Humanization and Pharmacokinetics of a Monoclonal Antibody with Specificity for Both E- and P-Selectin

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E- and P-selectin (CD62E and CD62P) are cell adhesion molecules that mediate leukocyte-endothelial cell and leukocyte-platelet interactions and are involved in leukocyte recruitment during inflammation. We previously developed a murine mAb, EP-5C7 (or mEP-5C7), that binds and blocks both E- and P-selectin. When used in humans, murine mAbs have short circulating half-lives and generally induce potent human anti-mouse Ab responses. We therefore engineered a humanized, complementarity determining region-grafted version of mEP-5C7 incorporating human γ4 heavy and κ light chain constant regions (HuEP5C7.g4). HuEP5C7.g4 retains the specificity and avidity of mEP-5C7, binding to human E- and P-selectin but not to human L-selectin, and blocking E- and P-selectin-mediated adhesion. Surprisingly, when administered to rhesus monkeys, HuEP5C7.g4 was eliminated from the circulation very rapidly, even faster than the original murine Ab. To isolate the cause of the short serum half-life of HuEP5C7.g4, several Ab variants were constructed. A chimeric IgG4 Ab was made by replacing the humanized V regions with murine V regions. A humanized IgG2 Ab, HuEP5C7.g2, was also made by replacing the human γ4 with a γ2 constant region. Results from pharmacokinetic studies in rhesus monkeys demonstrated that the chimeric IgG4 is also rapidly eliminated rapidly from serum, similar to the humanized IgG4 Ab, while the humanized IgG2 Ab displays a long circulation half-life, typical of human Abs. ***The Journal of Immunology,*** **1998, 160: 1029–1035.**

The selectin family consists of the L-, E-, and P-selectins expressed on leukocytes, activated endothelial cells, and activated platelets. The three selectins act in concert with other cell adhesion molecules to permit recruitment of leukocytes from the blood into inflamed tissues (1, 2). Current models suggest that the selectins mediate initial adhesive rolling of leukocytes on the endothelial vessel wall, a process followed by firm attachment and transmigration of leukocytes, which are mediated by other adhesion molecules.

Abs that block E- or P-selectin inhibit the inflammatory sequelae in several animal models of inflammation, including leukocyte-mediated tissue damage due to ischemia/reperfusion injury (3–9). Results from studies in which Abs to E- and P-selectin are used in combination and related studies in knockout mice have suggested that E- and P-selectin can work cooperatively together in some inflammatory conditions, and the absence of one may be compensated by the other (10–13). For therapeutic treatment of these inflammatory conditions, blockade of both selectins is preferred and could be provided by a cross-reactive mAb, such as mEP-5C7, that binds and blocks both E- and P-selectin (14).

Direct use of murine mAbs for human therapy is limited by the development of an immune response by the recipient patient against mouse-specific antigenic determinants, i.e., human anti-mouse Ab responses, as well as the presence of unwanted or the lack of desired human effector functions on mouse Abs. The human anti-mouse Ab response can result in potentially harmful reactions and the rapid clearance of circulating Ab (15–17). To reduce the immunogenicity of mouse Abs for human therapy, chimeric Abs were initially developed. Chimeric Abs retain the mouse V regions, and hence the specificity, of the parental rodent Abs, but replace the constant regions with the appropriate human heavy and light chain sequences. The use of chimeric Abs clearly addresses the issue of immunogenicity of the constant region caused by species differences, but V region differences remain a potential source of immunogenicity (18, 19).

Humanized Abs are generated by grafting complementarity-determining regions (CDRs)[2] of mouse Abs into a background of human framework regions and constant regions by recombinant DNA technology (20). Humanized Abs contain only ~10% mouse sequences and 90% human sequences, and thus further reduce or eliminate the immunogenicity while retaining the Ab specificities (21, 22). Using a computer-aided CDR-grafting method, we have successfully humanized several mouse Abs for potential treatment of various human diseases (23–27). Several humanized Abs, including humanized M195 (anti-CD33, a myeloid leukemia surface Ag) and humanized anti-Tac (anti-IL-2 receptor α subunit), have been evaluated in human clinical trials, demonstrating long half-lives and no evidence of immunogenicity when administered in multiple doses (24, 28). In this report we describe an unexpected outcome of the humanization of mEP-5C7; when injected into rhesus monkeys, the humanized Ab, HuEP5C7.g4, displayed a very short circulating half-life. A greatly lengthened circulating half-life was obtained, however, by replacement of the human IgG4 heavy chain constant region with a human IgG2 constant region.

