Monoclonal Antibodies with Identical Fc Sequences Can Bind to FcRn Differentially with Pharmacokinetic Consequences

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β2m, β2 microglobulin; Fab, fragment of antigen binding; Fc, fragment crystallizable; FcRn, neonatal Fc receptor; hFcRn, human FcRn; IV, intravenous; IVIVC, in vitro-in vivo correlation; KD, dissociation constant; mAbs, monoclonal antibodies; NHP, non-human primate; PBS, phosphate buffered saline; PK, pharmacokinetics; RU, response unit; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; t1/2, half-
life; Vss, volume of distribution at steady state
Abstract

The neonatal Fc receptor (FcRn) is a key determinant of IgG homeostasis. It binds to the Fc domain of IgG in a strictly pH-dependent manner and protects IgG from lysosomal degradation. The impact of FcRn salvage pathway on IgG monoclonal antibody (mAb) pharmacokinetics (PK) has been well established. In this report, a set of mAbs with wild-type human Fc sequences but different Fab domains were used to examine the potential impact of Fab domain on \textit{in vitro} FcRn binding and \textit{in vivo} PK. Surprisingly, mAbs with the same wild-type human Fc sequences but different Fab domains were shown to bind FcRn with considerable differences in both the binding at acidic pH and the dissociation at neutral pH, suggesting that the Fab domain may also impact FcRn interaction. For these mAbs, no relationship between the FcRn binding affinity at acidic pH and \textit{in vivo} PK was found. Instead, an apparent correlation between the \textit{in vitro} FcRn dissociation at neutral pH and the \textit{in vivo} PK in human FcRn mice, non-human primates and humans was observed. Our results suggested that the Fab domain of mAbs can impact their interaction with FcRn and thus their PK properties, and that \textit{in vitro} FcRn binding/dissociation assays can be a useful screening tool for PK assessment of mAbs with wild-type Fc sequences.
Introduction

Therapeutic monoclonal antibodies (mAbs) have proven to be a promising approach to treat many human diseases. There are currently more than 25 mAbs approved for therapeutic use, all belonging to the IgG family (Nelson et al., 2010). Human IgGs have an average half-life of 21 days in healthy human subjects, which is longer than any other known serum protein (Waldmann and Strober, 1969). The exceptionally long serum half-life of IgG has largely been attributed to the protective role of neonatal Fc receptor (FcRn), a membrane associated receptor shown to be responsible for IgG and albumin homeostasis (Ghetie and Ward, 2000; Chaudhury et al., 2003).

FcRn protein is a heterodimer consisting of two polypeptides: 50kDa class I major histocompatibility complex (MHC-I)-like protein (α-FcRn) and 15kDa β2 microglobulin (β2m). Each IgG molecule has an Fc domain and two antigen-binding Fab domains. Crystal structure has shown that FcRn binds to the Fc domain of IgG (Burmeister et al., 1994; Martin et al., 2001). The interaction between FcRn and IgG is strictly pH-dependent. It has been hypothesized that IgGs are subject to fluid-phase pinocytosis by many types of cells in the body (Akilesh et al., 2007; Montoyo et al., 2009). The pinocytosed IgGs are subsequently transported to the acidic endosomes where they encounter FcRn. FcRn binds to IgG with nanomolar affinities at slightly acidic endosomal pH (pH 5-6) and salvages IgG from lysosomal degradation. FcRn has no detectable binding to IgG at neutral pH, resulting in release of IgG upon encountering the extracellular milieu (Ghetie and Ward, 2000). Both the binding to FcRn at acidic pH and the release from FcRn at neutral pH are critical for maintaining the long circulation half-life of an IgG (Vaccaro et al., 2005). Interestingly, a recent population PK analysis of infliximab suggested that inter-subject variability in FcRn activity is an important covariance of infliximab PK, which
implies that the IgGs failed to be salvaged by FcRn consist of a significant portion to normal IgG elimination (Fasanmade et al., 2010).

Since FcRn plays an important role in IgG catabolism, one would expect that in vitro FcRn binding properties may be indicative of its in vivo pharmacokinetics (PK) properties. Such in vitro tools would be highly valuable during therapeutic mAb discovery and development processes. Indeed, in vitro FcRn binding properties have been used extensively to aid in Fc engineering of mAbs with varying Fc sequences (Fc mutants) for their optimal half-life potential in humans (Dall'Acqua et al., 2002, 2006; Vaccaro et al., 2005; Datta-Mannan et al., 2007a and 2007b; Yeung et al., 2009; Zalevsky et al., 2010).

Since FcRn is an Fc receptor, it had been commonly assumed that IgGs with same Fc sequences will bind to FcRn equally and be protected by FcRn similarly. This assumption has not been systematically challenged even though marketed therapeutic mAbs with wide-type human Fc sequence can exhibit a wide range of half-life and clearance values. While many of the observed PK differences can be rationalized by the known target-mediated clearance mechanism commonly associated with mAbs targeting membrane bound antigens (Mager, 2006), the reason for some of these discrepancies remains unknown. For example, the recently approved human anti-p40 antibody ustekinumab had a reported median half-life of 22 days (Zhu et al., 2009) while another similar anti-p40 antibody, briakinumab, only exhibited a half-life of 8-9 days (Gandhi et al., 2010). Interestingly, a recent report showed that mAbs and Fc-fusion proteins with the same Fc sequence can bind to FcRn differently, highlighting the potential importance of FcRn in regulating PK of therapeutic mAbs with wild-type Fc sequences (Suzuki et al., 2010).
In this report, we set out to investigate the impact of Fabs on *in vitro* FcRn binding and *in vivo* PK of IgGs with same human Fc sequences. A total of 15 therapeutic mAbs with wild-type human Fc sequences were included in our studies. We found that mAbs with wild-type human Fc sequences can interact with FcRn with considerable differences in both binding at acidic pH and dissociation at neutral pH, indicating that the Fab domain may also impact the FcRn interaction. Importantly, these *in vitro* FcRn binding differences, especially at neutral pH, can be correlated to the PK properties of these mAb in human FcRn mice, where no targeted mediated clearance mechanism is expected. A similar *in vitro-in vivo* correlation (IVIVC) was also observed for a limited number of mAbs with PK dataset available in non-human primates (NHPs) and humans, under the condition where target mediated clearance mechanism was minimized.
Materials and Methods

