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

Xing-Yue He, Zhenghai Xu, Jennifer Melrose, Alison Mullowney, Maximiliano Vasquez, Cary Queen, Vladimir Vexler, Corine Klingbeil, Man Sung Co and Ellen L. Berg

*J Immunol* 1998; 160:1029-1035;
http://www.jimmunol.org/content/160/2/1029

---

**References** This article cites 39 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/160/2/1029.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Humanization and Pharmacokinetics of a Monoclonal Antibody with Specificity for Both E- and P-Selectin

Xing-Yue He, Zhenghai Xu, Jennifer Melrose, Alison Mullowney, Maximiliano Vasquez, Cary Queen, Vladimir Vexler, Corine Klingbeil, Man Sung Co, and Ellen L. Berg

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 costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

---

1 Address correspondence and reprint requests to Dr. Ellen L. Berg, Protein Design Labs, Inc., 2375 Garcia Ave., Mountain View, CA 94043. E-mail address: eberg@pdl.com

2 Abbreviations used in this paper: CDR, complementarity-determining region; AUC0-tlast, area under the concentration-time curve; HRP, horseradish peroxidase.
Materials and Methods

Abs and cell lines

Mouse mononuclear 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 k) myeloma MOPC 21 and purified human (IgG4 k) 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(P-selectin), and Chinese hamster ovary (CHO) cell lines transfected with human E- or P-selectin gene (CHO(E-selectin) or CHO(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 2HH are mouse monoclonal anti-Id Abs generated against HuDREG-200 and HuEP5C7.g4, respectively. The hybridoma producing 2HH 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 dicymoculticate 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.). 2HH 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 G tails attached to the cDNA using terminal deoxytransferase (30). The sequences were determined using the dyeoxine 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 L. Hood (35). For the construction of humanized HuEP5C7.g4, the plasmid pVg4 was replaced by pVg2.M3.D.Tt (36). pVg2.M3.D.Tt incorporates the appropriate V region genes into the Xbcl site of the respective vectors. For the construction of chimeric Ab expression vectors, Xbcl fragments containing the murine Vh and Vl 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 Xbcl site of the respective pVc and pVg4 vectors. For the construction of humanized Ab expression vectors, Xbcl fragments containing the humanized Vh and Vl 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 EPSC7 Vh and Vl, generally using codons found in the mouse sequence. When natural 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, denatured, reannealed, and extended to create the final full-length dsDNA; and 3) 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 XbaI site for subsequent cloning.

The synthesized Xbal fragments were then inserted into the XbaI site of the respective pVc, pVg4, or pVg2.M3.D.Tt vectors. Hybrid Abs containing a humanized γ4 heavy chain were constructed by assembling (Cl1-24H11) or a chimeric γ4 heavy chain and a humanized light chain (ChHu/HuL) or 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 250 V and 25 μF. Hybridomas were used as 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(E-selectin) and L1-2(P-selectin) cells, respectively. Purified mEP-5C7 Ab was labeled with Na125I (Amersham, Arlington Heights, IL) using lactoperoxidase at 4 μCi/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 × 105 CHO(E-selectin) or L1-2(P-selectin) cells in 0.2 ml of binding buffer (PBS with 2% FCS and 0.1% sodium azide) at 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 free tracer Ab was calculated, and the avidities were calculated according to the formula: [X] = [EPC57] = (1/Ka)/(1/Kx), where Ka is the avidity of EP5C7, Kx is the avidity of the competitor X, brackets indicate the concentration of competitor Ab at which bound/free tracer binding is R0/2, and R0 is maximal bound/free tracer binding (38).

E- and P-selectin adhesion assays

HL-60 cell binding to CHO(E-selectin) or CHO(P-selectin) transfectant cell lines was performed as described previously (14). Confluent cultures of CHO(E-selectin) or CHO(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 reconstituted in assay buffer containing 0.25 μg/ml anti-CD18 Ab, NA8, at 2 × 10⁵ cells/ml. Assays were initiated by addition of 50 μl of HL-60 cells to CHO(E-selectin) or CHO(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(E-selectin) or CHO(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 (pre dose), 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 21.

