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CONSTRUCTION, EXPRESSION AND CHARACTERIZATION OF HUMANIZED ANTIBODIES DIRECTED AGAINST THE HUMAN α/β T CELL RECEPTOR

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Completely humanized antibodies with specificity for the human α/β TCR have been produced by genetic engineering. The L and H chain V region exons encoding the murine mAb BMA 031 CD regions and human EU framework regions were synthesized and replaced into previously isolated genomic fragments. These fragments were inserted into mammalian expression vectors containing the human κ and γ 1 C region exons. Two variants were constructed each containing selected BMA 031 amino acids within the human frameworks. The humanized genes were transfected into Sp2/0 hybridoma cells by electroporation and transfectomas secreting humanized antibody were isolated. Levels of antibody expression up to 7 pg/cell/24 h were obtained. The humanized antibody, BMA 031-EUCIV2, competed poorly with murine BMA 031 for binding to T cells. BMA 031-EUCIV3, however, bound specifically to T cells and competed effectively with both the murine BMA 031 antibody and a previously constructed chimeric BMA 031 antibody for binding to these cells. The relative affinity of BMA 031-EUCIV3 was about 2.5 times lower than BMA 031. The ability to promote antibody dependent cell-mediated cytolysis was significantly enhanced with the engineered antibodies as compared to murine BMA 031. Humanized BMA 031 is a clinically relevant, genetically engineered antibody with potential uses in transplantation, graft vs host disease, and autoimmunity.

mAb are emerging as a major modality for therapy of various pathologic conditions including malignant disease, cardiovascular disease, and autoimmune diseases. Some of these have demonstrated efficacy in treating colon carcinoma (1), B cell lymphomas (2), neuroblastoma (3), and in preventing transplant rejection (4, 5).

Clinical trials with murine antibodies, although encouraging, have indicated at least two fundamental problems of antibody therapy. First, murine IgG has a much shorter circulating half-life in man compared to what has been reported for human antibodies (6, 7), so that effective mAb therapy may require frequent multiple treatments with large amounts of murine antibody. Second, administration of murine IgG elicits a brisk HAMA[3] response that that can further reduce the circulating half-life of the mAb and produce allergic reactions including anaphylaxis (8–10).

Almost all of the murine mAb currently being used clinically provoke HAMA responses in patients. These include HAMA against both the C region and the V region (11). HAMA responses lead to altered pharmacokinetics of the injected mAb. The antibody is rapidly cleared from the serum and reduced antibody levels are attained (12). Although severe side effects are rare in patients with HAMA after retreatment with antibody, it is clear that if mAb are to be used therapeutically, reliable methods must be devised to reduce immune mediated complications or adverse reactions (13).

One approach to better immunotheraples currently being explored is to produce a truly human antibody. Unfortunately, human mAb technology has lagged far behind that of murine-based monoclonal technology. Human hybridomas are difficult to prepare, are often unstable, and secrete antibody at low levels (14, 15). The mAb generated are usually of the IgM class and of low affinity.

An attractive and viable strategy is to produce "humanized" versions of murine mAb through genetic engineering. Methods have been devised to replace all regions of a murine antibody with analogous human regions (16–18). Chimeric antibody technology has been applied to several therapeutically important antibodies (19–24) and has been useful in class switching and the production of isotypes with specific effector functions (25, 26). A chimeric antibody composed of the V regions of murine mAb 171A and the human γ 1C region has recently been used in patients with colon cancer. Whereas murine 171A has been used extensively in clinical trials and elicits a very pronounced HAMA response that alters its pharmacokinetics, antibody responses to chimeric 171A have been dramatically reduced. Moreover, the circulating half-life was increased relative to murine 171A and higher serum levels could be maintained at lower infused doses (27). Thus, with judicious genetic engineering, it is possible to manipulate antibody pharmacokinetics to minimize toxic side effects.

