J. Biochem. **144,** 115–120 (2008)
doi:10.1093/jb/mvn052

Humanization of an Anti-CD34 Monoclonal Antibody by Complementarity-determining Region Grafting Based on Computer-assisted Molecular Modelling

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Received January 25, 2008; accepted March 26, 2008; published online April 19, 2008

4C8 is a new mouse anti-human CD34 monoclonal antibody (mAb), which recognizes class II CD34 epitopes and can be used for clinical hematopoietic stem/progenitor cell selection. In an attempt to improve its safety profiles, we have developed a humanized antibody of 4C8 by complementarity-determining region (CDR) grafting method in this study. Using a molecular model of 4C8 built by computer-assisted homology modelling, framework region (FR) residues of potential importance to the antigen binding were identified. A humanized version of 4C8, denoted as h4C8, was generated by transferring these key murine FR residues onto a human antibody framework that was selected based on homology to the mouse antibody framework, together with the mouse CDR residues. The resultant humanized antibody was shown to possess antigen-binding affinity and specificity similar to that of the original murine antibody, suggesting that it might be an alternative to mouse anti-CD34 antibodies routinely used clinically.

Key words: CD34, hematopoietic stem cells, humanization, immunogenicity, monoclonal antibody.

Abbreviations: CDR, complementarity-determining region; FR, framework region; HAMA, human antimouse antibody; HSC, hematopoietic stem cell.

Transplantation of hematopoietic stem cells (HSCs) has been recognized as an effective treatment for hematologic malignancies, solid tumours and some non-malignant diseases (*1–3*). The CD34 molecule, which is expressed on all measurable hematopoietic stem and progenitor cells, has been the primary target used to isolate HSCs (*4–6*). Fluorescence-activated cell sorting and immunomagnetic selection systems utilize antibodies against CD34 antigen to enrich HSCs. The anti-CD34 monoclonal antibody (mAbs) have been categorized in three classes according to the differential sensitivity of their epitopes to enzymatic cleavage with neuraminidase, chymopapain and glycoprotease from *Pasteurella haemolytica* (*7*). Class I antibodies are directed against epitopes sensitive to all three enzymes. The epitopes detected by class II antibodies are sensitive to enzymatic cleavage with glycoprotease and chymopapain, and resistant to cleavage with neuraminidase. Class III antibodies recognize epitopes which are resistant to all three enzymes (*8*). Current selection methods usually utilize class I or class II antibodies, while class III antibodies are used for detection. In clinical transplantation, the QBEnd antibody, which belongs to class II mAbs, has been used most commonly to isolate HSCs (*9*).

Despite the widespread clinical use of CD34 antibodies for the purification of HSCs, all the current CD34 mAbs are murine antibodies, which have the potential to elicit human antimouse antibody (HAMA) immune response. An early attempt to reduce the immunogenicity is to generate chimeric antibodies which consist of murine antigen-binding variable regions fused genetically to human antibody constant regions (*10*, *11*). The clinical experiences demonstrated chimeric antibodies had a reduced immunogenicity in comparison with their mouse counterparts (*12*). However, chimeric antibodies could still evoke a strong HAMA response since murine variable regions were sufficient to trigger immune responses in humans (*13–16*). To further reduce the immunogenicity of chimeric antibodies, humanized antibodies have been generated by grafting the complementarity-determining regions (CDRs) of a murine antibody into the corresponding regions of a human antibody (*17*, *18*).

In our previous report, we generated three new mouse anti-human CD34 mAbs (5B12, 4C8 and 2E10), which, respectively, belonged to class I, class II and class III CD34 antibodies (*19*). Here we describe the humanization of the class II CD34 antibody 4C8. The humanized version of 4C8 shows a similar antigen-binding affinity and specificity compared with the parental mouse antibody, suggesting that it may have the potential to be used for clinical HSCs selection with better safety profiles.

