Polymerase chain reaction facilitates the cloning, CDR-grafting, and rapid expression of a murine monoclonal antibody directed against the CD18 component of leukocyte integrins

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ABSTRACT

Two novel approaches of recombinant PCR technology were employed to graft the complementarity determining regions from a murine monoclonal antibody (mAb) onto human antibody frameworks. One approach relied on the availability of cloned human variable region templates, whereas the other strategy was dependent only on human variable region protein sequence data. The transient expression of recombinant humanized antibody was driven by the adenovirus major late promoter and was detected 48 hrs post-transfection into non-lymphoid mammalian cells. The application of these new approaches enables the expression of a recombinant humanized antibody just 6 weeks after initiating the cDNA cloning of the murine mAb.

INTRODUCTION

The immunogenicity of murine-derived monoclonal antibodies (mAb) precludes the therapeutic use of these antibodies (1) in chronic or recurrent human diseases, such as inflammation or cancer. To minimize the unwanted human anti-mouse immune response, chimeric Abs were engineered which combined light and heavy chain variable (V_L, V_H) regions of murine origin and constant (C) regions from human sequences (2,3). Subsequently, Winter and colleagues (4) postulated that a 'humanized' version of a murine-derived mAb might reduce further the foreign character of the therapeutic Ab so as to be less immunogenic. A humanized Ab is one in which only the antigen-recognition sites or CDRs (complementarity-determining regions whose sequences are hypervariable, and thus antigen-specific, relative to the rest of the V regions) are of non-human origin, whereas all framework (FR) and C regions are products of human genes. In the construction of humanized Abs, several groups (4–7) have employed strategies requiring annealing, extension and ligation of many long synthetic oligos to graft human immunoglobulin (Ig) V_L, V_H containing rodent CDRs. In addition, once a suitable humanized Ab is identified and expressed, considerable time is required for drug selection and expansion of transfected clones prior to analysis of recombinant Ig.

We have devised a novel, rapid and effective means of substituting murine CDRs for their human counterparts through using overlapping polymerase chain reaction (PCR) fragments (8–13). Variations of this method are applicable to CDR-grafting regardless of the availability of human Ig V region clones. The grafted V regions are interchangeable in our expression vectors in cassette-like fashion. In addition, we have developed a transient expression assay in non-lymphoid mammalian cells which allows detection of recombinant human Abs (by ELISA) within 48 hours post transfection. Our system enables rapid expression for testing the folding, pairing assembly, secretion and resultant activity of intact recombinant humanized Abs.

MATERIALS & METHODS

Oligodeoxynucleotide synthesis

Oligos were synthesized on an Applied Biosystems 381A DNA synthesizer or Milligen Cyclone, removed from the resin by treatment with concentrated NH_4OH followed by desalting on a Pharmacia NAP-5 column (for oligos $\leq$40 bases in length) with H_20 elution or by the use of an OPC column (Applied Biosystems) eluting with 20% acetonitrile (for oligos >40 bases in length).

cDNA cloning

Two micrograms of total cellular RNA isolated (14) from 10^8 murine 1B4 (IgG2Aκ) myeloma cells (15) were reverse-transcribed for 30 min at 42°C using 200 units of Moloney MuLV reverse transcriptase (RT;BRL) and 10 pmol of the C region complementary strand primers representing either V_L (5'-TCTCAAGCTTTGGTGGCAAGAT(AG)GATACAGTTGG-TGCAGC-3') or V_H (5'-TCTCAAGCTTACCGATGG(AG)G-

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CTGTTGTTTTGGC-3') chain in 20 μl of a buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 20 units of RNAsin (Pharmacia). The RT was heat-inactivated at 95°C for 5 min and to each reaction was added 50 pmol of each of the paired primers (C region complementary-strand primers listed above plus FR1 primers for the kappa chain: [5'-TCTCGGATCCGA(CT)AT(CT)GTG(AC)T(CG)ACCC-AG-3']; or the IgG2A heavy chain: [5'-TTCTGGATCCG(CG-)AGGT(CGT)AAGCTGGTG(CG)AGTC(AT)GG-3')], 2.5 units of Taq DNA polymerase (Perkin Elmer Cetus) in 100 μl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200μM each dNTP). PCR was performed for 45 cycles (2', 94°C; 2', 55°C; 2', 72°C) followed by gel purification. The resulting 400 bp PCR product (see Figure 1) was phosphorylated, subcloned into the blunt-ended (SmaI cut) vector pSP72 (Promega) and sequenced using Sequenase® (United States Biochemical Corp.)