**MAbs**

A total of eight mAbs from Merck's internal pipeline and seven commercial mAbs were used to study the relationship between *in vitro* FcRn binding and *in vivo* PK. For human FcRn mouse and NHP studies, three IgG1/IgG2 pairs of Merck mAbs (MK-1/MK-2, MK-3/MK-4 and MK-5/MK-6) and five commercial mAbs with wild-type human IgG1 Fc sequences (adalimumab, basiliximab, bevacizumab, cetuximab and trastuzumab) were used. All Merck mAbs within the IgG1 or IgG2 subtype have identical wild-type human Fc sequences. Another two Merck mAbs (MK-A and MK-B) and three commercial mAbs (infliximab, omalizumab and palivizumab) were also added to study the relationship between *in vitro* FcRn binding and *in vivo* PK in humans.

All commercial mAbs were obtained from Myoderm (Norristown, PA).

**Expression and Purification of FcRn Proteins**

Soluble human, rhesus macaque and human FcRn mice ("hybrid", human $\alpha$ + mouse $\beta$) FcRn were engineered to be expressed using the Bac-to-Bac™ baculovirus expression system from Invitrogen (Carlsbad, CA). Briefly, the cDNAs of the $\alpha$-subunit were truncated to include only the leader peptide and extracellular domain (codons 1-290) to generate the soluble forms of FcRn-$\alpha$ (Popov et al., 1996). To facilitate purification, a His$_6$ tag was added to the C-terminus of each $\alpha$-subunit. The corresponding full-length $\beta$ chain ($\beta$2m) cDNA was co-expressed using a separate baculovirus expression vector. Viruses containing FcRn $\alpha$- and $\beta$-subunits (human $\alpha$ + $\beta$, rhesus $\alpha$ + $\beta$, or human $\alpha$ + mouse $\beta$) were co-transfected and expressed in Sf 9 cells for 72 h.
following which the FcRn-containing culture media was collected by centrifugation. The media was neutralized to pH 7.5 using 1 M Tris pH 8.0 and then filtered using 0.22 µm filterware. The heterodimeric FcRn proteins were isolated from media using a QIAGEN Ni-NTA Superflow (Valencia, CA) with a BioRad Econo-column (Hercules, CA) according to the manufacturer's recommendations. Purified FcRn proteins were filtered and concentrated using a Millipore Amicon-Ultra Centrifugal filter unit (Billerica, MA). SDS-PAGE was performed to confirm presence of the truncated α chain and β2m. The FcRn proteins were > 99% pure based on SDS-PAGE (data not shown). Purified FcRn proteins were dialyzed into 6 mM sodium phosphate, 100 mM NaCl, pH 7.4 and 0.05% surfactant P-20 (GE Healthcare), and dispensed into single-use aliquots and stored at -70 oC.

Surface Plasmon Resonance (SPR) Assay

The interaction between purified human IgG (mAbs) and FcRn was measured by SPR using a Biacore T-100 instrument (GE Healthcare Biosciences, Piscataway, NJ). Purified FcRn protein was immobilized onto a Biacore CM5 biosensor chip using an amine coupling kit (GE Healthcare) to reach a density of ~200 response units (RU). The kinetics experiment was conducted at 25 ºC using a pH 6.0 running buffer (50 mM NaPO4, 150 mM NaCl and 0.05% (v/v) Surfactant 20) with a flow rate of 30 µl/min. The mAbs were diluted with the pH 6.0 running buffer to 25, 50, and 100 nM. They were allowed to bind to FcRn for 3 min to reach equilibrium and then followed by 2 min of dissociation. Two 30-second pulses of pH 7.5 running buffer were used to regenerate the chip and the return of sensorgram to baseline was verified before next run. To determine FcRn binding affinity (dissociation constant, K_d) at pH 6.0, the data from all three concentrations was used simultaneously to fit a two state reaction model found in
the Biacore T-100 Evaluation software (BIAevaluation, GE Healthcare Biosciences, Piscataway, NJ).