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[2] Abbreviations used in this paper: CDR, complementarity-determining region; $AUC_{0\text{-}t_{last}}$, area under the concentration-time curve; HRP, horseradish peroxidase.

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Materials and Methods

Abs and cell lines

Mouse monoclonal anti-human E/P-selectin EP-5C7, anti-human P-selectin WAPS 12.2, anti-human E-selectin E-1E4, and anti-human CD18 NA8 Abs have been described previously (14). Mouse (IgG1 κ) myeloma MOPC 21 and purified human (IgG4 κ) were obtained from Sigma Chemical Co. (St. Louis, MO). SP2/0-Ag14, a mouse myeloma cell line, and HL60, a human promyelocytic cell line, were obtained from American Type Culture Collection (CRL 1581, and CCL 240, Rockville, MD). A mouse pre-B cell line, L1-2, transfected with human P-selectin, L1-2$^{\text{P-selectin}}$, and Chinese hamster ovary (CHO) cell lines transfected with human E- or P-selectin gene (CHO$^{\text{E-selectin}}$ or CHO$^{\text{P-selectin}}$ cells) were described previously (14). HuDREG-200, is a humanized IgG4 Ab (29), and binds human, but not rhesus, L-selectin (E. Berg, unpublished observations). D200Id and 2H9 are mouse monoclonal anti-Id Abs generated against HuDREG-200 and HuEP5C7.g4, respectively. The hybridoma producing 2H9 was generated by injecting BALB/c mice in the hind footpads with 10 μg of purified HuEP5C7.g4 prepared in monophosphoryl lipid A plus trehalose dicorynomycolate adjuvant (Sigma Chemical Co.), then 4 and 7 days later with Ab in PBS. Three days following the last immunization, fusion with P3X cells was performed, generally as previously described (14). Hybridoma supernatants were screened by ELISA on plates coated with HuEP5C7.g4 in the presence of 10% normal human serum (Sigma Chemical Co.). 2H9 binds HuEP5C7.g4 and mEP-5C7, but fails to bind a panel of other humanized Abs or purified human IgG4.

Cloning of V region cDNAs

The V domain cDNAs of the light and heavy chains of mEP-5C7 were cloned by the anchored PCR method using 3′ primers that hybridize with the C regions and 5′ primers that hybridize with the G tails attached to the cDNA using terminal deoxytransferase (30). The sequences were determined using the dideoxy termination method with an Applied Biosystems 373A automated sequencer (Foster City, CA).

Computer analysis

Three-dimensional models of the variable domains of the mEP-5C7 Ab were built with the help of the ABMOD and ENCAD programs created by Levitt and co-workers (31, 32). Sequence analysis, homology searches, and structural analysis of three-dimensional models were conducted with the program DELTA developed in our laboratory (33).

Construction of chimeric and humanized Abs

See Figure 3 for a description of the Abs constructed. Plasmids pVκ, containing the human Ck region (25, 34), and pVg4, containing the human γ4 constant region, were used for the construction of chimeric and humanized versions of mEP-5C7 of the human IgG4 isotype. pVg4 is identical with pVg1 except for containing heavy chain sequences from human γ4 instead of γ1 (25). The *Xba*I/*Bam*HI fragment containing the human γ4 constant region in the pVg4 expression vector was derived from phage provided by L. Hood (35). For the construction of humanized HuEP5C7.g2, the plasmid pVg4 was replaced by pVg2.M3.D.Tt (36). pVg2.M3.D.Tt incorporates two mutations into a human γ2 constant region (with Ala replacing Val and Gly at positions 234 and 237, using the EU numbering system) to reduce interaction of IgG2 with Fc receptors and minimize Ab effector activity. Heavy and light chain expression plasmids were constructed by inserting the appropriate V region genes into the *Xba*I site of the respective vectors.

For the construction of chimeric Ab expression vectors, *Xba*I fragments containing the murine V_L and V_H genes (including the 5′ signal peptide sequence and the 3′ splice donor signal) were constructed by PCR from their respective murine V region cDNAs and then inserted into the *Xba*I site of the respective pVκ and pVg4 vectors. For the construction of humanized Ab expression vectors, *Xba*I fragments containing the humanized V_L and V_H genes (including the 5′ signal peptide sequence and the 3′ splice donor signal) were constructed from assembling eight synthetic oligonucleotides.