CDR grafting

CDR grafting utilizing PCR recombination was accomplished by synthesizing eight oligos (4 primer pairs R1-R6, S1, S2) [See Figure 2. The appropriate primer pair (50 pmol of each) was combined with 10 ng of plasmid DNA representing the human FR (obtained from Winter and colleagues)(5), and 2.5 units of Taq DNA polymerase in 100 μl of PCR buffer. These primers directed PCR for 25 cycles (cycle periods, as above). The products of the four reactions were purified by agarose gel electrophoresis and electroelution, and 10 ng of each of the purified products were combined with terminal primers A1 and A2 (50 pmol of each) and 2.5 units of Taq DNA polymerase in 100 μl of PCR buffer and amplified for 25 cycles. CDR grafting via long oligos and PCR recombination (see Figure 3) was accomplished by adding 1 pmol of each of 4 long 100mers (G1-G4) together with 50 pmol terminal primers A3 and A4 and 2.5 units Taq DNA polymerase in 100 μl of PCR buffer and amplified for 25 cycles. The combined 400 bp fragment was purified by agarose gel electrophoresis and electroelution. In parallel, two DNA fragments representing amino-terminal sequences encoding a 5' untranslated sequence and the signal peptide and carboxy-terminal sequences encoding FR 4, splice donor, and a 5'-intronic sequence between V and C regions were used for PCR with primer pairs S1, G5 and S2, G6 (G5 and G6 contain an 18 base overlapping sequence between G1 and G4 respectively). PCR was performed with 50 pmol each primer, 10 ng plasmid DNA containing both the signal and intronic sequences, 2.5 units of Taq polymerase in 100 μl of PCR buffer for 25 cycles and the product purified as above. These two DNA fragments (10 ng of each) were combined with 10 ng of the amplified 1B4 CDR-grafted V_H region, 50 pmol terminal primers A1 and A2, and 2.5 units of Taq polymerase in 100 μl PCR buffer and amplified for 25 cycles.

Heavy chain expression vector construction

The vectors used for the expression of both H- and L-chain genes were derived from the pD5 eukaryotic expression vector (16) which contains the origin of adenovirus replication (ori), the SV40 enhancer, the adenovirus major late promoter (MLP), the adenovirus 2 tripartite leader, a 5' splice donor from the adenovirus third leader and a 3' splice acceptor derived from an Ig locus, a multiple cloning site (containing the sites BamHI, SpeI, SacI, NheI, ClaI, NdeI, and NotI), and the SV40 late polyadenylation signal.