**Pharmacokinetic analysis**

Compartment-independent pharmacokinetic analysis (39) was performed using the KaleidaGraph program for Macintosh (Developed by Abelbeck Software, distributed by Synergy Software, Reading, PA). The area under the concentration-time curve, AUC, was calculated by the trapezoidal rule. AUC was determined by extrapolating to infinity from Cₜₙ, the concentration at the last measured time point, and kₑ is the elimination rate constant of the terminal portion of the concentration-time curve. AUC was derived from AUCₖₑₑ + AUCₖₑₑₑₑ, where AUCₖₑₑₑₑ is the area under the elimination curve from a minimum of four last data points. The elimination half-life, t₁/₂ₑ, was derived from 0.693/kₑ.

**ELISAs**

Microtiter plates (Nunc Immunolon 1) were coated overnight with 100 μg/well of capture Abs. These included polyclonal anti-mouse IgG (H+L) (human Ig absorbed, Biosisco, Camarillo, CA), diluted 1/200, or anti-Id Abs against EP5C7 (2H9) or HuDREG-200 (D200Id), diluted 1/2000 in PBS. Plates were then blocked by incubating wells with 5% nonfat dry milk/PBS for 1 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 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 1/10000 dilution for HuEP5C7.g4, and chimeric and hybrid Abs against EP5C7 Ab. CHOE-selectin cells (14) were used as sources of E-selectin and P-selectin. The binding avidities were calculated 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. 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. CHOE-Selectin cells and L1-2P-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 × 10⁸ M⁻¹ for E-selectin, identical with that measured for mEP-5C7, while for P-selectin, HuEP5C7.g4 had an avidity of 1.5 × 10⁸ M⁻¹, and mEP-5C7 had an avidity of 6.7 × 10⁹ M⁻¹.

**Results**

Cloning and sequencing of V region cDNA

The murine EP-5C7 Ab heavy and light chain V regions 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 V₅ and V₄ cDNAs. For the heavy chain, a unique sequence was identified. For the light chain, two sequences were identified. One sequence had their respective signal peptide sequences.

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 V₅ sequences is III-3R (41) of Kabat’s subgroup III, with 72% identity in the framework region. The V₅ from III-3R was also used. This V₅ 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 V₅ domain, but was not required at any residues in the V₄ 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 (V₅) or subgroup I (V₄). This was performed at residues 61, 72, 82, and 99 of V₅, and at residues 1, 75, and 78 of V₄. 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.

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. CHOE-Selectin cells and L1-2P-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 × 10⁸ M⁻¹ for E-selectin, identical with that measured for mEP-5C7, while for P-selectin, HuEP5C7.g4 had an avidity of 1.5 × 10⁸ M⁻¹, and mEP-5C7 had an avidity of 6.7 × 10⁹ M⁻¹.
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-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-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 blocking of HL-60 cells to CHO-P-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/
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).

![Figure 4](image-url)  
**FIGURE 4.** Inhibition of HL-60 cell adhesion to CHO-E-selectin or CHO-P-selectin cells by mEP-5C7 (h), HuEP5C7.g2 (■), or HuEP5C7.g4 (Δ) Abs. Fluorescently labeled HL-60 cells were incubated with CHO-E-selectin (A and B) or CHO-P-selectin (C and D) cells in the presence of mEP-5C7 (h) and HuEP5C7.g2 (■, A and C) or HuEP5C7.g2 (■) and HuEP5C7.g4 (Δ, 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 HuH/ChL) 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>
<thead>
<tr>
<th>Antibody</th>
<th>AUC₀₋₅₅₅ (µg × h/ml)</th>
<th>mean ± SD</th>
<th>t½ elimination (days), mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEP-5C7</td>
<td>1905 ± 1018</td>
<td>2.52 ± 0.73</td>
<td></td>
</tr>
<tr>
<td>HuEP5C7.g4</td>
<td>708 ± 260</td>
<td>2.87 ± 1.29</td>
<td></td>
</tr>
<tr>
<td>HuEP5C7.g2</td>
<td>4693 ± 572</td>
<td>6.64 ± 0.87</td>
<td></td>
</tr>
<tr>
<td>ChEP5C7.g4</td>
<td>963 ± 289</td>
<td>2.40 ± 0.58</td>
<td></td>
</tr>
<tr>
<td>Hybrid Ch/HuH</td>
<td>531 ± 168</td>
<td>2.22 ± 0.99</td>
<td></td>
</tr>
<tr>
<td>Hybrid Hu/H/ChL</td>
<td>564 ± 171</td>
<td>2.57 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>HuDREG-200</td>
<td>1901 ± 723</td>
<td>6.54 ± 1.25</td>
<td></td>
</tr>
</tbody>
</table>

*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.

