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A rapid procedure for the humanization of monoclonal antibodies*

(Antibody humanization; site-directed mutagenesis; polymerase chain reaction; efficiency)

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SUMMARY

An efficient and rapid procedure for the humanization of murine monoclonal antibodies (MumAb) is described. It consists of site-directed mutagenesis (SDM) to transfer the murine complementarity-determining regions (MuCDR) onto human framework regions (HuFR), followed by polymerase chain reaction (PCR) of the SDM product. Using SDM/PCR, rapid and correct humanization of MumAb heavy chains is clearly demonstrated. Compared to current protocols this method considerably reduces the time and labour required to generate humanized mAb.

To test large numbers of HumAb for immunotherapy, a simple and efficient method for humanizing MumAb is required. Both SDM (Riechmann et al., 1988) and PCR (Lewis and Crowe, 1991) have been used separately to humanize MumAb. However, these procedures have either very low efficiency, or are time consuming. Described here is a new efficient humanization method, which consists of SDM followed by PCR of the SDM product (see Fig. 1). The effectiveness of the SDM/PCR humanization procedure is demonstrated by humanizing the V_H domain of MumAb (40-40) (Hudson et al., 1987) and DX48 (Lewis and Crowe, 1991). These two MumAb are specific for the hapten digoxin.

The DNA fragment (*Hin*dIII-*Bam*HI) coding for the heavy chain variable domain (HuV_{NP}) from the humanized mAb Hu_{NP} (specific for the hapten nitrophenactyl) was removed from the plasmid pSV_{gpt} HuV_{NP}-HuIgG (provided by Dr. Neuberger, Cambridge, UK), and cloned into the corresponding sites in M13mp18, to produce the construct $M13mp18HuV_{NP}$. The $M13mp18HuV_{NP}$ ss DNA was used as the template for the SDM. The oligos used for the SDM coded for MumAb DX48 or (40-40) V_H CDRs, and HuV_{NP} FRs. The SDM reactions were done using the modified method of Taylor et al. (1985). During each SDM either MuCDR1, or MuCDR1 and -2, or MuCDR1, -2 and -3 were transferred onto HuV_{NP} FRs. A portion (80%, v/v) of the SDM DNA was used to transform competent *E. coli* cells (TG1). The resulting colonies were screened with [^{32}P]oligos coding for (mAb (40-40) or DX48) V_H MuCDR1 (Sambrook et al., 1989). The number of bacterial colonies positive for V_H MuCDR1 for mAb (40-40) or DX48 are shown in Table I. Plasmid DNA from 10 MuCDR1-positive colonies was prepared, and the V_H domains were sequenced (Sanger et al., 1977; the results are shown in Table I). PCR of the SDM DNA (20%, v/v) was done using primers designed to generate the V_H domains of the HumAb (40-40) or DX48. The PCR DNA was digested with *Pst*I + *Bst*EII and an aliquot (10%, v/v) was inserted between the corresponding restriction sites in the expression plasmid pVHD1.3-VKD1.3tag1 (provided by Dr. G. Winter, Cambridge, UK). The ligated

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*On request, the author will supply detailed experimental evidence for the conclusions reached in this Brief Note.

Abbreviations: CDR, complementarity-determinining region(s); Ab, antibody(ies); FR, framework region; Hu, human; HuV_{NP}, nitrophenylacetyl-specific V_H; Ig, immunoglobulin; mAb, monoclonal Ab; Mu, murine; oligo, oligodeoxyribonucleotide; PCR, polymerase chain reaction; ss, single strand(ed); SDM, site-directed mutagenesis (sed); V_H, heavy-chain variable domain.