Nucleotide sequences were designed to encode the protein sequences of the humanized EP5C7 V_L and V_H, generally using codons found in the mouse sequence. Several degenerate codons were changed to create convenient restriction sites or remove undesired ones. To synthesize each V gene, four pairs of oligonucleotides (~80 bases in length) were synthesized (380B DNA Synthesizer, Applied Biosystems) with overlapping stretches of 20 nucleotides. Assembly and amplification of the genes were conducted in four steps: 1) the four pairs of complementary oligonucleotides were annealed and extended with Klenow fragment in separate reactions; 2) the resulting four dsDNA fragments were mixed pairwise, denatured, reannealed, and extended in two separate reactions; 3) the resulting two dsDNA fragments were mixed, denatured, reannealed, and extended to create the final full-length dsDNA; and 4) the resulting DNA was used for PCR amplification with two short primers, which correspond to the 5′ and 3′ ends of the template DNA and contain an *Xba*I site for subsequent cloning.

The synthesized *Xba*I fragments were then inserted into the *Xba*I site of the respective pVκ, pVg4, or pVg2.M3.D.Tt vectors. Hybrid Abs containing a humanized γ4 heavy chain and chimeric light chain (HuH/ChL) or a chimeric γ4 heavy chain and a humanized light chain (ChH/HuL) were also constructed for evaluation.

Production and purification of humanized and chimeric Abs

Cells producing humanized Abs (HuEP5C7.g4 or HuEP5C7.g2), chimeric Abs (ChEP5C7.g4), or hybrid Abs (Hybrid HuL/ChH, or Hybrid HuH/ChL) were generated by transfection of Sp2/0 cells with the appropriate plasmids by electroporation using a Gene Pulser apparatus (Bio-Rad, Hercules, CA) at 360 V and 25 μF capacitance according to the manufacturer's instructions. Before transfections, plasmids were linearized using *Pvu*II. All transfections were performed using 20 μg of plasmid DNA and 10^7 cells in PBS. The cells from each transfection were plated into four 96-well tissue culture plates. After 48 h, selective medium was applied. Cells were selected in DMEM/10% FBS/hypoxanthine/thymidine media supplement (Sigma Chemical Co.) and 1 μg/ml mycophenolic acid. Ab-producing clones were screened by assaying for the presence of Ab in the culture supernatant by ELISA. Abs from transfectants as well as hybridomas were purified from serum-free culture supernatants (Hybridoma-SFM, Life Technologies, Gaithersburg, MD) by passage over a column of staphylococcal protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ). The bound Abs were eluted with 0.2 M glycine-HCl, pH 3.0, and neutralized with 1 M Tris-HCl, pH 8.0. The buffer was exchanged for PBS by passing over a PD10 column (Pharmacia).

Avidity measurement

Binding avidities of mEP-5C7 and HuEP5C7.g4 with E- and P-selectin were determined by competitive binding of radiolabeled Ab to CHO$^{\text{E-selectin}}$ and L1-2$^{\text{P-selectin}}$ cells, respectively. Purified mEP-5C7 Ab was labeled with $Na^{125}I$ (Amersham, Arlington Heights, IL) using lactoperoxidase at 4 mCi/mg of protein (37). Increasing amounts of cold competitor Ab (mEP-5C7 or HuEP5C7.g4) were added to 2 ng of radiolabeled tracer mEP-5C7 Ab and incubated with 4×10^5 CHO$^{\text{E-selectin}}$ or L1-2$^{\text{P-selectin}}$ cells in 0.2 ml of binding buffer (PBS with 2% FCS and 0.1% sodium azide) for 2 h at 4°C with constant shaking. Cells were washed and pelleted, and the radioactivity associated with the cell pellets was measured. The ratio of bound and and free tracer Ab was calculated, and the avidities were calculated according to the formula: $[X] - [EP5C7] = (1/K_x) - (1/K_a)$, where K_a is the avidity of EP5C7, K_x is the avidity of the competitor X, brackets indicate the concentration of competitor Ab at which bound/free tracer binding is $R_0/2$, and R_0 is maximal bound/free tracer binding (38).