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.,...
HUMANIZATION OF AN ANTI-E/P-SELECTIN mAb

FIGURE 6. Two-week pharmacokinetic profiles of HuEP5C7.g2 in rhesus monkeys. Three rhesus monkeys were given 2 mg/kg of HuEP5C7.g2. At the indicated times, serum samples were prepared and analyzed for the presence of HuEP5C7.g2 as described in Materials and Methods. The mean and SD of the amount of Ab measured in triplicate serum samples taken from individual rhesus monkeys are shown. The pharmacokinetic parameters shown were determined by analyzing the 3-wk elimination profiles of HuEP5C7.g2 and HuDREG-200 and the 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.

BSA, insulin, and DNA) in vitro (not shown). In addition, tissue cross-reactivity studies on baboon tissues did not identify any unexpected tissue reactivity of HuEP5C7.g4 (V. Vexler, unpublished observations). To identify the probable cause of the rapid elimination of HuEP5C7.g4, four additional Abs were generated: a chimeric Ab (mouse V\text{H} and human V\text{L}), two hybrid Abs (mouse V\text{L} and humanized V\text{H}, or humanized V\text{L} and mouse V\text{H}) with human IgG4 constant regions, and a humanized Ab of the human IgG2 isotype, HuEP5C7.g2.

The serum half-lives of chimeric and hybrid Abs in rhesus monkeys were short, similar to that of HuEP5C7.g4 (Fig. 5). In contrast, changing the isotype of the constant regions from IgG4 to IgG2 resulted in a humanized Ab with a longer serum half-life (Figs. 5 and 6). This Ab, HuEP5C7.g2, was identical with IgG2 resulted in a humanized Ab with a longer serum half-life (47, 48). However, the short half-life of HuEP5C7.g4 cannot be due only to its V region either, because when the same humanized V region is combined with a human γ2 constant region in HUEP5C7.g2, a long half-life results. Moreover, the murine Ab mEP-5C7, with a V region of the same specificity and affinity as HuEP5C7.g4, has a half-life as long as can be expected for mouse Abs injected into primates. It is only the combination of the EP-5C7 V region binding site in either murine or humanized form together with the human γ4 constant region, whether in HuEP5C7.g4, the chimeric ChEP5C7.g4 Ab, or the hybrid humanized/chimeric Abs, that leads to rapid elimination from the serum (Fig. 5).

Possible explanations for these findings are 1) the mode of flexibility of the human γ4 hinge region may allow the EP-5C7 V region to bind with some affinity to other Ags in rhesus monkeys (perhaps to other lectin-like domains), leading to rapid clearance from the serum, or 2) the ability of the γ4 constant region to bind to certain Fc receptors on leukocytes together with the ability of the V region to bind to E- and P-selectin may create an interaction that leads to clearance of the Ab from the serum. The mutated γ2 region used here, which was engineered to eliminate binding to Fc receptors (36), would avoid this problem. These are but two of the number of potential explanations left after more obvious ones were ruled out by our experimental results. The rapid clearance of HuEP5C7.g4 from the serum is more likely due to Ab sequestration and not elimination from the body, since the rate of clearance of HuEP5C7.g4 observed during the first several days after administration slows down significantly after 4 days (Fig. 5).

Regardless of the reason for the short-half life in primates of HuEP5C7.g4, the availability of the humanized HuEP5C7.g2 Ab with its unique function of binding and blocking both E- and P-selectin and with a long half-life in the circulation presents a viable anti-inflammatory drug candidate for clinical evaluation.

Acknowledgments

Sincere thanks to Lisa Hernandez and Chuck Bullock for organizing the pharmacokinetic studies, to Heather Sernard and Paul Sauer for cell culture, to Brad Tada and Julie Mikkelsen for Ab purification, and to Brent Larsen, Mike Klein, and Eddie Kast for protein analysis to confirm the identity of the recombinant Abs.

References