The neutral pH dissociation experiment was conducted similarly except using a pH 7.3 running buffer. The mAbs were diluted to 100 nM using pH 6.0 running buffer, allowed to bind FcRn for 2 min and then quickly exposed to pH 7.3 running buffer for dissociation. The use of Biacore T-100 instrument (kinetics injection) and the impeccable maintenance of instrument are critical for the neutral pH dissociation assay. A series of report points during the dissociation phase were recorded. In particular, one report point (Binding) was inserted at 2 seconds before the pH 7.3 dissociation phase begins and another report point (Stability) was inserted at 5 seconds into the dissociation phase, and "%bound" was calculated as:

\[
\%\text{bound} = \frac{RU_{\text{Stability}}}{RU_{\text{Binding}}} \times 100 \quad (1)
\]

In addition, the whole dissociation process was captured by a series of reporting points from 2 to 105 seconds following dissociation. The resulting dissociation curve was found to be best described by a biexponential decline function:

\[
RU_t = A e^{-k_1 t} + B e^{-k_2 t} + C \quad (2)
\]

where t is time post dissociation, RU_t is RU at time t, A and B are initial values at time zero for the two (faster and slower) dissociation phases, k_1 and k_2 are the apparent first order rate constants for the faster and slower dissociation phases, and C is the RU at end of dissociation. Both %bound and the biexponential model derived parameters were used to establish potential correlations with in vivo PK

**PK Study in Human FcRn Mice**
The heterozygous Tg276 human FcRn mice used in this study was obtained from Jackson Laboratory (Bar Harbor, ME). It is deficient in mouse FcRn-α chain and carries a human FcRn-α chain gene instead (Petkova et al., 2006). For PK studies, each animal (3-4/group) received a single intravenous (IV) injection of mAb at 10 mg/kg via tail vein. Series of 10 μl of blood was collected at specified time points (every 24 h until day 4 and then every 2-3 day until day 15) via tail vein with a positive displacement pipette and the blood was mixed with 1 μl of 55 mM EDTA immediately. The mixture was then diluted with 90 μl of PBS and centrifuged. The resulting diluted plasma was subsequently used for immunoassays to quantify mAb levels. All studies were approved by the Merck Institutional Animal Care and Use Committee.

A qualified anti-human IgG immunoassay with GyroLab (Gyros, Uppsala, Sweden) was used to determine all mAb levels in blood (Roman et al., 2011). Briefly, a biotinylated mouse anti-human kappa or lambda chain monoclonal antibody (BD Pharmingen) was used for capture and an ALEXA-647 labeled mouse monoclonal antibody specific for human Fc domain (Southern Biotech) was used for detection. The mAb concentrations were extrapolated from standard curves. The assay range was established with spiked quality control (QC) samples. The accuracy and precision were within 100 ± 20% and lower limit of quantitation (LLOQ) was 100 ng/ml (Roman et al., 2011). The entire immunoassay was automated using the Gyros® Bioaffy workstation and Bioaffy 200 compact disks (Gyros, Uppsala, Sweden).

The blood mAb concentrations were converted to their corresponding plasma concentrations using a fixed mouse blood-to-plasma ratio of 0.59 as reported previously (Davies and Morris, 1993). The plasma concentration was used for noncompartmental PK analysis using WinNonlin (Enterprise Version 5.01, Pharsight Corp, Mountain View, CA). The mAb elimination phase
terminal half-life ($t_{1/2}$) was determined using data points from the terminal phase (usually between day 3 and day 15 post dose). These data points generally fitted well to a monoexponential decay function.

**PK Parameters in NHPs and Humans**

The PK parameters in NHPs and humans were obtained from either Freedom of Information or Merck's internal database. In case a mAb exhibited non-linear PK, only PK data at relatively high doses (saturating dose), where the target-mediated clearance mechanism are minimized or eliminated, were used.
Results

*Characterization of mAb-human FcRn interaction in vitro*

SPR allows real-time detection of biomolecular binding events and has been the most widely used technology to analyze the interaction between IgG and FcRn. We also developed in-house SPR assays using Biacore to examine the pH-dependent IgG-FcRn interaction, i.e. ability of IgG to bind FcRn at pH 6.0 and to dissociate at pH 7.3. To better mimic the in *vivo* situation and to compare the interaction among mAbs, purified human FcRn protein was immobilized onto a Biacore CM5 chip (ligand), and mAbs were flowed through to observe their pH-dependent interaction with FcRn (analyte).

We first examined the pH 6.0 FcRn binding. As shown by the sensorgrams in Fig 1A, the interactions between mAbs and FcRn are clearly multi-phasic under these conditions, indicating that it could not be described by a single reaction. This is consistent with many previous observations (Gurbaxani and Morrison 2006; Datta-Mannan et al, 2007a and 2007b; Yeung et al., 2009). The BIAevaluation software provided four kinetic model options to evaluate multi-phasic ligand-analyte interactions: bivalent analyte (A + B $\leftrightarrow$ AB; AB + B $\leftrightarrow$ AB2); heterogeneous analyte (A1 + B $\leftrightarrow$ A1B; A2 + B $\leftrightarrow$ A2B); heterogeneous ligand (A + B1 $\leftrightarrow$ AB1; A + B2 $\leftrightarrow$ AB2) and two state reaction (A + B $\leftrightarrow$ AB $\leftrightarrow$ AB*). To select the model most suitable for describing the observed multi-phasic IgG-FcRn interaction, we compared the human IgG dissociation curves following 15 seconds, 3 minutes and 15 minutes of FcRn binding and found them to be non-parallel, i.e. longer FcRn-binding time led to slower dissociation (data not shown). This result indicated that the IgG-FcRn complex on the Biacore chip can undergo conformation change that stabilizes the complex, which suggested that the two state reaction
model is more appropriate to describe the interaction between IgG and FcRn in our system. The BIAevaluation software uses Chi², the average squared residual, as a measure of closeness of fit for the fitted curve. Through comparison of Chi², we also confirmed that the two state reaction model fitted the pH 6.0 IgG-FcRn binding data significantly better than the other three kinetic models. Therefore we used it for all subsequent pH 6.0 K_D determinations as described in Materials and Methods. It needs to be pointed out that even the two state reaction model can only describe the IgG-FcRn interaction over a relatively narrow mAb concentration ranges, and the absolute K_D values can vary between runs depending on experimental conditions such as the amount of FcRn being immobilized onto a chip. Nevertheless, we were able to recapitulate many reported affinity changes of known Fc mutants in our assay (data not shown). Moreover, we showed in a recent report that our assay can reliably detect a relatively minor (~30%) affinity difference due to methionine oxidation in the Fc region and the in vitro FcRn binding affinities correlated well with methionine oxidation levels and in vivo PK (Wang et al., 2011).