Chimeric antibodies may be effective in lowering the HAMA response in patients and increasing serum half-lives, but these properties are still inferior to human

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[3] Abbreviations used in this paper: HAMA, human anti-murine antibody; ADCC, antibody dependent cell mediated cytolysis; FR, framework

antibodies. Inasmuch as chimeric antibodies are still 30% murine, enhanced efficacy may be obtained by humanizing the V regions. New technologies have recently been advanced to produce totally humanized antibodies by grafting the CDR of murine antibodies onto human FR (17, 28–30). The resulting antibodies when expressed with human C regions should be essentially human. This technology, although technically straightforward, is not always totally successful. Selected FR amino acids appear to be involved in Ag binding. Identification of important FR amino acids has been achieved, up to now, by the use of x-ray crystallographic data (17) and sophisticated computer modeling (30) and several totally humanized antibodies have been produced with affinities close to those of their parental antibodies (17, 28–30).

We report here the production of a humanized antibody, without the use of sophisticated structural data, which retains the affinity and specificity of BMA 031, a murine mAb directed against the human α/β TCR. Moreover, humanized BMA 031 displays enhanced ADCC activity. BMA 031 has been used successfully in preventing organ transplant rejection (5) and may have potential efficacy in other T cell-related disorders.

MATERIALS AND METHODS

Cell culture. The BMA 031 and Sp2/0-Ag14 hybridomas were cultured in DMEM media supplemented with 10% FCS, 2 mM L-glutamine, 10 mM HEPES, pH 7.3, 10 mM nonessential amino acids (GIBCO, Gaithersburg, MD), and 10 mM pyruvate. Chimeric and humanized BMA 031 transfectomas were grown in the above media containing 1 μg/ml mycophenolic acid, 50 μg/ml xanthine, and 500 μg/ml Geneticin (GIBCO). All lines were maintained at 37°C in 7% CO_2.

Computer analysis. Sequences were manipulated and homology searches were performed with the Genetics Computer Group Sequence Analysis Software Package (University of Wisconsin Biotechnology Center, Madison, WI)) using the National Biomedical Research Foundation databases.

Synthesis of VH and VL regions. The VH and VL exons were synthesized on an Applied Biosystems (Foster City, CA) model 380A DNA synthesizer. Each region was synthesized completely as *EcoRI-HindIII* fragments consisting of overlapping (10–15 nucleotide overlap) oligomers (75–110 nucleotides). The oligomers were deprotected and purified by electroelution from polyacrylamide gels. The oligomers were then mixed in equimolar amounts (30 pmol), phosphorylated, annealed, and ligated into pUC 19 previously digested with *EcoRI* and *HindIII*.

Nucleotide sequencing. DNA sequencing of the synthesized VH and VL regions was performed directly on pUC subclones using universal forward and reverse primers (31).

Construction of humanized genes. To ensure efficient expression, the synthesized V regions were inserted into previously isolated genomic fragments (24) in place of the murine V regions. The resulting 5.6-kb *EcoRI* VH fragment was cloned into a mammalian expression vector containing the human γ1 C region and the *gpt* gene for selection. The 3.0-kb *HindIII* VL fragment was cloned into a vector containing the human κ C region and the *neo* gene (see Fig. 5).

Transfection of DNA into Sp2/0 cells by electroporation. DNA was introduced into murine hybridoma Sp2/0-Ag14 cells by electroporation. The 1 to 2 × 10^7 actively growing Sp2/0-Ag14 cells were washed and resuspended in 1.0 ml of sterile PBS. A total of 15 μg of each humanized, Ig_κ and IgG1, plasmid (linearized with *BamHI*) was added to the cell suspension. The DNA/cells were transferred to a precooled shocking cuvette, incubated on tee at least 5 min and then a 0.5 kv/cm electric pulse was delivered for 10 ms (Transfector 300, BTX, San Diego, CA). After shocking, the DNA/cell mixture was returned to ice for 10 min and then diluted in 40 ml of supplemented DMEM and incubated at room temperature for 10 min. Finally, the cells were transferred to a 37°C incubator with 7% CO_2 for 48 h before plating in selective medium, containing 1 μg/ml mycophenolic acid, 50 μg/ml xanthine, and 1 mg/ml Geneticin. Cells were plated in 96-well plates at 3 × 10^4 cells/well.

