Engineered Human IgG Antibodies with Longer Serum Half-lives in Primates*

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The neonatal Fc receptor (FcRn) plays an important role in regulating the serum half-lives of IgG antibodies. A correlation has been established between the pH-dependent binding affinity of IgG antibodies to FcRn and their serum half-lives in mice. In this study, molecular modeling was used to identify Fc positions near the FcRn binding site in a human IgG antibody that, when mutated, might alter the binding affinity of IgG to FcRn. Following mutagenesis, several IgG2 mutants with increased binding affinity to human FcRn at pH 6.0 were identified at Fc positions 250 and 428. These mutants do not bind to human FcRn at pH 7.5. A pharmacokinetics study of two mutant IgG2 antibodies with increased FcRn binding affinity indicated that they had serum half-lives in rhesus monkeys 2-fold longer than the wild-type antibody.

Antibody therapy is coming of age, with 15 monoclonal antibodies approved for therapeutic use in the United States and many others currently undergoing clinical trials (1). The advent of antibody engineering over the past two decades has contributed to the recent clinical success of therapeutic antibodies. The development of chimeric (2) and humanized (3) antibodies not only reduced the potent immunogenicity of rodent antibodies in humans but also improved the serum half-lives and efficacy of such therapeutics compared with rodent antibodies. Phage display (4) and other display technologies have led to the ability to increase the affinity of antibodies for their target antigens. More recently, antibody engineering has been used to modify the effector functions of antibodies by altering their binding to C1q (5) and various Fc receptors (6).

The neonatal Fc receptor (FcRn) is a heterodimer that comprises a transmembrane α chain with structural homology to the extracellular domains of the α chain of major histocompatibility complex class I molecules, and a soluble light chain consisting of β2-microglobulin (β2m) (7). FcRn mediates both transcytosis of maternal IgG to the fetus or neonate and IgG homeostasis in adults (8). Evidence for the latter role initially came from studies indicating an unusually short serum half-life for IgG antibodies in β2m-deficient mice (9–11). This observation led to the generation of mutant mouse hinge-Fc fragments with enhanced binding to FcRn and increased serum persistence in mice (12). Recently, several studies have identified human IgG1 mutants with enhanced FcRn binding (6, 13), although no improvement in the serum half-lives of these mutants was observed in mice (13) or reported in primates.

The binding of IgG to FcRn is sharply pH-dependent; IgG binds to FcRn under mildly acidic conditions and is readily acidic conditions and is readily degraded in acidified endosomes, rescued from degradation in lysosomes, recycled back to the cell surface, and returned to the circulation (8). Mutagenesis studies have identified both the mouse (15, 16) and human (17) Fc residues believed to be important in mediating pH-dependent binding. The results of the mutagenesis studies are consistent with the interpretation of a crystallographic study of the Fc-FcRn interaction (18). In the current study, molecular modeling was used to identify residues in the human IgG Fc near the FcRn binding site that, when mutated, might alter binding to FcRn without affecting the pH dependence of this interaction. Following exhaustive mutagenesis at these positions, several IgG2 mutants were identified with improved binding to FcRn at pH 6.0 that retained the property of pH-dependent release. A pharmacokinetics study in rhesus monkeys showed that two mutant IgG2 antibodies with increased FcRn binding affinity had considerably longer serum half-lives than the wild-type antibody.

**EXPERIMENTAL PROCEDURES**

Molecular Modeling—Molecular models of the human Fc-FcRn complex were generated based on the crystal structures (18, 19) and a model (20) of the rat Fc-FcRn complex. First, the rat β2m and FcRn α chains of the complex were replaced, respectively, with the human β2m (21) and FcRn α chains (22). Next, the rat Fc residues of the complex were replaced with the corresponding human IgG1 Fc residues (23), and then energy minimization calculations were done using SEGMOD and ENCAD (24, 25) to produce a model of the human IgG1/FcRn complex. The process was repeated to produce a model of the complex of human FcRn and the M3 variant of human IgG1 (IgG1M3) (26).

IgG Mutagenesis, Expression, and Purification—The light and heavy chain cDNAs from a trioma cell line expressing the human anti-hepatitis B virus (HBV) antibody OST577 (27) were cloned by PCR. The light and heavy chain V-genes were converted into mini-exons and subcloned, respectively, into pV2, a derivative of pVK (28) containing the human α constant region, and the M3 variant of pVg2.D.T (26). Overlay expansion PCR (29) was used to generate random amino acid substitutions at positions 250, 314, or 428 (numbered according to the EU index (23)) in the heavy chain of OST577-IgG1M3, until each possible amino acid at these positions was obtained. The resulting PCR fragments were subcloned into pVAg2M3-OST577, a derivative of the M3 variant of pVg2.D.T containing the pUC18 replication origin (30).