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MATERIALS AND METHODS

Materials—Human acute myeloid leukaemia cell line, KG-1a, was obtained from ATCC (Manassas VA). This cell line stably expressed the CD34 antigen. c4C8 is the chimeric antibody derived from the mouse anti-CD34 antibody 4C8 (*19*). hu12F6 is an anti-human CD3 humanized antibody described previously (*20*).

Molecular Modelling of Variable Fragment (Fv) of 4C8—The Protein Data Bank (PDB) was searched for antibody sequences that had high sequence identity with 4C8 Fv. Two separate BLASTP searches were performed for light chain variable region (V_L) and heavy chain variable region (V_H) of 4C8. To construct the three-dimensional structure of the 4C8 Fv by homology modelling (INSIGHT II 2003, Accelrys, San Diego, CA), the sequences of V_L and V_H of 4C8 and their templates were aligned, respectively. The coordinates for the structurally conserved regions (SCRs) were assigned from the template and the loop regions were generated by Homology program. The new built structure was subjected to molecular dynamics simulations and then energy-minimized by 1000 steps of the steepest descent method and followed by conjugate gradient method using Discover program. Finally, the refined model was assessed by Profile-3D program.

Humanization of Anti-CD34 mAb 4C8—V_L and V_H of 4C8 were subjected separately to a BLASTP search against the entire non-redundant Genbank database (PDB, SwissProt, SPupdate, PIR). The human antibody variable region that was the most homologous in sequence to the 4C8 variable region was chosen as the human framework region (FR) for the humanized version of 4C8. The CDRs in humanized antibody were chosen to be identical to those in mouse 4C8 antibody. Because the transfer of mouse CDR residues alone into human FR usually results in a loss of antigen-binding activity, the key murine FR residues of potential importance to antigen binding must be substituted into the human acceptor framework to restore affinity. The molecular model of 4C8 Fv showed that some FR residues were close enough to the CDRs to either influence their conformations or interact directly with antigen. When these FR residues differed between 4C8 and its human framework, the residue in the humanized antibody was chosen to be the murine 4C8 residue rather than the human antibody residue. The light and heavy chain variable region genes of humanized versions of 4C8 were synthesized by overlapping PCR method. The PCR was performed with an initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 50 s, and then a final extension at 72°C for 10 min. The light and heavy chain expression vectors for humanized antibodies were constructed in an identical manner to the anti-CD34 chimeric antibody c4C8 as described in our previous report (*19*).

Antigen-binding Activity Assays—Appropriate light and heavy expression vectors for humanized antibodies were cotransfected into COS-7 cells using Lipofectamine 2000 reagent (Invitrogen, San Diego, CA) according to the manufacturer's instruction. After 48 h incubation, the supernatants were collected and analyzed by enzyme-linked immunosorbent assay (ELISA) for quantization of antibodies. The ELISA assay used goat anti-human IgG Fc (KPL, Gaithersburg, MD) as capture antibody and goat anti-human kappa horseradish peroxidase (HRP) (Southern Biotechnology Associates, Birmingham, AL) as detecting antibody. Flow cytometry analysis (FCM) was performed to determine the binding of humanized antibodies to KG-1a cells using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Briefly, KG-1a cells at 1×10^6 cells/ml were incubated with different dilutions of the culture supernatants containing humanized antibodies for 1 h at 4°C. The cells were washed and incubated with FITC-goat anti-human IgG (H + L) (Zymed, San Francisco, CA) for 1 h at 4°C. And then the cells were washed and analyzed by FCM.

Stable Expression and Purification of Humanized Aantibodies—Appropriate light and heavy expression vectors were cotransfected into Chinese hamster ovary (CHO)-K1 cells using Lipofectamine 2000 reagent. Stable transfectants were isolated using the same method as described previously (*19*). The culture supernatants from different transfectants were analyzed for antibody production by ELISA described above. The clones producing the highest amount of humanized antibodies were selected and grown in serum-free medium. The humanized antibodies were purified by Protein A affinity chromatography from the serum-free culture supernatant. Antibody concentration was determined by absorbance at 280 nm.