Cytofluorometric assay for affinity. To analyze the relative affinity of the BMA 031 antibody preparations, competitive immunofluorescence assays were carried out. PBMC were separated by Ficoll-Hypaque density gradient centrifugation and incubated *on* ice for 1 h in the dark with mAb at various concentrations (0.05–50 μg/ml) premixed with either FITC-BMA031 or FITC-BMAEUCIV3 (2 μg/ml). Unbound antibodies were removed by two washing steps. Cells from all experiments were analyzed either on an Ortho (Raritan, NJ) Cytofluorograph 50H/2150 Computer System or on a Becton Dickinson (Mountain View, CA) FACStar Plus as described elsewhere (32). The intensity of fluorescence was calculated by modified Ortho or standard FACStar Plus software and is expressed as mean channel number.

Cytotoxicity assays. To measure the cytolytic capacity of the BMA 031 antibody preparations, a 20 h [^{51}Cr] release assay was performed to measure ADCC and NK activity. [^{51}Cr]-labeled HPB-ALL target cells were incubated with (ADCC) or without (NK activity) various concentrations of antibodies for 20 h in the presence of Ficoll-separated PBL (effector cells). α/β TCR negative CEM cells were used as control target cells. The antibodies were allowed to bind first to target cells (30 min) before the effector cells were added. The E:T cell ratio varied from 1:1 to 50:1. Cytolysis in the absence of antibodies was considered to be due to NK activity. The percentage of specific lysis was calculated as described earlier (33). Spontaneous [^{51}Cr] release in the absence of effector cells and in the presence of the antibodies being tested was always less than 5%. All samples were analyzed in triplicate.

RESULTS

Designing humanized BMA 031 antibodies. To determine the optimal human sequence with which to humanize the murine BMA 031 antibody, the murine BMA 031 amino acid sequence was used to search the NBRF data base for the most homologous human antibody. Inasmuch as molecular models of antibodies show strong interactions between the H and L chains, we decided to use the H and L chain from the same human antibody. The human EU antibody turned out to be the best overall choice. The homology between the BMA 031 and EU FR (nos. 1–3) was 79% (67% identical) for the H chain and 81% (63% identical) for the L chain. The BMA 031 antibody uses JH3 and JK5. These are most homologous to human JH4 and JK4. A first generation humanized BMA 031 antibody would contain BMA 031 CDR, EU FR, and homologous human J regions. We refer to this antibody as BMA 031-EUCIV1 (Fig. 1).

A refinement to this basic humanized version can be made in the sequence immediately before and after the CDR. The CDR are assigned based on sequence homology data (34). Molecular models of antibodies have shown that the actual CDR loops can contain amino acids up to five amino acids away from the "Kabat" CDR (36). Also, Reichmann et al. (17) have shown the functional importance of a FR amino acid four residues from a CDR. Therefore, maintaining at least the major amino acid differences (in size or charge) within four amino acids of the CDR as murine may be beneficial. We refer to the antibody containing these changes as BMA 031-EUCIV2 (Fig. 1). Additionally, all differences within four amino acids of the CDR could be maintained murine. We refer to this antibody as BMA 031-EUCIV3.

Further refinements can be made, but, without complex computer modeling, it is difficult to prioritize their importance. For example, several amino acids are either BMA 031 specific or EU specific (i.e., different from the consensus sequence within their subgroups). Inasmuch as these amino acids presumably arose through somatic mutation to enhance their respective activities, it would seem logical to maintain the BMA 031-specific amino acids and change the EU-specific amino acids to the