Human kidney cell line 293-H (Invitrogen) was transiently cotransfected with the antibody expression plasmids using the LipofectAMINE 2000 reagent (Invitrogen). Culture supernatants were concentrated and buffer exchanged into PBS, pH 6.0, with Vivaspin centrifugal concentrators (Vivascience, Hanover, Germany). Mouse myeloma cell line Sp2/0 (American Type Culture Collection, Manassas, VA) was stably cotransfected by electroporation. OST577-IgG1M3 antibodies were pu...
FBB, pH 6.0, and resuspended in 120 μl/H9262 Protein Design Labs, Inc.) and detected with horseradish peroxidase-pDL208 by electroporation, yielding cell line NS0-HuFcRn. Cultures, Salisbury, Wiltshire, UK) was stably transfected with pDL172, a derivative of pVk containing human IgG heavy chain cDNAs were cloned by PCR from human peripheral blood mononuclear cells and subcloned into pDL172, a derivative of pVk containing a glycosylphosphatidylinositol linkage signal from human decay-accelerating factor (31) fused to the FcRn α chain, resulting in pDL208. Mouse myeloma cell line NS0 (European Collection of Animal Cell Cultures, Salisbury, Wiltshire, UK) was stably transfected with pDL208 by electroporation, yielding cell line NS0-HuFcRn.

**Competitive Binding Assays—**Transiently expressed IgG3-M3 wild-type and mutant antibodies were tested for binding to human FcRn in a single-point competitive binding assay. Briefly, 2 × 10⁶ NS0-HuFcRn cells/test were washed with FACS binding buffer (FBB) (PBS containing 0.5% bovine serum albumin, 0.1% Na₃H₂PO₄, pH 8.0, and then with FBB, pH 6.0, and resuspended in 120 μl of biotinylated OST577-IgG3-M3 (8.3 μg/ml) and concentrated supernatant (containing 8.3 μg/ml of competitor antibody) in FBB, pH 6.0. After 1 h on ice, the cells were washed with FBB, pH 6.0, and resuspended in 25 μl of 2.5 μg/ml streptavidin-conjugated R-PE (BioSource, Camarillo, CA) in FBB, pH 6.0. After 30 min on ice, the cells were washed with FBB, pH 6.0, resuspended in 1% formaldehyde in PBS, and analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

Purified IgG3-M3 wild-type and mutant antibodies were further tested in a competitive binding assay using purified competitor antibody (2-fold serial dilutions from 208 to 0.102 μg/ml) as described above. IC₅₀ values were calculated using GraphPad Prism, version 3.02 (GraphPaper Software, San Diego, CA).

**Results and Discussion**

Identification of IgG Mutants with Altered Binding to FcRn—Molecular models of the human Fc-FcRn complex (Fig. 1) guided the selection of positions 250, 314, and 428 of the human IgG heavy chain for mutagenesis. Although the wild-type amino acids at these positions are located near the Fc-FcRn interface, it does not appear likely that they directly contribute to the pH-dependent interaction between Fe and FcRn. Inspection of the molecular models suggested that amino acid substitutions at these positions might increase or decrease the affinity of Fe for FcRn, without disrupting pH-dependent binding and release, by affecting the conformation of Fc amino acids that do interact with FcRn. Since the amino acids at and around these positions are conserved among all four human IgG subtypes (23), it is reasonable to expect that the FcRn binding phenotype resulting from an amino acid substitution in one IgG subtype could be transferred to the other three IgG subtypes. In this study, an IgG3-M3 form (26) of the human anti-HBV monoclonal antibody OST577 (27) was chosen for mutagenesis because it would not be expected to bind either to antigen or Fcγ receptors in HBV-free primates.

PCR mutagenesis was used to generate all 19 single amino acid substitutions at each position. Transiently expressed OST577-IgG3-M3 mutants were screened for binding to NS0-HuFcRn cells in a single-point competitive binding assay (Fig. 2). Several of the mutants at positions 250 (e.g. Glu and Gln) (Fig. 2A) and 428 (e.g. Phe and Leu) (Fig. 2C) appeared to be stronger competitors in this assay than the wild-type antibody, indicating that these mutants have increased binding to FcRn at pH 6.0. At position 250, it appears that the presence of a hydrogen bond acceptor in the appropriate geometry (e.g. Glu or Gln) is important for better FcRn binding, since smaller but chemically related residues (e.g. Asp or Asn) reduced binding to FcRn. At position 428, a large hydrophobic amino acid confers better FcRn binding. None of the mutations at position 314 (Fig. 2B) resulted in increased binding to FcRn.

In a previous study (13), human IgG1 Fc position 428 was randomly mutated, and mutants were screened for binding to mouse FcRn by phage display; however, no mutants at this position with increased binding to mouse FcRn were obtained. This could be because of biases in the construction of the library, the loss of certain sequences during propagation in Escherichia coli, or incomplete sampling of the library during screening. Another explanation is that mouse rather than human FcRn was used to pan the human IgG1 Fc mutant library in the previous study (13). Because the amino acids at and around position 428 are not conserved between the mouse and human Fe regions, it follows that an Fc mutant at position 428 may interact differently with mouse and human FcRn.