The pH 6.0 binding assay was used to examine a group of mAbs from Merck's internal pipeline for their ability to bind human FcRn. Consistent with a previous report (Suzuki et al, 2010), we found that mAbs with identical wild-type human Fc sequences can bind to human FcRn with considerable differences (Fig 1A). As shown in Table 1, mAbs with identical wild-type IgG1 (MK-1, 3 and 5) or IgG2 (MK-2, 4 and 6) Fc sequences can bind to human FcRn with up to 3-fold differences in affinity at pH 6.0. Meanwhile, the IgG subtypes appeared to have only minor impact on FcRn binding, i.e. IgG1 and IgG2 mAbs with the same (MK-1 and 2; MK-3 and 4) or similar (MK-5 and 6) Fab domains bind to FcRn with similar affinities (20-25% higher for the IgG2s than corresponding IgG1s) (Table 1).
Since the rapid dissociation at neutral pH is also essential for FcRn function, we next compared the ability of these mAbs to dissociate from human FcRn at neutral pH. To mimic the hypothesized in vivo IgG-FcRn interaction process, the mAbs were allowed to reach equilibrium binding with FcRn in pH 6.0 running buffer and then quickly exposed to a pH 7.3 running buffer for dissociation. All tested mAbs had an extremely fast initial dissociation phase under these conditions (Fig 1B). However, following the initial rapid dissociation phase, different mAbs were found to have various amounts of "slow-dissociation" fractions. The difference in "slow-dissociation" fraction of a mAb can be quantified with a simple "%bound" parameter, i.e. percentage of mAb remaining bound to FcRn 5 seconds into the dissociation phase (Equation (1), Materials and Methods) and the results were also shown in Table 1.

The characteristics of a mAb's pH 7.3 dissociation appeared to be independent of its pH 6.0 binding affinity. For example, MK-5 and 6 exhibited higher %bound than mAbs with higher (MK-1 and 2) or lower (MK-3 and 4) pH 6.0 affinities (Table 1). We were unable to identify any unique biophysical properties associated with mAbs with relatively high % bound, e.g. isoelectric point, aggregation. For example, size-exclusion chromatography showed that all mAbs used in this study have greater than 97% monomer contents and no relationship between %bound and the trace amounts of aggregation levels could be found (data not shown). Similar to pH 6.0 binding affinity, the observed differences in %bound appeared to be related to the Fab domains rather the IgG subtypes (Table 1).

**Correlation of in vitro FcRn binding and in vivo PK in human FcRn mice**

The interaction between IgG and FcRn is species-specific. While human IgGs bind to NHP and human FcRn similarly, their interaction with rodent FcRn is significantly different (Ober et al.,
2001; Datta-Mannan et al., 2007a and 2007b). Human FcRn mice had recently been suggested as a valuable surrogate system for evaluating human IgG PK (Petkova et al., 2006; Zalevsky et al., 2010). The so-called human FcRn mice actually had a "hybrid FcRn", consisting of human \( \alpha \) chain \((\alpha\text{-FcRn})\) and mouse \( \beta \) chain \((\beta_2\text{m})\) of FcRn. Unlike mouse or rat FcRn, the "hybrid" FcRn was expected to have similar human IgG binding properties as that of human FcRn (Petkova et al., 2006). Therefore we decided to first investigate the potential IVIVC in human FcRn mice.

For this purpose, we used five readily-available commercial IgG1 mAbs (adalimumab, basiliximab, bevacizumab, cetuximab, and trastuzumab, all with wild-type human IgG1 Fc sequences) in addition to the six mAbs from Merck's internal pipeline (MK-1 to MK-6) described above. All mAbs used here either does not have known mouse ligand, or only recognize a mouse ligand in very low abundance. Therefore, the potential impact of FcRn interaction on PK can be studied with minimal interference from target-mediated clearance mechanisms.

The \textit{in vitro} interaction of these 11 mAbs with purified hybrid FcRn, both pH 6.0 binding and pH 7.3 dissociation, was first examined and compared to that with human FcRn. As shown in Figs 2A and 2B, all 11 mAbs exhibited very similar \( K_D \) at pH 6.0 and \%bound at pH 7.3 to hybrid and human FcRn, consistent with previous suggestions that the hybrid FcRn has comparable human IgG binding and dissociation characteristics as that of human FcRn.

We next determined the PK of these 11 mAbs in human FcRn mice. Each animal (3-4/group) received a single IV dose of mAb at 10 mg/kg. The 10 mg/kg dose was used to minimize potential residual impact of target-mediated clearance, intended or non-intended. Series of blood
samples was collected at specified time points and drug levels were determined using an anti-human IgG immunoassay. The PK parameters were summarized in Table 2. These 11 mAbs exhibited a range of terminal t1/2 and clearance values, despite of their wild-type human Fc sequences and the fact that no major impact of target-mediated clearance was expected.