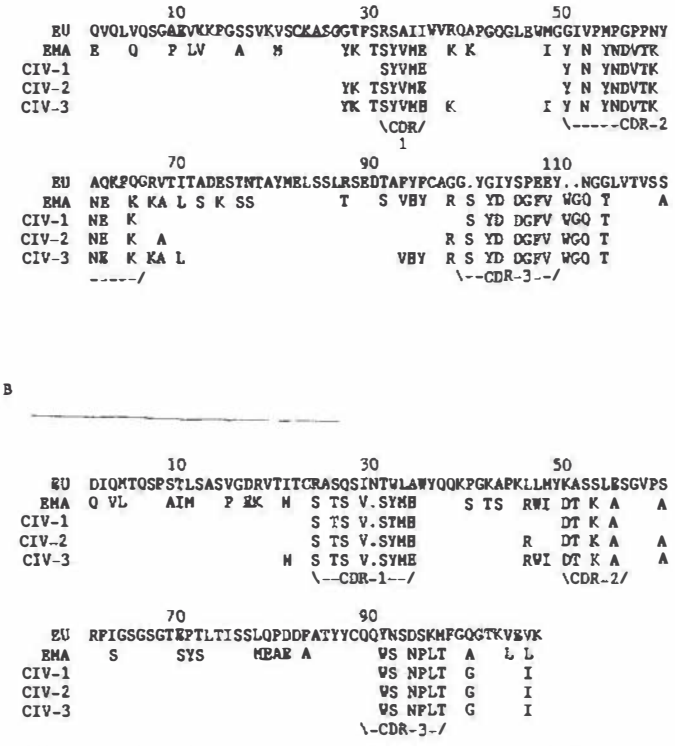

Figure 1. Amino acid sequences of EU, BMA 031, and humanized BMA 031 V regions. *A*, The VH region and *B*, the VL region. The positions of the CDR are indicated.

TABLE I

Amino acid (AA) differences between BMA 031 and EU and their consensus sequences

AA Position[a]	EU AA	Human AA	BMA031 AA	Mouse AA
H chain, EU specific				
70	Ile	[b]	Leu	Leu
72	Ala	[b]	Ser	Val
74	Glu	[b]	Lys	Lys
93	Phe	Val	Val	Val
95	Phe	Tyr	Tyr	Tyr
98	Gly	Arg	Arg	Arg
L chain, EU specific				
10	Thr	Ser	Ile	Ile
48	Met	Ile	Ile	Ile
63	Ile	Ser	Ser	Ser
70	Glu	Asp	Ser	Ser
81	Asp	Glu	Glu	Glu
H chain, BMA specific				
1	Gln	Gln	Glu	Gln
7	Ser	Ser	Ser	Pro
9	Ala	Ala	Pro	Ala
20	Val	Val	Met	Leu
40	Ala	Ala	Lys	Arg
72	Ala	[b]	Ser	Val
82	Glu	Glu	Glu	Gln
94	Tyr	Tyr	His	Tyr
L chain, BMA specific: None				

[a] Numbers correspond to those in Figure 1.
[b] Variable.

consequences. Changing an amino acid in one chain may cause changes in the interactions with other amino acids of that chain as well as with amino acids in the other chain. Therefore, extreme caution must be exercised to limit the number of changes. Table I outlines these potential changes. The residue numbers correspond to those in Figure 1. As can be seen, EU differs from the human VH-I subgroup consensus sequence in six positions (70, 95, and 98), and these are addressed in BMA-031-EUCIV3. In one position (no. 93) the human consensus sequence is the same as BMA 031. Moreover, the Phe_{93} in EU is highly unusual; this amino acid is only found in this position in one other human antibody in subgroup VH-III. One could rationalize changing this from EU to the human consensus, so we incorporated this change into BMA 031-EUCIV3. For the two remaining positions (nos. 72 and 74), there is no clear human consensus so we maintained the EU sequence. The L chain had five EU-specific amino acids. One is within four amino acids of the CDR (no. 48) and is maintained as BMA 031 in BMA 031-EUCIV3. In two positions (nos. 63 and 81) the human consensus is the same as BMA 031 and therefore could be changed to the human consensus. We decided not to make these changes at this time. The other two positions (nos. 10 and 70) were also not changed to limit the number of substitutions. There are eight BMA 031 specific amino acids in the H chain. In two positions (nos. 7 and 82) the BMA 031 sequence is the same as EU. His_{94} is unique to BMA 031. This position is considered "invariant" with Tyr_{94} occurring more than 98% of the time. Therefore, we decided to incorporate this change into BMA 031-EUCIV3. The remaining five positions (nos. 1, 9, 20, 40, and 72) were maintained EU to limit the number of changes. There are no BMA 031-specific amino acids in the L chain. The sequence is identical to the subgroup VI consensus. The changes in the human EU framework sequence back to BMA 031 are summarized in Table II. Twelve changes were made in the H chain; 5 in BMA 031-EUCIV2 and 7 more in BMA 031-EUCIV3. Five changes were introduced into the L chain; two in BMA 031-EUCIV2 and three more in BMA 031-EUCIV3.