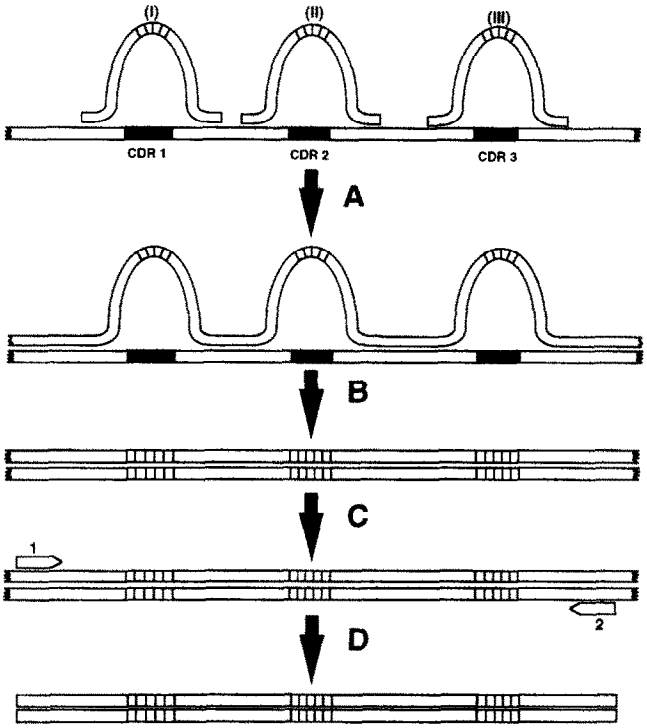

Fig. 1. The SDM/PCR humanization procedure. The oligos (I, II and III) coding for the MuCDRs and HuFRs were annealed to the HuV_{NP} ss DNA. **Step A**: second ss synthesis was done with phage T4 DNA polymerase using [α-^{35}S]dCTP in the presence of phage T4 DNA ligase to generate the mutant heteroduplex DNA. **Step B**: the first ss does not have the [α-^{35}S]dCTP and, therefore, can be nicked with *NciI* and digested with exonuclease III. The first ss was repolymerised with *E. coli* DNA polymerase I, and the two DNA ends were ligated with phage T4 DNA ligase. **Step C** depicts the annealing of the PCR primers 1 and 2, to the double-stranded DNA generated by the SDM reactions. In **Step D**, DNA suitable for cloning into a plasmid expression vector was produced by PCR.

DNA was transformed into *E. coli* DH5, and the resulting colonies were screened using [^{32}P]oligos coding for (mAb (40-40) or DX48) V_H MuCDR1. The number of MuCDR1-positive clones for mAb (40-40) or DX48 are shown in Table I. Plasmid DNA from ten MuCDR1-positive colonies was made, and the V_H domains were sequenced (see Table I). Most of SDM DNA (80%, v/v), but only a small portion of PCR DNA (2% (v/v) of the original SDM DNA) was used to transform *E. coli*. The number of V_H MuCDR1-positive colonies obtained for mAb (40-40) and DX48 is higher by the SDM/PCR protocol compared to SDM procedure alone. For example, using SDM to graft the three V_H MuCDRs of mAb (40-40) onto HuFRs, no MuCDR1-positive colonies were detected. However, using SDM/PCR, 30 MuCDR1-positive colonies were detected, and 10 had the correct V_H nt sequence. These data clearly demonstrates the advantage of PCR amplification of the low copy number of the SDM DNA coding for the humanised V_H. The data in Table I show that as the number of MuCDRs transferred in a single SDM or SDM/PCR increases (from 1 to 3) the number of colonies positive for mAb 40-40 or DX48 MuCDR1 with the correct nt sequence decreases.

TABLE I

E. coli colonies generated by the SDM and SDM/PCR humanization procedure

	Number of MuCDRs transferred onto HuFRs in each SDM[a]		
	1	1 and 2	1, 2 and 3
Heavy chain (40-40)[b]			
SDM	100 (80%)	50 (60%)	0 (0%)
SDM/PCR	210 (90%)	100 (70%)	30 (30%)
Heavy chain (DX48)[c]			
SDM	150 (70%)	65 (50%)	4 (25%)[d]
SDM/PCR	360 (70%)	190 (60%)	55 (30%)

Aliquot (80%, v/v) of the DNA from each SDM was used to transform *E. coli* TG1. The remaining 20% (v/v) of the SDM DNA was used for PCR. Aliquot (10%, v/v) of the PCR DNA was used for ligation and transformation of *E. coli* DH5. The number of colonies positive for mAb (40-40) or DX48 V_H MuCDR1 obtained by SDM and SDM/PCR are shown. The V_H domain of 10 muCDR1-positive colonies from each SDM and SDM/PCR was sequenced and the percentage of colonies with the correct DNA sequence are shown in bracket.

[a]In each SDM or SDM/PCR either MuCDRs 1, or 1, 2 or 1, 2, 3 were transferred onto HuFRs.

[b]Heavy chain of the antidigoxin mAb 40-40.

[c]Heavy chain of the antidigoxin mAb DX48.

[d]Plasmid DNA was available from only four colonies, and one colony had the correct V_H nt sequence.

After humanization of DX48 and (40-40) heavy chain by SDM and SDM/PCR, the V_H of MuCDR1-positive colonies was sequenced. The sequence data showed that for both SDM and SDM/PCR humanization protocols, the number of sequence errors in the humanized (40-40) and DX48 V_H DNA was similar. Therefore, the PCR step, does not significantly increase the number of sequence errors in the humanized V_H DNA.

REFERENCES

Hudson, N.W., Medgett-Hunter, M., Panka, D.J. and Margolies, M.N.: Immunoglobulin chain recombination among antibodies by hybridoma-hybridoma fusion. J. Immunol. 130 (1987) 2715–2723.

Lewis, A.P. and Crowe, J.S.: Immunoglobulin complementarity-determining region grafting by recombinant polymerase chain reaction to generate humanised monoclonal antibodies. Gene 101 (1991) 297–302.

Riechman, L., Clark, M., Waldmann, H. and Winter, G.: Reshaping human antibodies for therapy. Nature 332 (1988) 323–327.

Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Sanger, F., Nicklen, S. and Coulson, A.R.: DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74 (1977) 5463–5467.

Taylor, J.W., Ott, J. and Eckstein, F.: The rapid generation of oligonucleotide directed mutations at high frequency using phosphorothioate-modified DNA. Nucleic Acids Res. 13 (1985) 8764–8785.