E- and P-selectin adhesion assays

HL-60 cell binding to CHO$^{\text{E-selectin}}$ or CHO$^{\text{P-selectin}}$ transfectant cell lines was performed as described previously (14). Confluent cultures of CHO$^{\text{E-selectin}}$ or CHO$^{\text{P-selectin}}$ transfectant cells, grown in 96-well plates, were washed and incubated for 15 min with 50 μl of assay buffer (10% adult bovine serum/10% normal rabbit serum/10 mM HEPES (pH 7.2)/RPMI) containing various test and control Abs (Sigma Chemical Co.) at 10 μg/ml. Fluorescently labeled HL-60 cells were prepared as previously described (14) and resuspended in assay buffer containing 0.25 μg/ml anti-CD18 Ab, NA8, at 2×10^6 cells/ml. Assays were initiated by addition of 50 μl of HL-60 cells to CHO$^{\text{E-selectin}}$ or CHO$^{\text{P-selectin}}$ cells for a final volume of 0.1 ml while plates were rotated at 40 rpm (Innova 200 orbital shaker, New Brunswick, Inc., Edison, NJ). After 15 min at room temperature, unbound cells were removed by washing plates four times with 0.2 ml RPMI/well. Bound cells were fixed to the CHO$^{\text{E-selectin}}$ or CHO$^{\text{P-selectin}}$ cells by addition of 100 μl 1% paraformaldehyde (Sigma) in PBS. Plates were analyzed using a Microplate Fluorometer (model 7620, Cambridge Technology, Inc., Watertown, MA), and the relative number of cells bound per well was calculated from the total amount of fluorescence measured at 530 nm, using an excitation at 485 nm.

Pharmacokinetics of murine and humanized Abs in rhesus monkeys

Pharmacokinetic studies were performed at the California Primate Research Center (Davis, CA). Rhesus monkeys (3–5 kg; three per group) were injected with 2 mg/kg of the indicated Abs. Serum samples were collected relative to Ab injection at −7 days, 0 (predose), 0.5, 1.5, 2, 6, 12,

and 18 h and 1, 2, 3, 4, and 7 days. Samples from some animals were also obtained on days 14, 15, and/or 21.

Pharmacokinetic analysis

Compartment-independent pharmacokinetic analysis (39) was performed using the KaleidaGraph program for Macintosh (Developed by Abeldeck Software, distributed by Synergy Software, Reading, PA). The area under the concentration-time curve, $AUC_{0\text{-}t_{last}}$, was calculated by the trapezoidal rule. $AUC_{t_{last}\text{-}\infty}$ was determined by extrapolating to infinity from $C_{t_{last}}$ assuming exponential decay using the equation $AUC_{t_{last}\text{-}\infty} = C_{t_{last}}/k2$, where $C_{t_{last}}$ is the concentration at the last measured time point, and k_2 is the elimination rate constant of the terminal portion of the concentration-time curve. $AUC_{0\text{-}\infty}$ was derived from $AUC_{0\text{-}t_{last}} + AUC_{t_{last}\text{-}\infty}$. The parameter k_2 was estimated by linear regression analysis of the terminal portion of the curve from a minimum of four last data points. The elimination half-life, $t_{1/2}$, was derived from $0.693/k_2$.

ELISAs

Microtiter plates (Nunc Immunolon 1) were coated overnight with 100 μl/well of capture Abs. These included polyclonal anti-mouse IgG (H+L) (human Ig absorbed, Biosource, Camarillo, CA), diluted 1/200, or anti-Id Abs against EP5C7 (2H9) or HuDREG-200 (D200Id) at 2 μg/ml in PBS. Plates were then blocked by incubating wells with 0.5% nonfat dry milk/PBS for 1 to 2 h and subsequently washed twice in 0.05% Tween-20/PBS. Serum samples diluted appropriately (1/10 to 1/2000) or standards diluted and prepared in 0.5% milk/PBS were added to wells, and plates were incubated overnight at 4°C. Following four washes in 0.05% Tween-20/PBS, secondary Abs were applied. These included horseradish peroxidase (HRP)-conjugated Fc-specific anti-mouse Ig (The Jackson Laboratory, Bar Harbor, ME) at a 1/4000 dilution for evaluation of mEP-5C7, HRP-conjugated anti-human IgG4 (The Binding Site, Birmingham, U.K.) at a 1/1000 dilution for evaluation of HuEP5C7.g4, and chimeric and hybrid IgG4 Abs and HRP-conjugated anti-human IgG2 (The Binding Site) at a 1/400 dilution for evaluation of HuEP5C7.g2. After 1 h, plates were again washed in 0.05% Tween-20/PBS. Bound Abs were detected by addition of 100 μl/well TMB substrate (Kirkegaard and Perry, Gaithersburg, MD), color development for 10 min, termination of the reaction by addition of 100 μl/well 2 M H_2SO_4, and then measurement of absorbance at 450 nm.