Competitive Binding Assay—KG-1a cells at 1×10^6 cells/ml were incubated with a subsaturating concentration of FITC-conjugated mouse anti-CD34 mAb 4C8 (FITC-4C8) and increasing concentrations of purified competing antibodies for 1 h at 4°C. The cells were washed and analyzed by FCM. The IC_{50} values of competitors were calculated using a four parameter algorithm.

RESULTS

Molecular Modelling of 4C8 Antibody—We searched the PDB for antibody sequences with high sequence identity with the 4C8 antibody variable regions. The crystallographic resolution and the source of Top 10 sequences for the V_H and the V_L of 4C8, respectively, were assessed (Table 1). The V_L and V_H of the Diels-Alder catalytic antibody 39-A11 (PDB No.1A4J), respectively, show 87% and 79% identity with the V_L and V_H of 4C8. The crystallographic resolution of Diels-Alder catalytic antibody 39-A11 is 2.1 Å and we selected it as the template of 4C8. Then the molecular model of the 4C8 Fv was obtained by Insight II molecular modelling software (Fig. 1).

Humanization of 4C8 Antibody—The V_H (GenBank No. EU330525) and V_L (GenBank No. EU330526) of the 4C8 antibody were, respectively subjected to BLASTP searches against the entire non-redundant Genbank database. The V_H of the human antibody AAC18206 (GenBank No. AAC18206) was 68% identical to the 4C8 heavy chain variable region and the V_L of human antibody BAC01734 (GenBank No. BAC01734) was 80% identical to the 4C8 light chain variable region. We selected the variable regions of these two human antibodies as frameworks for the V_H and V_L of humanized 4C8 antibody, respectively. Firstly, the three CDRs from

Table 1. **Ranking of the PDB antibody variable domain sequences with high sequence identity with 4C8.**

Ranking	PDB-code	Identity	Resolution (Å)	Source
Light chain				
1	2DQT	95	1.8	mouse
2	1KEL	94	1.9	mouse
3	1NQB	94	2.0	mouse
4	1JEL	93	2.5	mouse
5	2PCP	93	2.2	mouse
6	1FL6	93	2.8	mouse
7	2DDQ	93	2.35	mouse
8	1I9I	93	2.72	mouse
9	1CL7	92	3.0	mouse
10	1JP5	91	2.7	mouse
40	1A4J	87	2.1	mouse
Heavy chain				
1	1NCA	86	2.5	mouse
2	1IAI	84	2.9	mouse
3	1NCB	83	2.5	mouse
4	2ADF	83	1.9	mouse
5	2BRR	81	1.95	mouse
6	1A4J	79	2.1	mouse
7	1UWX	77	2.2	mouse
8	1DBA	77	2.8	synthesis
9	1N64	75	2.34	mouse
10	1MHH	75	2.1	mouse

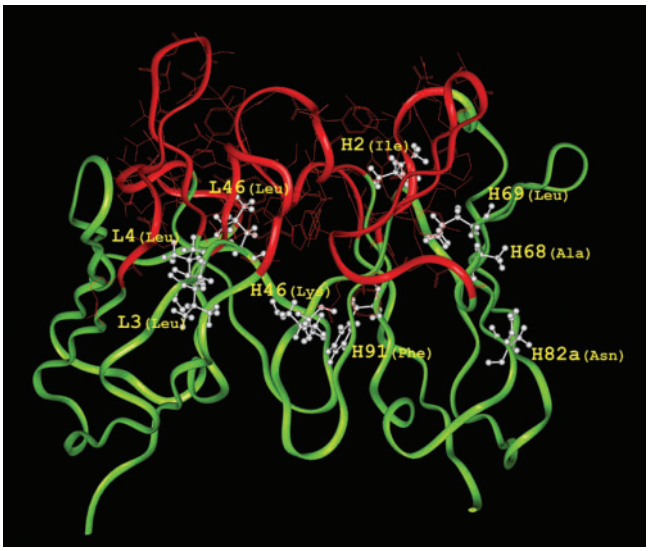

Fig. 1. **Molecular model of the 4C8 variable regions.** The 4C8 variable regions are shown by a solid ribbon representation. The FRs are shown in green and the CDRs are shown in red. Nine FR residues, L3, L4, L46, H2, H46, H68, H69, H82a and H91, which are within about 5 Å of the CDRs and are of potential importance to the antigen binding, are coloured white.