The adenovirus ori was removed by EcoRI and KpnI digestion and replaced by two fragments representing the Neo^R selectable marker gene (17) derived from plasmid pCMVIE-AK1-DHFR (18) as an EcoRI/BamHI 1.8 Kbp fragment and the Ig H chain enhancer sequence (19) following digestion with BglII and KpnI. The Ig H chain enhancer fragment was obtained as a 300 bp amplified fragment using PCR applied to human genomic DNA as template, the 2 primers: (5'-TTTTAGATCTGTCGACAG-ATGGCCGATCAGAACCAG-3' and 5'-TTGGTCGACGGT-ACCAATACATTTTAGAAGTCGAT-3') and standard procedures described above. The resultant expression vector was found to lack a small portion of the TK promoter responsible for the transcription of the Neo^R gene. The promoter was reconstituted by insertion into the EcoRI site of a 140 bp PCR-amplified fragment derived from pCMVIE-AK1-DHFR using the following primers: 5'-TATAGAATTCGGTACCCTTCATCC-CCGTGGCCCG-3' and 5'-TGCGTGTTCGAATTCGCC-3'. This resulted in the Neo-selectable vector and was designated pD5/IgH-En/Neo. The 1B4 CDR-grafted V_H region PCR product was digested with BglII and BamHI, purified, cloned into pD5/IgH-En/Neo (BamHI cut) and subjected to DNA sequence analysis to determine orientation and verify the sequence of the reconstructed V region. The appending of the human IgG4 (hIgG4) C region to the variable region was performed as follows. Plasmid pAT84 (20) containing the human genomic IgG4 C region as a 6.7 Kbp HindIII fragment was used as template DNA to amplify a 1.8 Kbp IgG4 fragment containing only the exons encoding C_{H1}, C_{H2} and C_{H3} and the introns separating them. PCR was performed with the following primers: 5'-ATTTGG-ATCCTCTAGACATCGCGGATAGACAAGAAC-3' and 5'-AATAATGCGGCCGCATCGATGAGCTCAAGTATGTA-GACGGGGTACG-3'. Following digestion with BamHI and NotI (contained on the 5' and 3'-primers, respectively), the IgG4 fragment was cloned into the BamHI and NotI sites of pD5/IgH-En/Neo containing the grafted V_H region. The resulting plasmid designated pD5/IgH-En/Neo/VH/Human Cγ4 (Figure 4) encodes an entire Ig H chain.

Light chain expression vector construction

The 1B4 CDR-grafted V_L region PCR product was digested with HindIII and XbaI, purified from an agarose gel and subcloned into these same sites of vector pSP72 (Promega) which contained the human kappa C_L region (21), obtained as follows. DNA (1μg) purified from a human B cell line (GM01018A; NIGMS Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ 08103) was used as a template with primers (5'-TCTCGGATCCTCTAGAAGAATGGCTG-CAAAGAGC-3' and 5'-TCTCGCTAGCGGATCCTTGCAG-AGGATGATAGGG-3') to PCR amplify a 920 bp fragment containing the splice acceptor for the human kappa C_L domain, the exon and a portion of its 3'-untranslated region. The PCR product was purified by agarose gel electrophoresis, digested with BamH1, and cloned into BamH1-digested pSP72. The pSP72-based intermediate vector containing both the 1B4 grafted V_L region and the human C_L region was digested with SpeI and ClaI resulting in a 1.5 Kbp fragment (containing the CDR-grafted variable and human constant region of the kappa L chain) and purified by agarose gel electrophoresis.

The Neo-selectable vector (pD5/IgH-En/Neo) for H chain expression was converted into one expressing the hygromycin B (Hyg B) selectable marker for L chain expression as follows: The Neo^R cassette was removed by digestion first with EcoRI

followed by DNA polymerase-directed fill in of the 5′ overhang, then subsequent SalI digestion. The Hyg B expression cassette (1.9 Kbp BamH1 fragment) containing the TK promoter and TK polyadenylation signal flanking the Hyg B gene from plasmid pLG90 (22) was removed from the plasmid pAL2 (Albert Lenny, personal communication) by BamHI digestion and subcloned into the BamHI site of the vector pSP72. The Hyg B cassette was removed from pSP72/Hyg B by digestion with SmaI and SalI and subcloned into the expression vector linearized as described above. This resulted in the Hyg B-selectable vector and was designated pD5/IgH-En/Hyg B. The vector was digested with SpeI and ClaI, purified and ligated to the 1.5 Kbp SpeI/ClaI L chain fragment purified from above. The resulting plasmid designated pD5/IgH-En/Hyg B/Vκ/Human Cκ (Figure 4) encoded an entire Ig kappa L chain.