Results

Cloning and sequencing of V region cDNA

The murine EP-5C7 Ab heavy and light chain V region cDNAs were amplified using an anchored PCR method (30), then cloned into pUC18 for sequence determination. Nucleotide sequences were obtained from several independent clones for both V_L and V_H cDNAs. For the heavy chain, a unique sequence was identified. For the light chain, two sequences were identified. One sequence had a nucleotide missing at the end of the V gene, causing a frame shift at the V-J junction, and was identified as the nonproductive allele from the fusion partner cells. The other sequence is typical of a mouse κ chain V region. The nucleotide and translated amino acid sequences of the coding light and heavy chain V regions of mEP-5C7 are shown in Figure 1. Sequence analysis indicates that the light chain V region gene of mEP-5C7 belongs to mouse κ-chain subgroup I, and the heavy chain gene belongs to mouse heavy chain subgroup III (40).

Modeling and design of humanized sequences

For humanization, the approach of Queen et al. (23) was followed. First, sequences of human V regions most similar to mEP-5C7 were identified. Among the best V_H sequences is III-3R (41) of Kabat's subgroup III, with 72% identity in the framework region. The V_L from III-3R was also used. This V_L belongs to the Kabat subgroup I of κ-chains and has 61% identity with mEP-5C7 in the framework region.

With the help of the three-dimensional model, a number of framework positions were identified that differed between mEP-5C7 and the chosen human III-3R sequence and whose location in three-dimensional space relative to the hypervariable regions, or CDRs, makes it likely that they could influence CDR conformation, and thus binding affinity. Amino acids from the murine EP5C7 sequence were used in those positions. This was performed at residues 69 and 70 in the V_L domain, but was not required at any residues in the V_H domain. Furthermore, in agreement with the protocol outlined previously (23), a number of amino acids in the III-3R human sequences that were unusual at their positions for their respective (human) subgroups were also identified. Those amino acids were changed to correspond to consensus human sequences of human subgroup III (V_H) or subgroup I (V_L). This was performed at residues 61, 72, 82, and 99 of V_L and at residues 1, 75, and 78 of V_H. The final amino acid sequences of the humanized EP-5C7 heavy and light chain V regions are shown compared with the murine sequences in Figure 2.

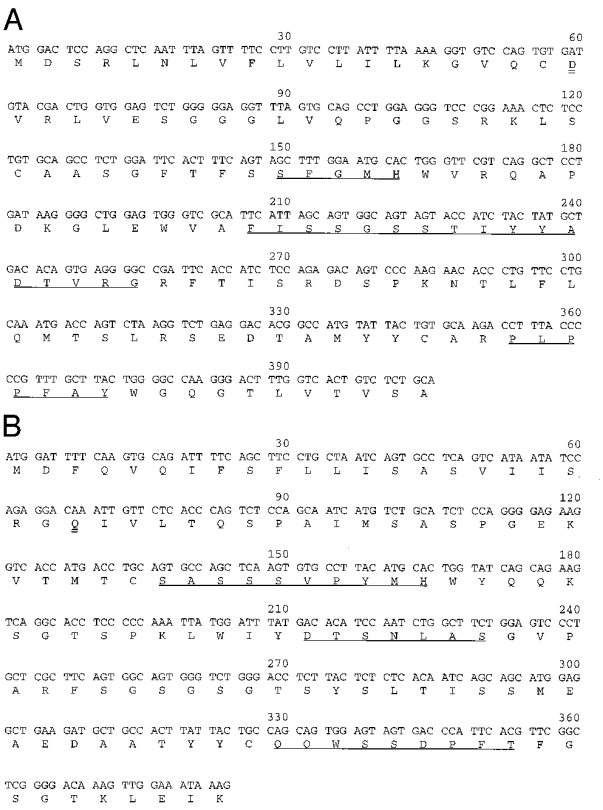

FIGURE 1. cDNA and translated amino acid sequences of the heavy chain (*A*) and light chain (*B*) V regions of the murine EP-5C7 Ab. The three CDRs in each chain are underlined. The first amino acid of the mature light and heavy chains are double underlined, preceded by their respective signal peptide sequences.

Avidity measurement

Recombinant Abs containing mouse or humanized V regions and mouse or human constant regions, as shown in Figure 3, were produced as described in *Materials and Methods*. The avidities of the humanized Ab, HuEP5C7.g4, for E-selectin and P-selectin were determined and compared with those of mEP-5C7 by competition with the radioiodinated mEP-5C7 Ab. $CHO^{E\text{-selectin}}$ cells and $L1\text{-}2^{P\text{-selectin}}$ cells (14) were used as sources of E-selectin and P-selectin. The binding avidities were calculated as described in *Materials and Methods*. HuEP5C7.g4 had an avidity of 3.3×10^8 M^{-1} for E-selectin, identical with that measured for mEP-5C7, while for P-selectin, HuEP5C7.g4 had an avidity of 1.5×10^8 M^{-1}, and mEP-5C7 had an avidity of 6.7×10^8 M^{-1}.

