-based reshaped and control Campath-1H antibodies were ineffective (Fig. 3).

## DISCUSSION

We have described here the successful reshaping of the CD4 antibody Campath-9. This result, together with the previously described reshaping of other therapeutic antibodies (9, 32), demonstrates the feasibility of applying this concept to the many rodent-derived monoclonal antibodies with clinical potential. The Campath-1H antibody has already been used successfully in clinical studies of lymphoma therapy (33) and in the treatment of an autoimmune disorder (10). In this later case, the Campath-1H antibody was combined with the Campath-9 antibody to give a long-term remission in a case of systemic vasculitis that appeared intractable prior to Campath-9 antibody treatment. Although the relative contributions and importance of the Campath-1H and Campath-9 antibodies cannot be ascertained from this single case study, the availability of Campath-9 as a reshaped antibody, Campath-9H (the KOL-based version), should provide for further useful clinical studies.

Our reshaping of Campath-9 into Campath-9H raises interesting questions regarding general strategies for the reshaping of rodent antibodies. At present, there are only four additional reports of reshaped antibodies and in no case has the effectiveness of two different human antibody framework regions been compared (9, 30–32). Three of these antibodies had reshaped  $V_H$  regions based on NEW (9, 30, 31), two of which had reshaped  $V_L$  regions based on REI (9, 31), regardless of whether closer homologies existed in the sequence data bases. In the fourth example, the antibody was reshaped based on a homologous human framework, but 12 of the 29 residues that differed between the human and mouse  $V_H$  region frameworks were left as in the mouse sequence, and no data were presented that compared this reshaped antibody with a form in which all of the framework residues were derived from the human sequence (32). For the reshaping of Campath-9, we have shown that the selection of a particular human framework can be important in the retention of antibody avidity. We have made two reshaped antibodies that differ only in their usage of human  $V_H$  region

framework sequences, KOL and NEW, and found one form to be far superior to the other. The KOL-based CD4 antibody retained biological activity in ADCC assays and had a relative binding avidity only slightly reduced from the unaltered V region sequences of the chimeric CD4 antibody. In contrast, the NEW-based CD4 antibody, though still retaining specificity for CD4, had a considerably reduced relative binding avidity and had no biological activity. The KOL  $V_H$  region has a 72% homology to Campath-9  $V_H$  region, whereas the NEW  $V_H$  framework has only 47% homology. In this case then, it would seem that the selection of a human V region framework that was highly homologous to the rodent V region was the best strategy for framework selection. We have also successfully reshaped a CD3 antibody by the same approach (E.G.R., unpublished data), so this strategy may prove to be generally applicable to antibody reshaping. Arguably, although such a *bestfit* strategy has been applied here to whole antibody V regions, it might also be applied separately to individual variable, diversity, and joining recombining segments of an antibody V region.

The different avidities we observe when reshaping with KOL- and NEW-based frameworks are likely to be due to the complex intrachain associations between CDR and framework residues. Alternatively, different interchain V region associations between these two heavy chains and the REI-based reshaped light chain may also play a role. One possible structural explanation for the differences between the KOL- and NEW-based reshaped antibodies is described by Trantomano *et al.* (34). In studies of a series of solved immunoglobulin structures they observed that the conformation of the heavy chain CDR 2, "H2 loop," is dependent upon the length and sequence of this loop and its interaction with the framework residue at position 71 (Kabat numbering system). KOL and NEW have distinctly different structures in this region and a different residue at position 71. The H2 loop of the Campath-9 antibody is very similar to that of KOL and both contain an arginine residue at the relevant framework position (this is position 74 in Fig. 1A). Perhaps the change of this residue upon reshaping to the NEW-based framework accounts in part for its low avidity. However, it should be noted that Campath-1H was successfully reshaped to the NEW-based framework despite having a substantially different H2 loop length and sequence and also a different framework residue at position 71 (34). Clearly the ability to use structural features like this to reliably predict a suitable strategy for reshaping will benefit from more examples where different strategies are experimentally compared.

In previous studies, genes encoding reshaped antibodies were produced either by total synthesis of the desired sequence (32) or by *in vitro* mutagenesis of a human V region sequence to incorporate the rodent CDRs (9, 30, 31). We propose the following strategy based on our successful reshaping experiments whereby the isolated rodent V region is used as a starting point for constructing reshaped V regions. Here, human framework sequences are transferred to the rodent V regions by means of *in vitro* mutagenesis. When coupled with the *bestfit* method from above, mutagenic oligonucleotides can be highly homologous to the rodent frameworks, and hence the efficiency of mutagenesis is high. In the case of Campath-9H, this was accomplished in a single mutagenesis reaction with five oligonucleotides 33–58 bases in length. This strategy should be readily applicable to any monoclonal antibody for which the cDNA has been cloned and for which a homologous human framework can be identified in the sequence data bases. It is also interesting to note that expression in the nonlymphoid Chinese hamster ovary cell line results in an antibody with demonstrable activity in ADCC. The capacity of this cell line for high-level expression of reshaped antibodies (16) should facilitate the large-scale production of this antibody for clinical studies.

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