Expression and assay of recombinant antibody

Expression plasmids encoding the 1B4 CDR-grafted H and L chain were purified through CsCl gradients. The plasmids were co-transfected (10 μg of each) into human 293 cells and monkey COS-7 and CV1P cells (2.5×10^6 cells in 100 mm plates) by standard calcium phosphate precipitation methods (23,24). The culture supernatants were assayed 48 hrs post-transfection by a sandwich ELISA described as follows: Immulon-2 (Dynatech Labs.) 96-well plates were coated overnight with a 5 μg/mL solution of mouse anti-human Cκ mAb (cat. #MC009, The Binding Site, Inc., San Diego, CA) in 0.1 M $NaHCO_3$ buffer (pH 8.2) at 4°C, and blocked with 1% bovine serum albumin (BSA) in 0.1M $NaHCO_3$ for 1h at 25°C. After this and all subsequent steps, washing was performed with phosphate buffered saline (PBS). The wells then were incubated with culture supernatants containing recombinant humanized 1B4 IgG4-antibody, or with predetermined quantities of hIgG4 purified by protein A Sepharose (Pharmacia Fine Chemicals) chromatography from hIgG4 myeloma serum (cat. # BP026, The Binding Site, Inc.) All samples were diluted in PBS containing 0.05% (w/v) Tween-20. 100-μL aliquots were incubated for 1h at 37°C in triplicate, and standard calibration curves were constructed using hIgG4 concentrations of 10−100 ng/mL. Bound and fully assembled hIgG4 (either native or humanized IgG) were detected with 100-μL aliquots of a 1:500 dilution of mouse anti-hIgG4 Fc mAb conjugated to alkaline phosphatase (cat #05−3822, Zymed Laboratories, Inc., South San Francisco, CA) in 0.1 M Tris, 0.15 M NaCl ph 7.5 containing 1% (w/v) BSA. After incubation for 1h at 37°C and subsequent washing, the quantities of bound conjugate were detected by incubating the wells with a 1mg/mL solution of p-nitrophenyl phosphate in 0.1M 2,2′ amino-methyl-propanediol buffer, pH 10.3, for 30 min at 25°C and measuring the adsorbance at 405 nm with a UV Max plate reader (Molecular Devices).

RESULTS

cDNA cloning of an anti-CD18 mAb

The V_H and V_L regions of murine 1B4 anti-CD18 mAb (15) were cloned using PCR and was similar to the approach taken by other groups (26,27). Analysis of the murine 1B4 immunoglobulin with an isotyping ELISA kit (ScreenType, Boehringer Mannheim, Indianapolis, IN) indicated it is comprised of an IgG2A H chain and a kappa L chain (15). The 5′ primers for PCR were chosen from conserved N-terminal FR1 kappa and IgG2A V region sequences (28). The H chain 5′ primer was 24-fold degenerate and the L chain 5′ primer 16-fold (Figure 1). Both 3′ PCR primers were chosen from conserved regions of either C_{H1} or C_L, each being 2-fold degenerate. Agarose gel analysis (Figure 1) of the PCR products of the V_H and V_L regions clearly indicates fragments migrating at approximately 400 bp as was predicted from the chosen primers. These PCR products were subcloned and sequenced and the CDR and FR regions were delineated based on the locations of the hypervariable CDR domains in available sequenced V genes (28).

Grafting of murine anti-CD18 mAb CDRs onto human V region FRs

Once the murine FR and CDRs had been identified, two general approaches were devised to graft these CDRs onto human V region FRs. The first approach uses a rearranged genomic DNA