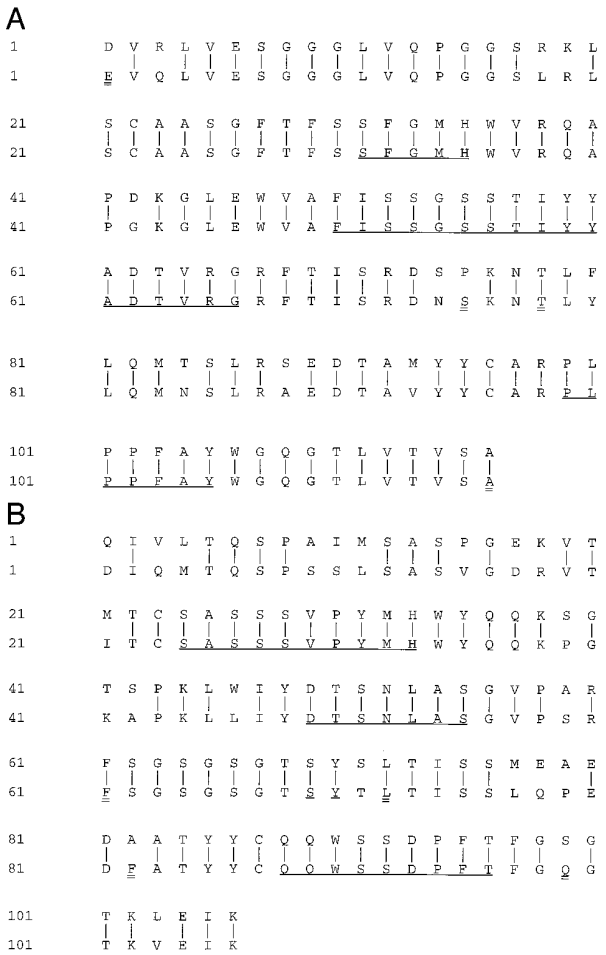

FIGURE 2. Amino acid sequences of the mature heavy chain (*A*) and light chain (*B*) V regions of the humanized EP-5C7 (lower lines) and the mouse EP-5C7 (upper lines) Abs. The three CDRs in each chain are underlined once. Residues in the framework that have been replaced with mouse amino acids are also underlined once, and consensus human amino acids, different from those in III-3R, in the humanized Ab are doubly underlined.

Name (Heavy Chain Isotype)	Variable Region Heavy Chain	Variable Region Light Chain	Structure
HuEP5C7.g4 (human IgG4)	humanized	humanized	
mEP-5C7 (murine IgG1)	murine	murine	
HuEP5C7.g2 (human IgG2)	humanized	humanized	
ChEP5C7.g4 (human IgG4)	murine	murine	
Hybrid HuL/ChH (human IgG4)	murine	humanized	
Hubrid HuH/ChL (human IgG4)	humanized	murine	

FIGURE 3. Humanized and murine Abs. The species, isotype, and type of V region heavy and light chains for each Ab are listed. A representational figure for each Ab is also shown. Regions containing murine sequences are shown in black, human κ light chain constant regions (C_κ) are gray with black outlines, humanized V_L and V_H are white, human IgG4 heavy chains are horizontally striped, and human IgG2 heavy chains are gray.

Blocking of E-selectin- and P-selectin-mediated adhesion

To compare the abilities of HuEP5C7.g4 and HuEP5C7.g2, the humanized EP5C7 Ab of IgG2 isotype, with that of mEP-5C7 to inhibit binding of E-selectin to its counter-receptor, these Abs were tested for their ability to block binding of HL-60 cells to E-selectin transfectant cells. Assays of the adhesion of HL-60 cells with $CHO^{E\text{-selectin}}$ cells were performed as previously described (14) in the presence of the various Abs at the indicated concentrations. Figure 4 (*A* and *B*) shows that both HuEP5C7.g2 and HuEP5C7.g4 blocked binding of HL-60 cells to $CHO^{E\text{-selectin}}$ transfectants as well as or slightly better than mEP-5C7.

To measure the abilities of HuEP-5C7.g2, HuEP5C7.g4, and mEP-5C7 to inhibit binding of P-selectin to its counter-receptor, the binding of HL-60 cells to $CHO^{P\text{-selectin}}$ transfectants in the presence of these Abs was determined. Figure 4 (*C* and *D*) shows that HuEP5C7.g2 and HuEP5C7.g4 block binding of HL-60 cells to P-selectin transfectants as well as mEP-5C7. Isotype-matched control Abs had no effect on binding in this assay (14) (data not shown).