4C8 light chain or heavy chain were directly grafted into human antibody light chain or heavy chain frameworks to generate humanized antibody genes. The CDR-grafted antibody light chain (h4C8VLa) and heavy chain genes (h4C8VHa) were synthesized by overlapping PCR. A schematic diagram of the PCR strategy is shown in Fig. 2. The PCR primers for h4C8VLa and h4C8VHa

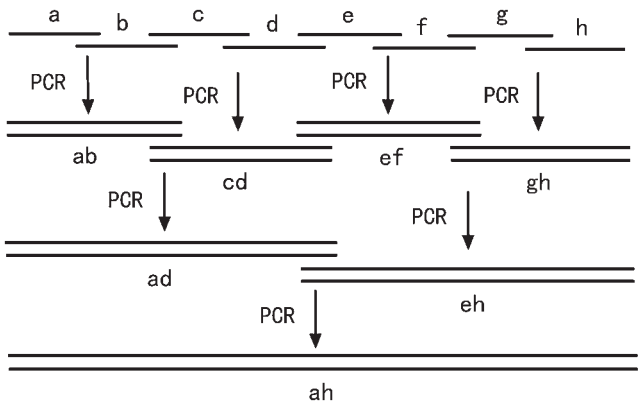

Fig. 2. **Schematic diagram of the PCR strategy.** Overlapping PCR was used to create genes encoding the heavy and light chain variable regions of the CDR-grafted antibody. Eight primers ranging from 70 to 75 bases were used for each heavy or light chain variable region.

were shown in Supplementary Table 1 and 2, respectively. The amino acid sequences of h4C8VHa and h4C8VLa were shown in Fig. 3. The resultant humanized V_H gene h4C8VHa was cloned into expression vector, and was co-expressed with 4C8 chimeric antibody light chain (c4C8VL) expression vector in COS cells, yielding the antibody h4C8VHa/c4C8VL. This antibody in COS cell culture supernatant was quantified by ELISA and the binding of h4C8VHa/c4C8VL to KG-1a cells was determined by FCM. The results indicated that this antibody had almost totally lost its antigen-binding activity (Fig. 4). This suggested that some key FR residues in the humanized version must be back-mutated to the corresponding murine residues in the mouse 4C8 antibody to reconstitute the antigen-binding affinity. When comparing the 4C8 antibody with its human templates, 12 different FR amino acids within 5 Å of the CDRs were found and collected as candidates to mutate back. Out of them, six V_H FR residues and three V_L FR residues were observed to probably affect the structure of the CDRs with the help of graphic manipulation (Fig. 1). A number of humanized light and heavy chain versions were generated to evaluate the contribution of each of the nine different FR residues to antigen-binding activity and their amino acid sequences were shown in Fig. 3. As shown in Fig. 4, installation of four murine FR residues into h4C8VHa (V68A, F69L, S82aN and Y91F) created humanized heavy chain h4C8VHc, which could bind to KG-1a with high affinity when combined with c4C8VL. However, h4C8VHc/c4C8VL showed much weaker antigen-binding activity than h4C8VHb/c4C8VL, suggesting that the murine V_H FR residues 2 and 46 also had important effects on antigen-binding activity. The humanized light chain versions were evaluated by combination with the humanized heavy chain h4C8VHb. As illustrated in Fig. 4, h4C8VHb/h4C8VLa was shown to be unable to bind to KG-1a cells, suggesting that some key V_L FR residues of h4C8VLa should be back-mutated. Our results indicated that each of the three murine V_L FR residues (3, 4 and 46) was of critical significance to antigen-binding (Fig. 4). Back-mutation of all of the three