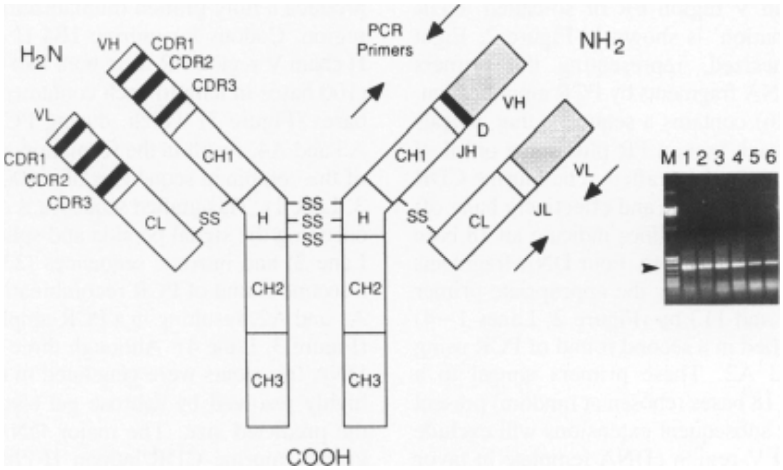

Figure 1. Schematic of the coding sequence for an antibody molecule depicting the position where PCR primers were chosen to clone the anti-CD18 V_H and V_L regions. Arrow indicates size of fragments on the agarose gel depicted on the right after RNA/PCR with primers shown (see text). CDR: complementarity-determining region; FR: Framework region; V: variable region; C: constant region; L: light chain; H: heavy chain; D: diversity; J: joining; NH_2: amino terminus; COOH: carboxyl terminus; -S-S-: disulfide bridge; lanes 1−4, V_H RT/PCR products; lanes 5−6, V_L RT/PCR products; M: λ HindIII, pBR322 MspI.

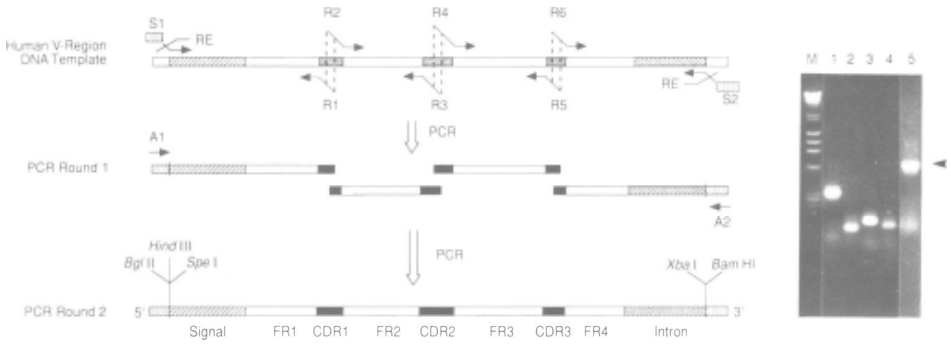

Figure 2. Strategy for CDR-grafting utilizing PCR recombination. Primer pairs S1-R1, R2-R3, R4-R5, and R6-S2 generate 4 PCR products in round 1 as shown in the figure and lanes 1−4 on the agarose gel depicted on the right. The 4 fragments are combined and PCR amplified with external amplifiers A1 and A2 in round 2 (see text). The arrow pointing to the band in lane 5 depicts a murine CDR-grafted/human L chain FR of 600 bp. RE: restriction endonuclease sites; M: λ HindIII, ϕ X174 HaeIII.

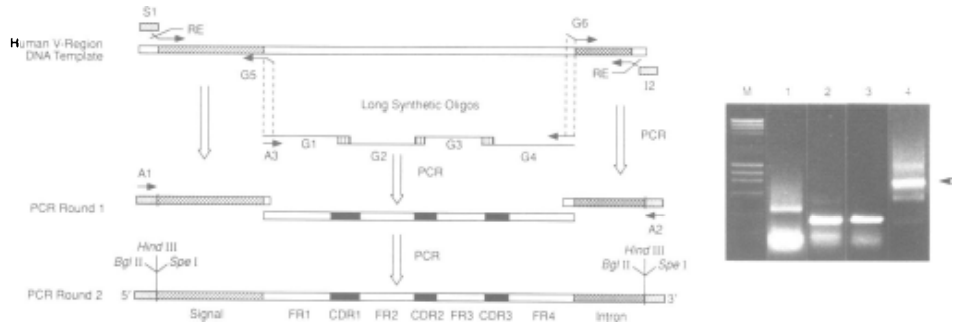

Figure 3. Strategy for CDR-grafting utilizing long oligos and PCR recombination. In round 1 long oligos G1, G2, G3, G4 and short terminal primers A3 and A4 generate the PCR product as shown in the figure and lane 1 on the agarose gel depicted on the right. In parallel, signal peptide (primers S1 and G5) and intron (primers G6 and S2) PCR products are generated as shown (lanes 2 and 3, respectively). The 3 fragments are combined and PCR amplified with external amplifiers A1 and A2 in round 2 (see text). The arrow pointing to the band in lane 4 depicts a CDR-grafted/heavy chain FR of 850 bp.