To examine whether there is any relationship between \textit{in vitro} FcRn binding and \textit{in vivo} PK, we plotted \textit{in vivo} terminal t1/2 in human FcRn mice against hybrid FcRn pH 6.0 binding affinity K_D and %bound at pH 7.3 for these mAbs. No apparent relationship between terminal t1/2 and K_D at pH 6.0 could be seen (Fig 2C). Interestingly, an apparent trend was observed when terminal t1/2 was plotted against the %bound at pH 7.3: mAbs with higher %bound tend to have shorter terminal t1/2 \textit{in vivo} (Fig 2D). The trend was similar for mAbs in either IgG1 or IgG2 backbones.

Terminal t1/2 was used as the primary PK parameter here because less clearance data at saturating dose in NHPs and humans are available. A similar trend in opposite direction can also be seen when %bound was plotted against clearance, i.e. mAbs with higher %bound tend to show higher clearance than mAbs with relatively lower %bound (Table 2).

\textit{Correlation of in vitro FcRn binding and in vivo PK in NHPs}

We next examined whether this potential IVIVC is also true in NHPs as NHPs are the most commonly used species for human PK prediction of mAbs (Ling et al., 2009; Wang and Prueksaritanont, 2010). For 8 out of the 11 mAbs used in our human FcRn mice study, we were able to collect, from either internal database or Freedom of Information, their NHP (rhesus or cynomolgus) terminal t1/2 data at relatively high doses (saturating dose), where the relative impact of target-mediated clearance was minimized or eliminated. There is only 1 amino acid difference between rhesus and cynomolgus monkey FcRn (the third amino acid in \(\beta_2m\) was an
arginine in rhesus and proline in cynomolgus) and we found no difference in their human IgG binding characteristics for rhesus and cynomolgus FcRns (data not shown). Therefore, we used rhesus monkey FcRn as the representative NHP FcRn in our in vitro FcRn binding/dissociation assays and compared its human IgG binding and dissociation characteristics to that of human FcRn. As shown in Figs 3A and 3B, all 11 mAbs exhibited very similar $K_D$ at pH 6.0 and %bound at pH 7.3 to rhesus monkey and human FcRn, confirming that NHP FcRn also exhibited comparable binding and dissociation characteristics to that of human FcRn.

The NHP terminal $t_{1/2}$s were plotted against $K_D$ at pH 6.0 and %bound at pH 7.3 respectively in Figs 3C and 3D. Similar to our findings in human FcRn mice, no relationship between $K_D$ at acidic pH and in vivo terminal $t_{1/2}$ could be found (Fig 3C). In contrast, a similar trend was observed between dissociation at neutral pH and in vivo PK: mAbs with higher %bound tend to have shorter terminal $t_{1/2}$ in vivo (Fig 3D). Only five of the mAbs had NHP clearance data at saturating dose available and a similar trend was observed from this very limited dataset: mAbs with higher %bound (MK-5 and 6) did show 2- to 3-fold higher clearance than mAbs with relatively low %bound (adalimumab, bevacizumab and trastuzumab).

*Correlation of in vitro FcRn binding and in vivo PK in human*

Subsequently, we explored the relationship between human FcRn binding/dissociation and mAb PK in humans. Many of the 11 mAbs we used for human FcRn mouse and NHP studies did not have human PK data at saturating dose (MK-1 to MK-6, basiliximab and cetuximab). So we used a different set of mAbs, which included all readily available commercial mAbs (infliximab, omalizumab and palivizumab in addition to adalimumab, bevacizumab and trastuzumab) and
internal mAbs (MK-A, MK-B) with human terminal $t_{1/2}$ at saturating dose available, for this purpose.

The "%bound" parameter is a simple way to measure a mAb's dissociation from FcRn at pH 7.3. However, one caveat of this method is its variability. Due to the extremely fast initial dissociation process and possibly experimental factors we did not fully understand, the absolute %bound values can vary from experiment to experiment. When a group of mAbs across a relatively wide range of %bound, such as ones we used to study IVIVC in hFcRn mice and NHPs, were studied, the overall trend as reported in Figs 2D and 3D was highly reproducible. However when the mAbs are all within a relatively narrow range of %bound, such as ones we used to examine human PK correlation, we experienced some difficulty in establishing a stable correlation between %bound at pH 7.3 and terminal $t_{1/2}$ in humans.

To better define the human IVIVC, we tried to use model fitting for more accurate quantification of the differences in pH 7.3 FcRn dissociation for mAbs. As shown in Fig 1B, all human IgGs we examined exhibited extremely fast dissociation from FcRn following exposure to pH 7.3 buffer. This initial phase (0-2 sec) can only be described by a linear function and is similar among all mAbs. A more pronounced difference in the dissociation process between mAbs can be seen following the initial linear phase, and the subsequent dissociation curve (2-105 sec) could be described by Equation (2) (Materials and Methods). The biexponential function could describe the dissociation curves for mAbs with either high or low %bound values with $R^2 >0.99$ (Fig 4A), suggesting that all mAbs have both fast and slow dissociation phases, and they only differ in parameters describing these two phases.
We next investigated whether the biexponential model derived parameters, either alone or in combinations, could lead to a better IVIVC in humans. Following extensive evaluation, $k_2/B$ was identified as the most promising combination. Since $k_2$ is the apparent first order rate constant for the slower dissociation phase and $B$ is the intercept of the slower dissociation phase at time zero, either lower $k_2$ or higher $B$ will lead to higher %bound. Apparently, lower $k_2$ or higher $B$ will also lead to lower $k_2/B$. Therefore $k_2/B$ should be inversely related to %bound. But unlike %bound, $k_2/B$ was derived from the whole dissociation curve, so it can better describe the overall neutral pH FcRn dissociation property. In fact, we did find the correlation trend between \textit{in vitro} human FcRn binding/dissociation and human terminal $t_{1/2}$ significantly more reproducible using $k_2/B$ than using %bound. When mAbs with relatively big difference in %bound were evaluated, the advantage of $k_2/B$ over %bound was not as apparent (data not shown). A correlation between $k_2/B$ and \textit{in vivo} terminal $t_{1/2}$ in human was shown in Fig 4B. The results suggested that there is a similar IVIVC in humans as what we observed in human FcRn mice and NHPs.