Determination of DNA sequence for humanized V regions. The amino acid sequence of the V regions were reverse translated using the actual BMA 031 codons wherever possible and BMA 031 codon preferences everywhere else. To aid in future modifications, unique restriction enzyme sites were engineered into the sequence at approximately 60-bp intervals by making use of the degeneracy of the genetic code. Finally, convenient restriction enzyme sites 5′ and 3′ of the coding region of BMA 031 were identified and this flanking sequence was incorporated into the final humanized sequence to be syn-

TABLE II

Amino acid (AA) changes in EU FR

AA Position[a]	EU AA	BMA031 AA	CIV2 AA	CIV3 AA
H chain				
27	Gly	Tyr	Tyr	Tyr
28	Thr	Lys	Lys	Lys
30	Ser	Thr	Thr	Thr
38	Arg	Lys	Arg	Lys
48	Met	Ile	Met	Ile
67	Arg	Lys	Arg	Lys
68	Val	Ala	Ala	Ala
70	Ile	Leu	Ile	Leu
93	Phe	Val	Phe	Val
94	Tyr	His	Tyr	His
95	Phe	Tyr	Phe	Tyr
98	Gly	Arg	Arg	Arg
L chain				
21	Ile	Met	Ile	Met
46	Leu	Arg	Arg	Arg
47	Leu	Trp	Leu	Trp
48	Met	Ile	Met	Ile
60	Ser	Ala	Ala	Ala

thesized. The final DNA sequences of BMA 031-EUCIV2 VH and VL, excluding the *Eco*RI and *Hin*dIII cloning ends, are shown in Fig. 2.

Synthesis of humanized BMA 031 V regions. The L and H chain V region exons encoding the humanized antibodies were synthesized completely as *Eco*RI-*Hin*dIII fragments consisting of 10 to 15 overlapping (10–15 nucleotide overlap) oligomers (75–110 nucleotides). The oligomers were phosphorylated, annealed and ligated into a pUC vector previously cut with *Eco*RI and *Hin*dIII. The assembled fragments were sequenced to verify accuracy of synthesis.

Reconstruction of BMA 031 genomic fragments with humanized V exons. To increase the probability of efficient expression of the synthesized coding regions, the humanized sequences were replaced into the previously isolated 5.6-kb *Eco*RI VH and 3.0-kb *Hin*dIII VL genomic fragments of BMA 031 (Fig. 3). Due to the lack of unique restriction enzyme sites, several subclonings were necessary. To achieve this goal, four vectors, each containing modified genomic subfragments, were constructed. The first vector, pUCBMAVH-1.0HΔN was constructed by subcloning the 1.0-kb *Hin*dIII BMA 031 VH fragment into pUC19 with subsequent deletion of the 5′-*Nsi*I site. The second vector, pUCΔHBMAVH-5.6RΔH, was derived by cloning the 5.6-kb *Eco*RI BMA 031 VH fragment into a pUC19 vector with a previously deleted *Hin*dIII site. The 5′-*Hin*dIII site of the insert was then deleted to complete the construction. The third vector, pUCBMAVL-1.4RH2, was constructed by subcloning the 1.4-kb *Eco*RI-*Hin*cII BMA 031 VL fragment into pUC19. The fourth vector, pUCΔRSBMAVL-3.0H, was made by cloning the 3.0-kb *Hin*dIII BMA 031 VL fragment into a pUC19 vector that had a previous deletion from the *Eco*RI site to the *Sal*I site in the polylinker.