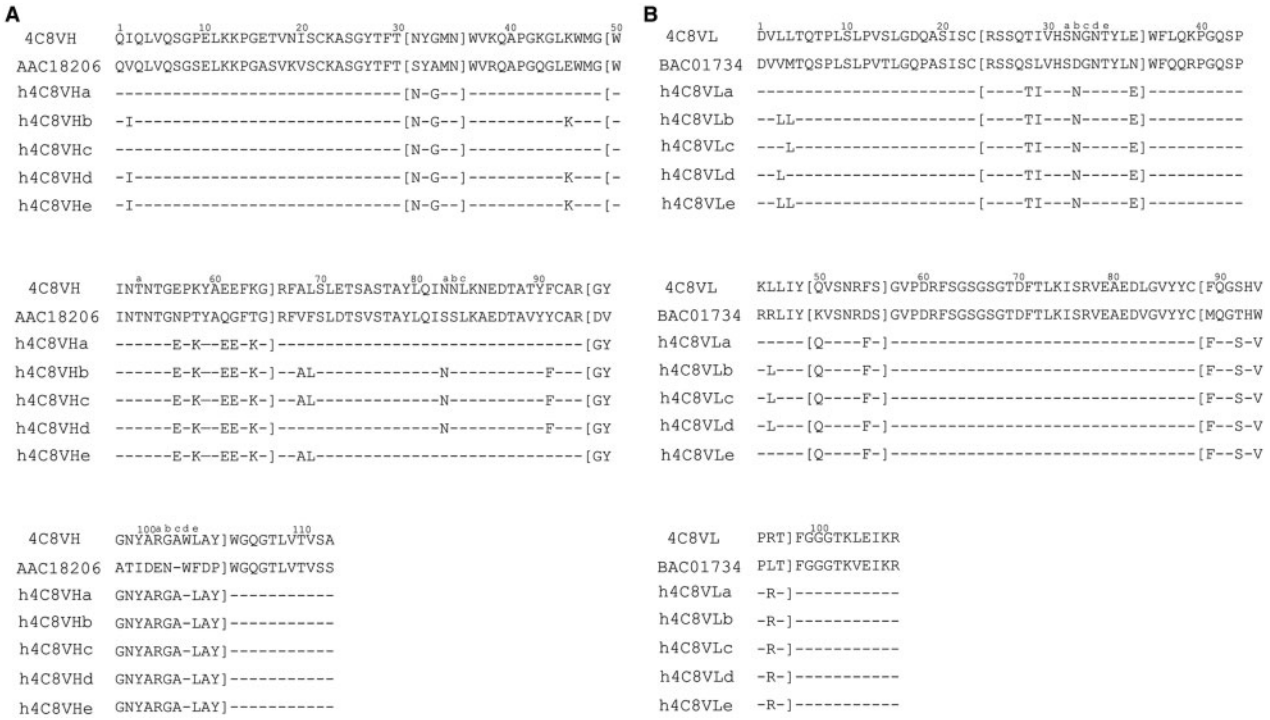

Fig. 3. **Amino acid sequences of humanized 4C8 heavy (A) and light (B) chain variable regions.** 4C8VH and 4C8VL, respectively, indicate heavy and light chain variable regions of murine 4C8 mAb. The V_H of the human antibody AAC18206 was chosen as framework for the humanized heavy chain and the V_L of human antibody BAC01734 was chosen for the humanized light chain. h4C8VHa, h4C8VHb, h4C8VHc, h4C8VHd and h4C8VHe indicate different versions of humanized heavy chain variable regions. h4C8VLa, h4C8VLb, h4C8VLc, h4C8VLd and h4C8VLe indicate different versions of humanized light chain variable regions. The dashes represent amino acids that are the same as the corresponding residues in human antibodies AAC18206 or BAC01734. The CDRs are enclosed with brackets. Amino acids (in one-letter notation) are numbered according to Kabat (*21*).