\textit{Application of in vitro FcRn tools in mAb lead optimization program}

The potential IVIVC we identified here is highly valuable for therapeutic mAb development. An example of its application in mAb lead optimization process was shown here. In an internal mAb program, a large number of mAb leads were generated from fully human phage display libraries. All leads positive in \textit{in vitro} functional assays (>50) were screened using the pH 7.3 FcRn binding/dissociation assay as described above. Among them, ten leads were selected and their PK in hFcRn mice was determined using the same methodology as described. The IVIVC of these ten leads and two control antibodies was shown in Fig 5. We only evaluated %bound here because these early mAb leads had relatively big difference in their pH 7.3 FcRn dissociation.
Under this situation, %bound is sufficient to differentiate the mAbs and it is a much easier parameter to generate than $k_2/B$. As shown in Fig 5, the results with novel mAb leads confirmed the trend we observed previously, i.e. mAbs with higher %bound tend to have shorter terminal $t_{1/2}$ \textit{in vivo}. The pH 7.3 FcRn dissociation assay is especially effective in screening out leads with undesired PK properties. This experience greatly improved our confidence about the value of this \textit{in vitro} FcRn tool for mAb lead optimization process. It is noteworthy that, mAbs in this set recognize a relatively abundant mouse ligand with a wide range of affinities, and therefore may be subjected to some target-mediated clearance mechanism under the studied conditions.
Discussion

The role of FcRn in extending the half-life of IgG has been well established and the impact of Fc mutants on *in vitro* FcRn binding and *in vivo* PK had also been extensively characterized (Ghetie and Ward, 2000; Dall'Acqua et al., 2002 and 2006; Roopenian and Akilesh, 2007; Zalevsky et al., 2010). However, since FcRn binds to the Fc domain, IgGs with identical Fc sequences had widely been assumed to interact with FcRn similarly. In this study, we systematically examined the interaction between IgG and FcRn using a group of mAbs with identical Fc sequences and showed that the Fab domain can indeed impact both pH 6.0 FcRn binding and pH 7.3 FcRn dissociation. Importantly, we identified a potential IVIVC between pH 7.3 FcRn dissociation and terminal t$_{1/2}$. The steady state location of FcRn is endosomal, where it binds to IgG at acidic pH with high affinity and protects it from lysosomal degradation; the FcRn-bound IgGs are recycled to the cell surface and released upon exposure to neutral pH (Ghetie and Ward, 2000). The exocytosis of FcRn and its IgG cargo had been observed with multifocal plane microscopy, where a tubular was seen to extend from sorting endosomes to exocytic sites at the plasma membrane, the tip of the tubule merged with the plasma membrane in an exocytic event that lasts for only approximately 0.3 seconds, and the tubule retracted quickly back to the sorting endosome after exocytosis (Prabhat et al., 2007). Based on this observation, one can expect that mAb molecules failed to dissociate from FcRn at neutral pH within a very short time window may lose its chance to be released back into circulation and be targeted for lysosomal degradation instead. This provides a potential explanation on why mAbs with more slow dissociation factions have shorter terminal t$_{1/2}$ *in vivo*. Our finding is also consistent with previous observations that the lack of binding at neutral pH is essential, if not more important.
than binding at pH 6.0, for IgGs to be salvaged by FcRn (Vaccaro et al., 2005; Datta-Mannan et al., 2007a and 2007b).

Our results confirmed the previous finding that mAbs with the same Fc sequence can bind to FcRn differently (Suzuki et al., 2010). However, unlike what reported by Suzuki et al., we did not find any correlation between pH 6.0 FcRn binding affinity and in vivo PK for mAbs with wild-type Fc sequences. In fact, a closer examination of the results in Suzuki’s report suggested that the observed correlation can largely be attributed to the FcRn-binding affinity differences between Fc-fusion proteins and mAbs, and no such correlation among the tested mAbs was apparent. From the way FcRn functions, one can fully anticipate that both acidic pH binding and neutral pH dissociation are important for the function of FcRn. For mAbs with wild-type Fc sequence but different Fabs, our results reported here suggested that the neutral pH dissociation could have more impact on in vivo PK. On the other hand, we did observe that pH 6.0 binding affinity appears to be a better predictor of in vivo PK for mAbs with same Fab and different Fc sequences (Wang et al., 2011; unpublished data).

The correlation trend between in vitro FcRn binding/dissociation and in vivo PK we observed here is far from being perfect, although not that unexpected. Besides the known challenges associated with macromolecule bioanalysis and inherited variability of in vivo studies, FcRn is apparently not the only factor that can impact mAb elimination. Factors shown to influence mAb PK include residual target-mediated disposition (intended or non-intended, despite our effort to minimize it), intrinsic mAb stability, glycosylation and immunogenicity (Wang et al., 2008). Recently, Igawa et al. also suggested a potential role of isoelectric point in mAb PK (Igawa et al., 2010). Nevertheless, our results clearly suggested that mAbs with wild-type human Fc sequences
can interact with FcRn differently. This difference in FcRn interaction can have profound impact on mAb PK and should be monitored closely.