The cloning scheme to replace the humanized sequences into the genomic fragments is outlined in Figure 4. The newly synthesized *Sau*I-*Nsi*I BMA 031-EUCIV2 VH fragment was isolated from the pUC19 subclone and cloned into pUCBMAVH-1.0HΔN. Then, the 1.0-kb *Hin*dIII fragment was isolated and cloned into pUCΔHBMAVH-5.6RΔH. Finally, the 5.6-kb *Eco*RI fragment was isolated and subcloned into the mammalian expression vector containing the human γ 1 C region and the *gpt* gene for selection (Fig. 5).

The newly synthesized *Sau*I-*Hin*cII BMA 031-EUCIV2 VL fragment was isolated and cloned into pUCBMAVL-1.4RH2. Then, the 1.4-kb *Eco*RI-*Hin*cII fragment was isolated and cloned into pUCΔRSBMAVL-3.0H. Finally, the 3.0 *Hin*dIII fragment was isolated and cloned into the mammalian expression vector containing the human κ C region and the *neo* gene for selection (Fig. 5).

The BMA 031-EUCIV3 constructs were prepared in the same manner as BMA 031-EUCIV2. Replacement oligomers incorporating the coding changes for BMA 031-EUCIV3 were synthesized and cloned into the pUCBMA-EUCIV2 constructs. The final clone was sequenced to ensure accuracy of the coding sequence. The BMA 031-EUCIV3 V regions were replaced into the original BMA 031 genomic fragments and these fragments were cloned into the mammalian expression vectors described above.

Expression and purification of humanized BMA 031 antibodies. The humanized genes were transfected into Sp2/0 hybridoma cells by electroporation and selected in media containing both mycophenolic acid and Geneticin. Transfectomas secreting humanized BMA 031 antibodies were identified by ELISA. Secretion levels up to 7 pg/cell/24 h were obtained. The best clone from each transfection (CIV2 and CIV3), with respect to secretion level and growth characteristics, was expanded for further study.

The BMA 031-EUCIV2 and -EUCIV3 antibodies were partially purified by protein A-Sepharose column chromatography. Analysis of the antibodies by reducing and nonreducing SDS-PAGE showed a high degree of purity (data not shown). Analysis by a series of ELISA assays showed that the antibodies contained human κ and γ 1 C

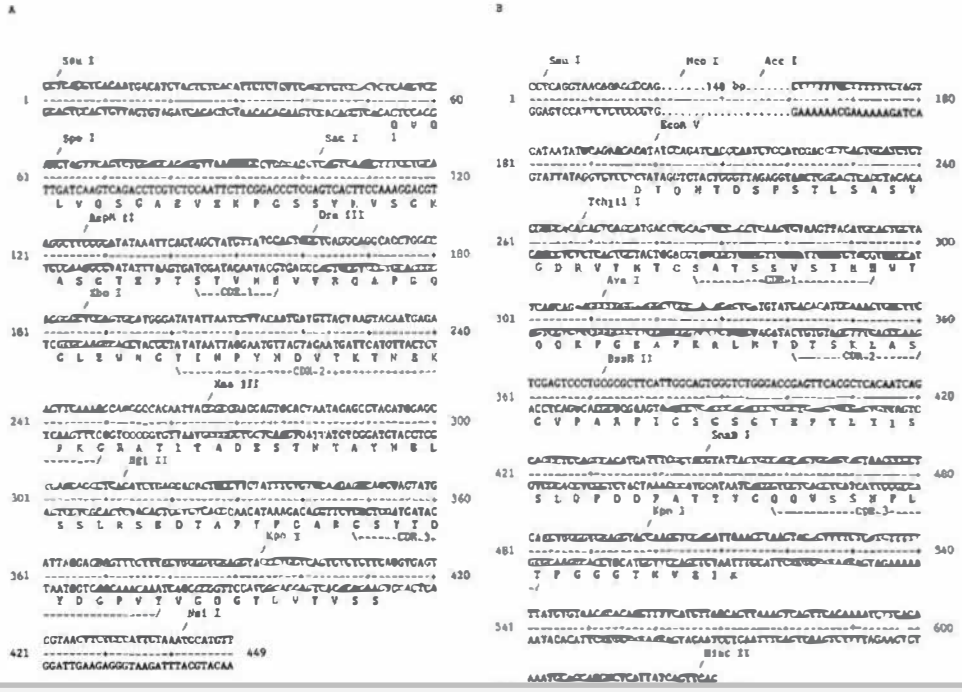

Figure 2. DNA sequences of the V regions of BMA 031-EUCIV2. *A*, The BMA 031-EUCIV2 VH region and *B*, the BMA 031-EUCIV2 VL region.