clone (containing intact VDJ for H chain, VJ for L chain, each flanked by signal peptide and intron, and V-C intron sequences) of a human V region as template for PCR. Both FRs contained a leader sequence and 3′ intronic sequences. The grafting of murine CDRs onto a human V region FR or so-called 'CDR grafting via PCR Recombination' is shown in Figure 2. Eight oligo primers were synthesized, representing the primers necessary to generate four DNA fragments by PCR amplification. Each internal primer (R1-R6) contains a sequence that anneals to the beginning or end of each human FR plus some or all of the murine 1B4 CDR sequence to be grafted. The murine CDR sequences do not anneal to the template and effectively hang off the human FRs as shown. The dotted lines indicate an 18 base complementary sequence between primers. Four DNA fragments generated in the first round of PCR by the appropriate primer pairs of sizes 314, 97, 134, and 113 bp (Figure 2, Lanes 1−4) were recombined and amplified in a second round of PCR using external amplifiers A1 and A2. These primers anneal to a complementary sequence of 18 bases (chosen at random) present on primers S1 and S2, their subsequent extensions will exclude amplification of the original V region cDNA template in favor of the CDR grafted recombinant, resulting in a PCR-amplified 600-bp DNA fragment. As depicted in figure 2 (Lane 5), a DNA fragment encoding a fully grafted murine CDR/human L chain FR was generated, demonstrating the success of this novel technique.

Often the protein sequence of antibodies is available (ie, from protein databases) when DNA clones encoding human FRs are not. Therefore, a second approach was devised which uses long synthetic oligos in conjunction with PCR recombination to produce a fully grafted (humanized) human FR/murine CDR V region. Codons for murine 1B4 H chain CDRs and the human H chain V region FR were built into the design. The 4 long oligos (100 bases in length) each contained complementary ends of 18 bases (Figure 3) which, during PCR using external amplifiers A3 and A4, result in the formation and subsequent amplification of the combined sequences of a 400-bp DNA fragment (Figure 3, Lane 1). This grafted sequence is combined with PCR products encoding the signal peptide and splice donor (225 bp, Figure 3, Lane 2) and intronic sequences (230 bp, Figure 3, Lane 3) in a second round of PCR recombination using external amplifiers A1 and A2, resulting in a PCR-amplified 850-bp DNA fragment (Figure 3, Lane 4). Although three other minor PCR-amplified DNA fragments were generated in this round, they were not as highly resolved by agarose gel eletrophoresis and were not of the predicted size. The major DNA fragment encodes a fully grafted murine CDR/human H chain FR thereby definitively demonstrating a second successful application of PCR recombination in CDR grafting.

The fully grafted 1B4 CDR/human H- and L-chain FR-encoding DNAs generated by the two approaches were subcloned and sequenced to determine the accuracy of PCR recombination.

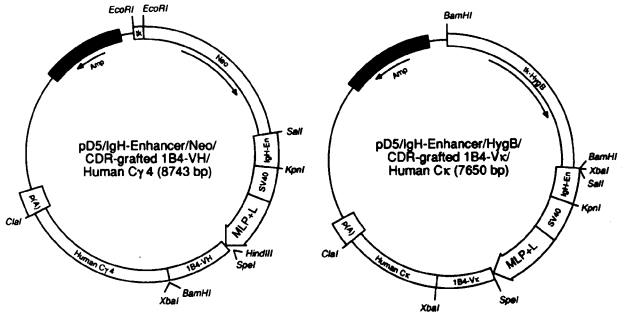

Figure 4. Vectors used for L and H chain Ig gene expression. IgH-En: Immunoglobulin heavy chain enhancer; SV40: SV40 origin and enhancer; MLP+L: Adenovirus major late promoter and tripartite leader; p(A): SV40 late polyadenylation signal; ori: pBR322 origin of replication; Amp: ampicillin resistance gene; tk: herpes simplex virus thymidine kinase promoter; Neo: neomycin (G418) resistance gene; Hyg B: hygromycin B resistance gene: 1B4-VH: CDR-grafted/H chain FR; 1B4-Vκ: CDRγ-grafted/L chain FR; Human Cγ4: human gamma-4 C region; Human Cκ: human kappa C region.