This potential IVIVC was first showed in human FcRn mice. Human FcRn mouse is a rodent species with human-like FcRn, thus giving us a surrogate system to study the impact of human or human-like FcRn on human IgG PK. The model also gives us the convenience of studying potential impact of FcRn interaction on PK with minimal interference from target-mediated clearance, because we have access to more mAbs that either does not have a known mouse ligand, or only recognize one in very low abundance. One caveat of this model is that the FcRn expression pattern and its relative expression levels are not known. In addition, human FcRn mice only have mouse IgG in the circulation. Mouse IgGs bind to human FcRn with much lower affinity than that of mAbs with human Fc sequences (Ober et al., 2001) and thus would not provide a competitive environment for mAbs like endogenous NHP or human IgGs would do in NHPs or humans. Nevertheless, the PK data obtained from the human FcRn mouse can be used for screening purpose, reflecting likely relative t1/2 and clearance values of mAbs in NHPs and humans. Besides previous literature reports (Petkova et al., 2006; Zalevsky et al., 2010), our internal data also demonstrated reasonably good terminal t1/2 and clearance correlation between human FcRn mice and NHPs/humans for mAbs at saturating doses (unpublished data).

Consistent with the predictive value of human FcRn mice, a similar correlation between pH 7.3 FcRn dissociation and terminal t1/2 was also shown in NHPs (Fig 3D) and humans (Fig 4B), which confirms the finding in human FcRn mice (Fig 2D).

The exact mechanism of how the Fab domain could impact FcRn interaction is not clear. One possibility is that the Fab domain affects FcRn interaction through its impact on the tertiary or quaternary structure of IgG. A comparison of free and human Fc-bound rat FcRn crystal
structure had revealed quaternary structure rearrangements distant from the FcRn binding site, resulting in an altered inter-domain relationship (Burmeister et al., 1994). We hypothesize that different Fabs may impact this type of rearrangement differently and therefore affect the FcRn interaction. Another interesting possibility is that the Fab domain may impact the ability of FcRn to form dimer. Using computer docking and modeling, the Fab domain of human IgG was found to be quite close to the proposed FcRn dimer interface (Andrei A Golosov, unpublished result). Previous work had shown that rat FcRn only binds to human IgG with high affinity as a dimer (Raghavan et al., 1995). Even though there is no direct evidence suggesting human FcRn can form dimer by itself, it has been suggested that human FcRn may form dimer upon binding to IgG (West and Bjorkman, 2000; Martin et al., 2001). Finally, even though we were unable to identify any biophysical characteristics that uniquely associated with the mAbs showing relatively higher %bound, it is possible that the Fab domain may impact FcRn interaction indirectly, i.e. through local charge or some pro-aggregation conformation that cannot be detected by standard analytical methods.

Regardless of the mechanism of how the Fab domain might impact FcRn binding, it is highly valuable to have an in vitro system that can be indicative of in vivo PK behavior of therapeutic mAbs. Modulating PK is an important aspect of therapeutic mAb development. All therapeutic mAbs are administered parenterally. The ability to develop a mAb with extended half-life and thus less frequent dosing is often crucial for the success of a product. In addition, some types of therapeutic mAbs, e.g. those for immunotoxicotherapy, may benefit from a shorter half-life. Since engineering of the conserved Fc sequences for half-life purpose may raise additional immunogenicity concern, the possibility of modulating mAb half-life through Fab region is an attractive alternative. As we have shown here, in vitro FcRn binding assay could be a highly
valuable complementary tool for mAb PK assessment when incorporated into early lead optimization process, where they can help to identify mAb leads with desired PK properties. It is conceivable that these *in vitro* FcRn tools can also help human PK projection together with interspecies scaling.
DMD #39453

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Authorship Contributions

Participated in research design: Wang, Lu, Hochman, and Prueksaritanont

Conducted experiments: Wang, Lu, Fang, Hamuro, and Pittman

Contributed new reagents or analytic tools: Fang, Hamuro, and Carr

Performed data analysis: Wang, and Lu

Wrote or contributed to the writing of the manuscript: Wang, and Prueksaritanont
References


DMD #39453

Footnotes

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Legends for Figures

Figure 1. SPR sensorgrams of the pH-dependent interaction between mAbs (100nM) and immobilized human FcRn. (A) pH 6.0 binding. (B) pH 7.3 dissociation.

Figure 2. (A) Comparison of human IgG (mAbs) pH 6.0 binding affinity (K_D) to human and hybrid FcRn. (B) Comparison of human IgG (mAbs) pH 7.3 dissociation (%bound) to human and hybrid FcRn. (C) Terminal t_1/2 of mAbs in human FcRn (hFcRn) mice plotted against hybrid FcRn binding affinity (K_D) at pH 6.0. (D) Terminal t_1/2 of mAbs in hFcRn mice plotted against hybrid FcRn %bound at pH 7.3.

Figure 3. (A) Comparison of human IgG (mAbs) pH 6.0 binding affinity (K_D) to human and rhesus FcRn. (B) Comparison of human IgG (mAbs) pH 7.3 dissociation (%bound) to human and rhesus FcRn. (C) Terminal t_1/2 of mAbs in NHPs plotted against rhesus FcRn binding affinity (K_D) at pH 6.0. (D) Terminal t_1/2 of mAbs in NHPs plotted against rhesus FcRn %bound at pH 7.3.