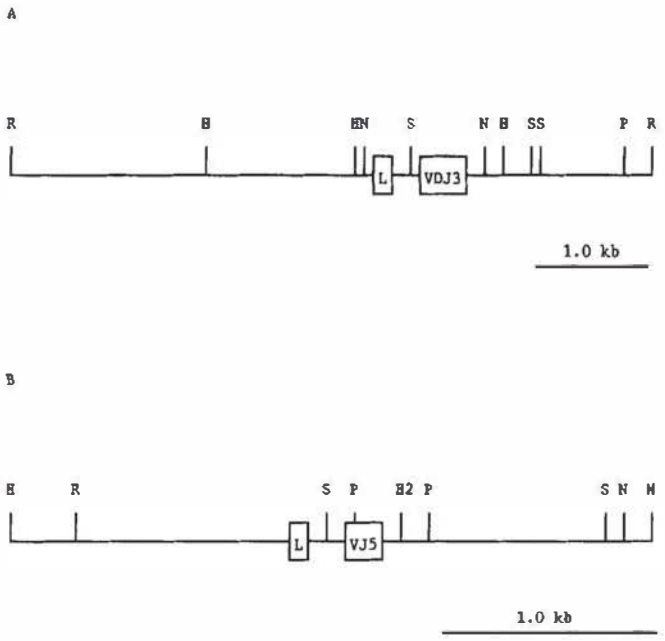

Figure 3. Partial restriction enzyme maps of BMA 031 V regions. *A*, The 5.6-kb *EcoRI VH* fragment containing the VDJ_3 exon. *B*, the 3.0-kb *HindIII* VL fragment containing the VJ_3 exons. *H*, *HindIII*; *H2*, *HincII*; *N*, *NstI*; *P*, *PstI*; *R*, *EcoRI*; *S*, *SauI*.

regions. Moreover, the antibodies did not react with anti-murine antibodies (data not shown).

Characterization of humanized BMA 031 antibodies. The BMA 031-EUCIV2 antibody bound poorly to T cells. In contrast, BMA 031-EUCIV3 shows an identical specificity as murine BMA 031. They both bind specifically to T cells and show no reactivity toward monocytes, E, or granulocytes (data not shown).

The relative affinities of murine BMA 031, chimeric BMA 031 (human IgG1), and the humanized variants were compared by competitive immunofluorescence assays. The data shown in Figure 6 indicate that both the murine BMA 031 antibody and the previously constructed chimeric BMA 031-G1 antibody block the binding of BMA 031-FITC in the same dose-dependent manner. BMA 031-EUCIV3 was about 2.5 times less efficient than murine BMA 031. BMA 031-EUCIV2 was unable to totally block BMA 031-FITC binding, even at concentrations as high as 50 μg/ml.

BMA 031 has been shown to be poor at mediating ADCC using human effector cells. To evaluate the ADCC capacity of the humanized antibodies, we compared them to rabbit anti-GH-1 antiserum. This antiserum was the best of eight rabbit anti-human T cell globulins in ADCC capacity. As shown in Figure 7, both the chimeric BMA 031 antibody and the BMA 031-EUCIV3 antibody were very efficient at ADCC. Even at very low effector:target cell ratios (Fig. 7*A*) or extremely low antibody concentrations (Fig. 7, *B* and *C*), the engineered antibodies are highly potent at mediating killing of the HPB-ALL cells.

DISCUSSION

We have joined the DNA segments containing the CDR from the BMA 031 mAb specific for the α/β TCR and the FR from the human EU antibody to the DNA segments encoding human γ-1 and κ C regions. When the humanized genes were introduced into non-Ig producing Sp2/0 cells, functional humanized antibodies specific for T cells were assembled and secreted.