FR residues (V3L, M4L and R46L) generated the humanized light chain h4C8VLb, which showed the antigen-binding activity comparable to that of the anti-CD34 chimeric antibody c4C8 when combined with humanized heavy chain h4C8VHb (Fig. 4). This humanized anti-CD34 antibody (h4C8VHb/h4C8VLb) was designated as h4C8.

Competitive binding assay—The humanized antibody h4C8 was expressed in CHO cells and the CHO cell transfectants that stably produced antibodies were obtained by a positive selection procedure. Finally, h4C8 was purified from the CHO cell serum-free culture supernatant by Protein A affinity chromatography. In the competitive binding assay, h4C8 could effectively compete with 4C8 for binding to KG-1a cells (Fig. 5). The avidity (mean $IC_{50} \pm SD$) of h4C8 was about equal to that of 4C8 (Table 2), suggesting that this humanized antibody possessed affinity and specificity similar to that of the original murine antibody.

DISCUSSION

To reduce the immunogenicity of murine mAbs, humanized antibodies have been developed by grafting the CDRs of a mouse antibody into the corresponding regions of a human antibody. However, the transfer of murine CDRs alone usually results in a significant loss of antigen binding, because certain FRs are critical for preserving the CDR conformations or are directly involved in antigen binding. In most cases, the successful design of humanized antibodies requires that these key murine FRs be reintroduced into the human framework to restore affinity (*20*, *22–27*). Computer-assisted molecular modelling of the antibody can be used to identify such structurally important framework residues. With the aid of computer modelling, some groups have successfully developed various high-affinity humanized antibodies (*28–32*).

In the present study, a reliable three-dimensional model of the variable regions of the mouse anti-CD34 antibody 4C8 has been built using computer-aided homology modelling. Nine framework amino acids within about 5 Å of the CDRs of 4C8 were identified as key FR residues, which intimately interacted with CDR residues and might affect the structure of the antigen-binding site. Then we generated a humanized version of 4C8 by transferring these mouse key framework residues onto a human framework that was selected based on homology to the mouse framework, together with the mouse CDR residues. Antigen-binding assay showed that the resultant humanized anti-CD34 antibody (h4C8) completely restored the binding avidity of its murine counterpart. The relative affinity difference between the humanized antibody and the parental mouse antibody can be assessed by comparing the IC_{50} values of the two antibodies in the competitive binding assay (*16*). In this study, h4C8 was shown to have