The best competitors among the mutants at positions 250 and 428 were stably expressed either alone (T250Q, M428L) or in combination (T250Q/M428L), and purified antibodies were compared in a competitive binding assay to human FcRn (Fig.
3). Comparison of the IC\textsubscript{50} values indicated that the single mutants T250Q and M428L showed an increase in binding to human FcRn at pH 6.0 of \textasciitilde 3- and 7-fold, respectively, whereas the double mutant T250Q/M428L showed an increase in binding of \textasciitilde 28-fold. To confirm that binding was pH-dependent, the antibodies were allowed to bind to NS0-HuFcRn cells at pH 6.0 and were removed by washing the cells at pH 6.0, 6.5, 7.0, 7.5, or 8.0. As the pH value of the washes in successive samples was raised from pH 6.0 to 8.0, the binding of the wild-type and mutant antibodies to human FcRn was comparably diminished, with essentially no binding observed at pH 7.5 or above (data not shown). Similar results were obtained when these mutant antibodies were tested for binding to rhesus FcRn. The binding of the T250Q, M428L, and T250Q/M428L Ig\textsubscript{G\textsubscript{M3}} mutants to rhesus FcRn at pH 6.0 was \textasciitilde 4-, 8-, and 27-fold better than the wild-type antibody, respectively, and binding to rhesus FcRn was pH-dependent (data not shown).

**Pharmacokinetics of IgG Wild-type and Mutant Antibodies in Rhesus Monkeys—**The PK behavior of the OST577-Ig\textsubscript{G\textsubscript{M3}} wild-type, M428L, and T250Q/M428L antibodies was examined in rhesus monkeys. The PK profiles of the two mutants are clearly distinct from that of the wild-type (Fig. 4). The mean serum antibody concentrations of the M428L and T250Q/M428L mutants were maintained at higher levels than wild-type OST577-Ig\textsubscript{G\textsubscript{M3}} at all time points. Because the mean maximum serum antibody concentration (C\textsubscript{max}, Table I) was very similar among the three test groups, indicating that the administered antibodies were distributed to the circulation in a similar manner, the higher concentrations of the mutant Ig\textsubscript{G\textsubscript{M3}} antibodies thereafter are attributable to their increased persistence in the serum. Analysis of the mean clearance (CL) indicated that this was the case. The mean CL, the volume of serum antibody cleared per unit of time, was \textasciitilde 1.8-fold lower for the M428L mutant (0.0811 ± 0.0384 ml/h/kg; \( p = 0.057 \)), and \textasciitilde 2.8-fold lower for the T250Q/M428L mutant (0.0514 ± 0.0075 ml/h/kg; \( p = 0.029 \)) compared with wild-type OST577-Ig\textsubscript{G\textsubscript{M3}} (0.144 ± 0.047 ml/h/kg) (Table I), indicating a significant decrease in the clearance of the OST577-Ig\textsubscript{G\textsubscript{M3}} M428L and T250Q/M428L mutants.

The PK profiles of the OST577-Ig\textsubscript{G\textsubscript{M3}} wild-type and mu-
Pharmacokinetic parameters were calculated using WinNonlin (Pharsight). The group mean ± S.D. is shown for each parameter. Mann-Whitney tests were done using GraphPad Prism (GraphPad Software) to compare the statistical significance of differences in the pharmacokinetic parameters between the wild-type (WT) group and each mutant group. $C_{\text{max}}$, maximum serum antibody concentration; $t_{1/2}$, elimination (β-phase) half-life. *, indicates a significant difference ($p < 0.060$).

| antibody   | $C_{\text{max}}$ | CL       | AUC      | $t_{1/2}$ |
|------------|------------------|----------|----------|-----------|
| WT         | 36.7 ± 12.8      | 0.144 ± 0.047 | 7710 ± 3110 | 351 ± 121 |
| M428L      | 36.5 ± 20.1      | 0.0811 ± 0.0384* | 15200 ± 8700* | 642 ± 205 |
| T250Q/M428L| 39.9 ± 6.8       | 0.0514 ± 0.0075* | 19800 ± 2900* | 652 ± 28* |

**Table I**

Summary of pharmacokinetic parameters

Engineered Antibodies with Longer Serum Half-lives

Engineered antibodies with increased serum half-lives might prove valuable in antibody therapy. For example, it may be possible to reduce the frequency of administration of such antibodies. This will be a great benefit to patients undergoing long-term antibody therapy. Based on the results described in this study, it is reasonable to expect that human IgG3, IgG3, and IgG antibodies with longer serum half-lives also may be engineered by transferring the M428L or T250Q/M428L mutations into these IgG subtypes. In addition, it should now also be possible to alter the serum half-lives of other IgG-related therapeutics such as IgG Fc fusion proteins using this approach. Provided that the mutations described in this study do not substantially increase the immunogenicity of these therapeutics in humans, IgG antibodies and Fc fusion proteins with longer serum half-lives should represent a potent new class of human therapeutics.

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