Table 1. Expression level of recombinant antibodies

Cell Line	ng/mL
293	385 ± 38
COS-7	82 ± 1.2
CV1-P	50 ± 3.9

N=2

The Taq polymerase was found to be error prone during PCR recombination. Indeed, many clones lacked a completely intact open reading frame (ORF). For expediting the selection process of clones with intact ORFs, we screened clones by sequencing with a single dideoxy termination lane (A track) alone. In one case, we were able to quickly eliminate 18/31 prospective clones using this simple screening method. The lack of fidelity of the polymerase has been addressed in the context of our recombination strategy, and improvements on the method have been found which greatly diminish the number of undesirable clones (manuscript in preparation).

Expression and activity of recombinant CDR-grafted Ig genes

The vectors for IgG expression driven by the adenovirus MLP and tripartite leader are depicted in Figure 4. Both L and H chain expression vectors are identical, except that the L chain vector carries the Hyg B^R gene and the H chain vector the Neo^R gene. Transfections for transient expression were performed by co-transfection of equal amounts of each plasmid into three primate kidney cell lines; human 293 cells and monkey COS-7 and CV1-P cells. The culture supernatants were assayed 48 hours post-transfection by a trapping ELISA that specifically measures secreted human kappa L chain linked to a human IgG4 H chain. As shown in Table 1, recombinant IgG4κ antibodies were synthesized by all three cell lines. Human 293 cells expressed the highest transient level while the 2 monkey cell lines expressed significantly lower levels and this was not suprising since human 293 cells constitutively express the adenoviral E1A protein (29) which stimulates transcription directed from the adenovirus major late promoter (30).

In preliminary binding experiments, medium from transiently transfected 293 cultures containing fully-grafted humanized 1B4

Table 2. Comparative effects of unlabeled fully-grafted humanized 1B4 versus murine 1B4 on the binding of ^{125}I-murine 1B4 to stimulated human polymorphonuclear leukocytes (PMNs)

	Quantity of ^{125}I-Murine 1B4 Bound (CPM)	
Molarity IgG Added	Humanized 1B4	Native Murine 1B4
1×10^{-12}	1941	2211
1×10^{-10}	1761	1697
1×10^{-9}	858	783
5×10^{-9}	460	99

Purified murine 1B4 was radiolabeled with ^{125}I to a specific activity of 17 uCi/ug using chloramine-T. PMNs were isolated and activated with phorbol myristate acetate as previously described (25). Aliquots of PMNs were incubated in 1.8×10^{-11} M ^{125}I-murine 1B4 in the presence of various concentrations of either fully grafted humanized 1B4, or unlabeled murine 1B4, and the quantity of ^{125}I-murine 1B4 bound to the cells determined as described (25). For humanized 1B4, various dilutions of conditioned medium from 293 cultures transfected 48hr previously with the plasmids encoding the fully grafted 1B4 were used to compete with ^{125}I −1B4 binding to PMNs. The concentration of humanized 1B4 present in this medium was determined by ELISA as described in materials and methods.

was observed to compete with the binding of ^{125}I-murine 1B4 to CD18-containing receptors on stimulated human polymorphonuclear leukocytes (Table 2). The level of competition was very similar to that observed when unlabeled native 1B4 was used as the competitor. Similar results were obtained with CV1-P transfected cultures, while media from mock transfected cells or purified human IgG4 were completely uninhibitory (data not shown).