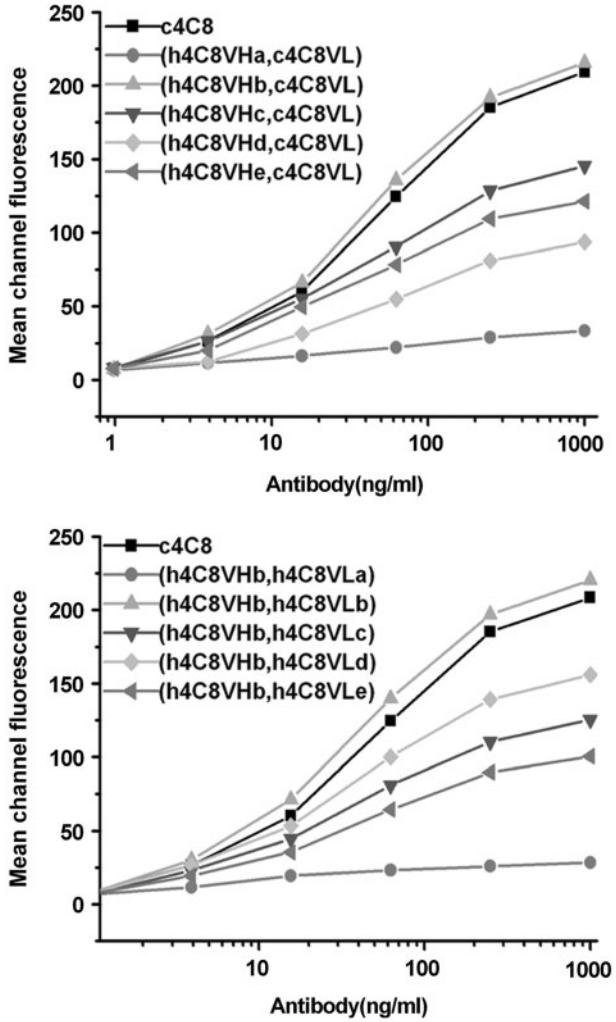

Fig. 4. **Antigen binding assays for 4C8 humanized antibodies.** Human KG-1a cells were incubated with serial log dilutions of c4C8 or different humanized versions for 1 h at 4°C. Cells were washed and incubated with FITC-goat anti-human IgG (H+L) for 1 h at 4°C. Cells were then washed and analysed by FCM. All data were expressed as the mean of triplicate samples.

similar IC_{50} values as compared to 4C8, suggesting that the relative affinities of the two antibodies were about equal. Back-mutation of a large number of murine residues may increase the immunogenicity of the humanized antibody. In this study, only nine mouse framework amino acids were back-mutated to generate the high-affinity humanized antibody. Previous clinical studies have indicated that humanized antibodies, which retained the similar number of murine framework residues as did h4C8, are in general much less immunogenic than mouse or chimeric antibodies, and are safe and well tolerated in humans (*16*).

In summary, our results have indicated that h4C8 possesses antigen-binding affinity and specificity comparable to that of the original mouse antibody. Due to the expected better safety profiles, h4C8 has the potential to be an alternative to mouse anti-CD34 antibodies routinely used for HSCs selection.

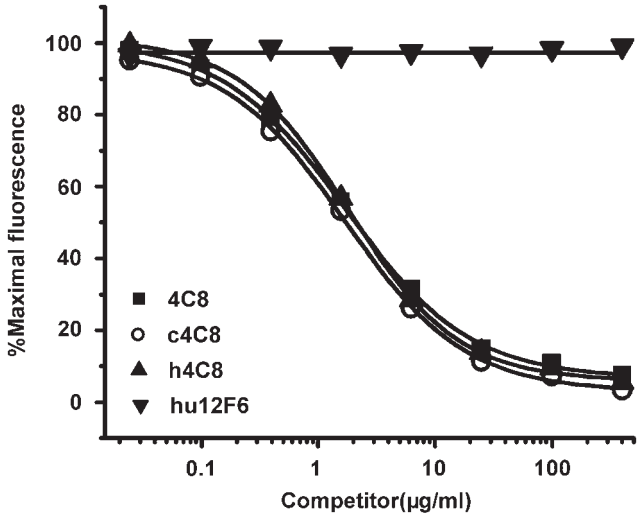

Fig. 5. **Binding of 4C8-FITC to human KG-1a cells in the presence of increasing concentrations of 4C8, c4C8 or h4C8.** KG-1a cells were incubated with a subsaturating concentration of 4C8-FITC and increasing concentrations of competing antibodies for 1 h at 4°C. The cells were then washed and analyzed by FCM. Maximal fluorescence means the mean channel fluorescence obtained in the absence of competitor antibodies. hu12F6 is a humanized anti-CD3 mAb that does not compete with anti-CD34 mAbs. All data were expressed as the mean of triplicate samples.

Table 2. **Competitive binding of 4C8 Abs to KG-1a cells.**

Types of antibodies	IC_{50} (μg/ml)[a]	SD	*n*
4C8	1.723	0.145	3
c4C8	1.692	0.137	3
h4C8	1.764	0.158	3

[a]Student's unpaired *t*-test shows that the IC_{50} value of h4C8 is not significantly different from that of 4C8.

Supplementary data are available at *JB* online.

This work was supported by grants from National Natural Science Foundation of China, Ministry of Science & technology of China (973 & 863 program projects), and Shanghai Commission of Science & Technology. The authors thank Ms. Yang Yang and Ms. Jing Xu for their technical assistance.

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