Pharmacokinetics in rhesus monkeys

The elimination profiles for 1 wk following injection in rhesus monkeys of mEP-5C7, HuEP5C7.g4, HuEP5C7.g2, chimeric or hybrid IgG4 Abs, or a control humanized IgG4 Ab, HuDREG-200, that does not bind to any Ag in rhesus monkeys are shown in Figure 5. HuEP5C7.g4 and chimeric and hybrid IgG4 versions of EP5C7 all cleared rapidly from the circulation, so their serum levels were <1 μg/ml after 72 h, while HuEP5C7.g2 displayed a long serum half-life, with a level of >10 μg/ml at 72 h. The calculated terminal elimination half-lives calculated from the last several time points and averaged for three rhesus monkeys are shown in Table I. The terminal elimination half-life calculated for HuEP5C7.g2 (6.6 ± 0.8 days) was similar to that for the control humanized IgG4 Ab, HuDREG-200 (6.5 ± 1.2 days). However, while the half-life calculated for mEP-5C7 was as short as the half-lives calculated for the IgG4 versions of EP5C7 (2.5 ± 0.7 vs 2.2–2.9 days), inspection of Figure 5 shows that mEP-5C7 behaves more like HuEP5C7.g2 and the control humanized IgG4, HuDREG-200, up until day 4. The 2-wk elimination profiles for each of three individual rhesus monkeys given HuEP5C7.g2 at 2 mg/kg are shown in Figure 6.

Discussion

We have previously described the identification of a mAb that binds and blocks the adhesive functions of both E- and P-selectin (14). Blockade of these receptors may have clinical utility in situations of leukocyte-mediated tissue damage, such as ischemia/

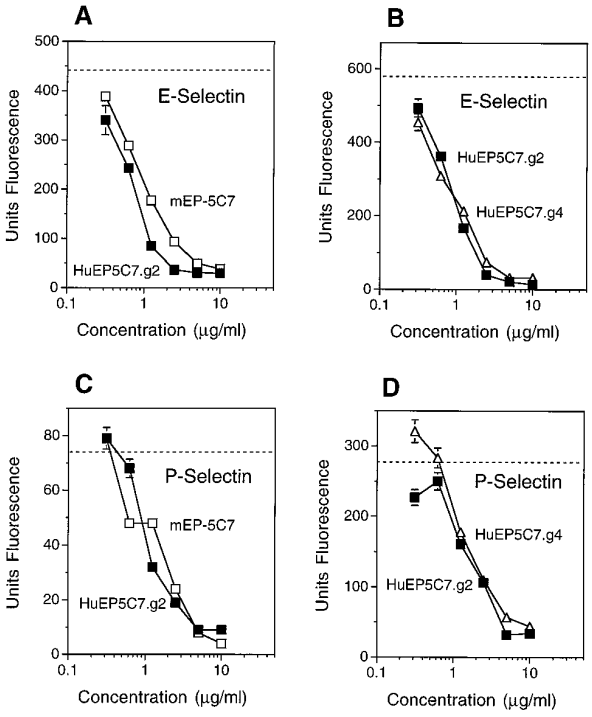

FIGURE 4. Inhibition of HL-60 cell adhesion to $CHO^{E\text{-selectin}}$ or $CHO^{P\text{-selectin}}$ cells by mEP-5C7 (□), HuEP5C7.g2 (■), or HuEP5C7.g4 (△) Abs. Fluorescently labeled HL-60 cells were incubated with $CHO^{E\text{-selectin}}$ (*A* and *B*) or $CHO^{P\text{-selectin}}$ (*C* and *D*) cells in the presence of mEP-5C7 (□) and HuEP5C7.g2 (■; *A* and *C*) or HuEP5C7.g2 (■) and HuEP5C7.g4 (△; *B* and *D*) at the indicated concentrations for 15 min. After washing, the relative number of bound cells was determined as described in *Materials and Methods*. The results from representative experiments performed with each sample in quadruplicate (± SD) are shown.