Figure 4. (A) Using a biexponential decline function (Equation (2)) to fit the pH 7.3 human FcRn dissociation curve. (B) Terminal t_1/2 of mAbs in humans plotted against model derived pH 7.3 dissociation parameters k_2/B.

Figure 5. Terminal t_1/2 of new mAb leads and control mAbs in hFcRn mice plotted against hybrid FcRn %bound at pH 7.3.
Tables

Table 1. *In vitro* human FcRn binding and dissociation parameters of MK-1 to MK-6

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MK-1</th>
<th>MK-2</th>
<th>MK-3</th>
<th>MK-4</th>
<th>MK-5</th>
<th>MK-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>isotype</td>
<td>IgG1</td>
<td>IgG2</td>
<td>IgG1</td>
<td>IgG2</td>
<td>IgG1</td>
<td>IgG2</td>
</tr>
<tr>
<td>Fab</td>
<td>A</td>
<td>A</td>
<td>B</td>
<td>B</td>
<td>C</td>
<td>C'</td>
</tr>
<tr>
<td>K_D (nM) at pH 6.0</td>
<td>56.3</td>
<td>73.0</td>
<td>18.5</td>
<td>24.7</td>
<td>25.7</td>
<td>33.2</td>
</tr>
<tr>
<td>%bound at pH 7.3</td>
<td>0.4%</td>
<td>0.0%</td>
<td>2.5%</td>
<td>2.9%</td>
<td>12.9%</td>
<td>9.9%</td>
</tr>
</tbody>
</table>

#: MK-1 and MK-2 have identical Fab domain A, MK-3 and MK-4 have identical Fab domain B, MK-5 and MK-6 have closely related, but slightly different Fab domains C and C'
**Table 2. Noncompartmental PK parameter estimates in hFcRn mice following 10 mg/kg IV dosing. Data are means ± S.D. (n = 3-4)**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Clearance (mL/h/kg)</th>
<th>Vdss (mL/kg)</th>
<th>Terminal t1/2 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK-1</td>
<td>0.58±0.18</td>
<td>100±14</td>
<td>129±31</td>
</tr>
<tr>
<td>MK-2</td>
<td>0.76±0.21</td>
<td>130±28</td>
<td>127±34</td>
</tr>
<tr>
<td>MK-3</td>
<td>0.78±0.03</td>
<td>105±11</td>
<td>93±7</td>
</tr>
<tr>
<td>MK-4</td>
<td>0.95±0.08</td>
<td>132±10</td>
<td>104±4</td>
</tr>
<tr>
<td>MK-5*</td>
<td>1.56±0.06</td>
<td>--</td>
<td>44±6</td>
</tr>
<tr>
<td>MK-6</td>
<td>3.32±0.53</td>
<td>153±28</td>
<td>44±3</td>
</tr>
<tr>
<td>adalimumab</td>
<td>0.86±0.07</td>
<td>81±12</td>
<td>86±16</td>
</tr>
<tr>
<td>basiliximab</td>
<td>0.76±0.04</td>
<td>117±9</td>
<td>120±5</td>
</tr>
<tr>
<td>bevacizumab</td>
<td>1.04±0.05</td>
<td>110±13</td>
<td>76±7</td>
</tr>
<tr>
<td>cetuximab</td>
<td>1.49±0.15</td>
<td>111±14</td>
<td>66±4</td>
</tr>
<tr>
<td>Trastuzumab*</td>
<td>1.49±0.24</td>
<td>--</td>
<td>81±1</td>
</tr>
</tbody>
</table>

*: The Vss of MK-5 and trastuzumab were not reported as their drug levels at the first time point (1h) significantly deviated from the theoretical values. Their clearance values were also expected to be less accurate, but the terminal t1/2 values were less likely to be impacted.
Figure 1.
Figure 2

A

$K_0$ at pH 6.0, human FcRn (nM)

B

%bound at pH 7.3, human FcRn (%)

C

Terminal $t_{1/2}$, hFcRn mice (h)

D

Terminal $t_{1/2}$, hFcRn mice (h)

$R^2 = 0.9959$

$R^2 = 0.9719$
Figure 3

A

$K_d$ at pH 6.0 human FcRn (nM) vs $K_d$ at pH 6.0 rhesus FcRn (nM)

$R^2 = 0.9763$

B

%bound at pH 7.3 human FcRn (%) vs %bound at pH 7.3 rhesus FcRn (%)

$R^2 = 0.9874$

C

Terminal $t_{1/2}$ NHP (h) vs $K_d$ at pH 6.0 rhesus FcRn (nM)

MK-2, MK-3, MK-5, bevacizumab, trastuzumab, basiliximab, adalimumab

D

Terminal $t_{1/2}$ NHP (h) vs %bound at pH 7.3 rhesus FcRn (%)

MK-3, MK-2, MK-6, bevacizumab, trastuzumab, basiliximab, adalimumab, MK-5
Figure 4.

A

\[ RU_t = A e^{-k_1 t} + B e^{-k_2 t} + C \]

B

Terminal \( T_{1/2} \)-human (h) vs. \( k_2/B \)

- Omalizumab
- Palivizumab
- Bevacizumab
- Infliximab
- Trastuzumab
- Adalimumab
- MK-A
- MK-B
Figure 5.