DISCUSSION

In this report, we have demonstrated novel adaptations of PCR to the successful cloning, humanization, and expression of a murine mAb directed against the CD18 component of leukocyte integrins (15). Notably, PCR has enabled us to expediently carry out the process of cDNA cloning of murine Ig V regions, CDR-grafting onto human FRs, and expression as measured by ELISA of a fully assembled recombinant human antibody in as little as six weeks. The novel technical approaches we have taken enable a considerable time and cost savings as compared to more traditional methods. In addition, the preliminary competitive binding experiments described here also suggest that the humanized CDR-grafted 1B4 molecule has an avidity for CD18 at the surface of activated PMNs nearly comparable to that of the native murine 1B4 mAb. The choice of particular human V_L and V_H region FRs used in the humanization of the murine 1B4 mAb play a critical role in the outcome of the humanization and will be reported elsewhere.

It must be emphasized that the techniques we describe necessitated a substantial amount of DNA sequencing in order to distinguish those clones that contained an intact ORF from those containing insertions, deletions, or substitutions. Initially, the method of CDR-grafting using DNA templates for PCR was much more successful for producing clones that contained intact ORFs than the synthetic long oligo method. Indeed, the majority of the clones derived from the latter approach had significant deletions of greater than 6 bp, as well as numerous point mutations. Retrospectively, upon sequence analysis using a dyad symmetry computer program (Intelligenetics) to ascertain predicted regions of stable secondary structure, it was noted that the particular sequences subject to deletion gave hairpin loops with ΔG values of −13 to −26 Kcal/mol. Furthermore, the stem-

loops predicted in the sequence by the symmetry program closely approximated the region of observed deletions in size and location. Subsequently, the codon choice was altered in this region of the long oligonucleotides. This resulted in a decrease in the predicted ΔG values and a reduction in the frequency of deletions during PCR recombination was observed in the product. In addition, a decrease in cycle number has also resulted in an increase in fidelity and a reduction in the number of deletions. Other parameters (i.e., cycle time, temperature, etc.) are being investigated actively. Although we do not know the upper limit of either the length or the number of fragments for which PCR recombination will be practical, we have been successful at combining up to 5 fragments and up to a total of approximately 1700 bp. PCR recombination need not be limited to CDR grafting, but will lend itself to a variety of other applications such as domain shuffling, domain editing, construction of hybrid molecules, etc. Thus, it is a general method to insert specific new sequences into other molecules.

We have generated expression vectors which enable us to exchange CDR-grafted FR regions as a single casette. This affords us the opportunity to combine a variety of human FRs with identically grafted murine CDR regions, and thus to explore the effect of the FR regions on the avidities of a particular human recombinant antibody. In this way we can test the hypothesis that the recipient FRs for CDR-grafting are generic, ie, any FR region is capable of supporting all CDR loops. Likewise, we can easily, rapidly and systematically introduce amino acid changes in the CDR or FR regions as part of a structure-function analysis of antibody/antigen interaction.

Our rapid transient expression system in monolayer culture has served a two-fold purpose. Firstly, due to the high frequency of deletions during PCR recombination, it provides a rapid screen that allows us to ascertain whether or not the CDR-grafted recombinant contains an intact ORF. Secondly, upon expression, the activity (avidity) of the recombinant antibody can be quickly determined and evaluated.

It is hopeful that CDR-grafting of the murine V-regions would reduce the immunogenicity of the mAb in the context of the human immune system. Experiments performed in mice (31) suggest that this would indeed be the case, in that a significant antigenic response was produced against the foreign V-region in a chimeric antibody. Although it is beyond the scope of this paper, it will be necessary to produce sufficient quantities of the recombinant humanized anti-CD18 mAb for clinical evaluation in humans. The establishment of stable mammalian expressor cell lines is currently in progress and will subsequently be followed by scale-up production. Ideally, the reduction in immunogenicity would result in increased serum half-life of the therapeutic mAb requiring reduced dosage to achieve efficacy. Furthermore, it might enable repeated administration of the recombinant humanized mAb for longer time periods.

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