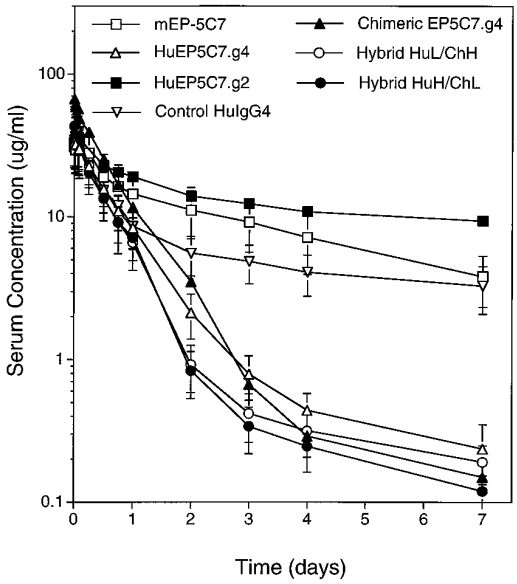

FIGURE 5. One-week pharmacokinetic profiles of murine, humanized, and chimeric Abs in rhesus monkeys. Rhesus monkeys (three per group) were given 2 mg/kg of each of the following Abs: mEP-5C7, HuEP5C7.g4, HuEP5C7.g2, HuDREG-200 (control HuIgG4), or chimeric (ChEP5C7.g4) or hybrid (Hybrid HuL/ChH or Hybrid HuHChL) EP5C7 Abs with human IgG4 constant regions. At the indicated times, serum samples were prepared and analyzed for the presence of the indicated Ab as described in *Materials and Methods*. The mean and SD of the serum concentration of Ab measured in three animals are shown.

Table I. *Terminal elimination half-lives in rhesus of murine and humanized Abs tested*[a]

Antibody	$AUC_{0-\infty}$ (μg × h/ml), mean ± SD	$t_{1/2}$ elimination (days), mean ± SD
mEP-5C7	1905 ± 1018	2.52 ± 0.73
HuEP5C7.g4	708 ± 260	2.87 ± 1.29
HuEP5C7.g2	4693 ± 572	6.64 ± 0.87
ChEP5C7.g4	963 ± 289	2.40 ± 0.58
Hybrid ChL/HuH	531 ± 168	2.22 ± 0.99
Hybrid HuL/ChH	564 ± 171	2.57 ± 0.28
HuDREG-200	1901 ± 723	6.54 ± 1.25

[a] The pharmacokinetic parameters shown were determined by analyzing the 3-wk elimination profiles of HuEP5C7.g2 and HuDREG-200 and 1-wk elimination profiles of other Abs in rhesus monkeys (three per group) given 2 mg/kg. Data were analyzed as described in *Materials and Methods*.

reperfusion injury (myocardial infarction or stroke), trauma, and shock, as well as chronic inflammatory conditions, such as rheumatoid arthritis or psoriasis. For therapeutic purposes, Abs have certain advantages over conventional small molecule drugs, e.g., relatively long serum half-lives and often more attractive safety profiles. Abs are bivalent, and the relatively spacious Ag-combining site naturally provides for a number of Ag contact points. Furthermore, the ability to "humanize" high affinity murine mAbs has resulted in improved therapeutic efficacy of Abs by reducing immunogenicity and improving serum half-lives.

The techniques of Ab humanization have evolved over the past 9 yr, so that in our laboratory, humanization of murine Abs specific for single protein Ags is nearly always successful. The case described here, however, is the first report of the humanization of an Ab with reactivity against two Ags, the E- and P-selectins. Although these two proteins share homology, there are significant differences between them (the various domains share 34–62% amino acid identity) (42, 43). This additional constraint made the likelihood of success of the humanization process less predictable.

Humanization of mEP-5C7 was successful in that the avidity of the humanized Ab, HuEP5C7.g4, for both E- and P-selectin was substantially retained. Interestingly, the murine Ab slightly favors P-selectin, while HuEP5C7.g4 slightly favors E-selectin. These slight differences in avidity are not likely to affect the efficacy of the humanized Ab in vivo, since they are not accompanied by changes in functional blocking activity (Fig. 4).

Since mEP-5C7 and HuEP5C7.g4 bind to the E- and P-selectins of nonhuman primates, including rhesus and baboon (44) (data not shown), pharmacokinetic studies were performed in both species. Unexpectedly, HuEP5C7.g4 displayed a very short elimination half-life, clearing from the circulation in both rhesus and baboons even more rapidly than mEP-5C7, particularly in the first 4 days (Fig. 5) (V. Vexler, unpublished observations). Several possibilities could account for a more rapid elimination profile of a humanized Ab than the original murine Ab, including chemical or physical instability, or creation of a novel binding site for another Ag. Such a novel binding site might have specificity for a single widely expressed Ag or might be nonspecific, binding many Ags but with low affinity (as do some natural Abs) (45).

HuEP5C7.g4 exhibited no obvious physical or chemical instability (data not shown), nor did it bind nonspecifically to Ags (e.g.,

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