Functional antibody, however, was dependent on substitution of various murine FR amino acids into the human FR. The identification of important FR amino acids in the absence of structural data or computer models is difficult but, by careful analysis of antibody sequence homologies, it is possible to generate a humanized sequence with a high probability of maintaining Ag binding. Our method consists of three parts. First, and possibly most important, is starting with the human antibody most homologous to the murine antibody under

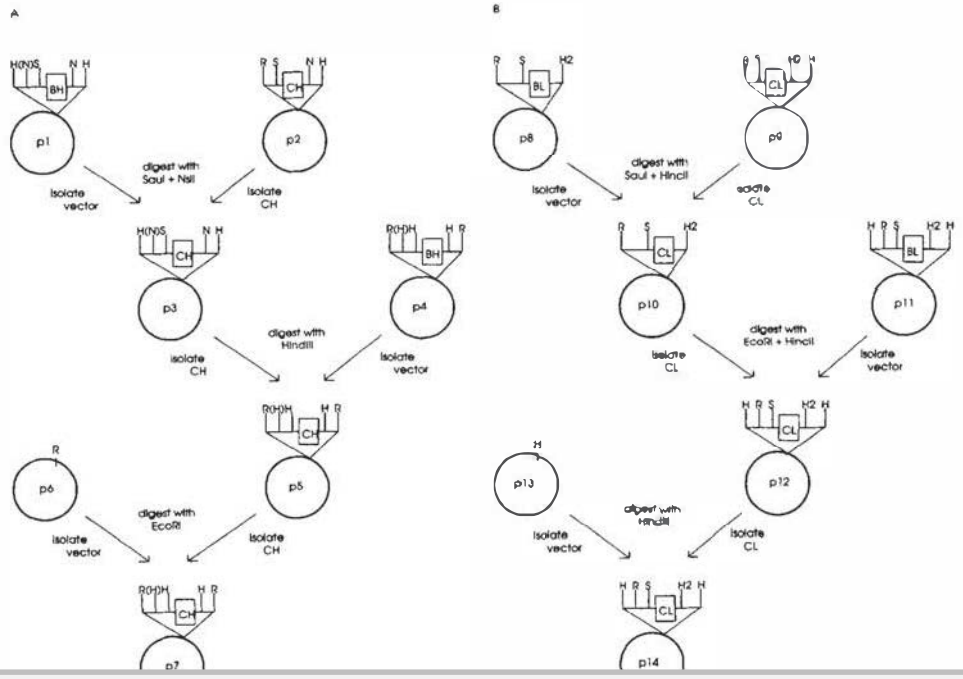

Figure 4. The cloning scheme to regenerate the BMA 031 genomic fragments with the humanized V regions. *A*, Substituting the humanized VH region into the 5.6-kb *EcoRI* VH fragment. *BH*, BMA 031 VH exon; *CH*, humanized BMA 031 VH exon; *p1*, pUCBMAVH-1.0HΔN; *p2*, pUCBMACIVH; *p3*, pUCBMACIVH-1.0HΔN; *p4*, pUCΔHBMAVH-5.6RΔH; *p5*, pUCΔHBMACIVH-5.6RΔH; *p6*, pSV2gpt-huγ1; *p7*, pSV2gpt-BMACIVH-huγ1. *B*, Substituting the humanized VL region into the 3.0 *HindIII* VL fragment. *BL*, BMA 031 VL exon; *CL*, humanized BMA 031 VL exon; *p8*, pUCBMAVL-1.4RH2; *p9*, pUCBMACIVL; *p10*, pUCBMACIVL-1.4RH2; *p11*, pUCΔRSBMAVL-3.0H; *p12*, pUCΔRSBMACIVL-3.0H; *p13*, pSV2neo-huκ; *p14*, pSV2neo-BMACIVL-huκ. Restriction enzyme sites identified are; *H*, *HindIII*; *H2*, *HincII*; *N*, *NstI*; R, *EcoRI*; S, *SauI*.

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