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Differences in promiscuity for antibody–FcRn interactions across species: implications for therapeutic antibodies

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Abstract

Preclinical tests of therapeutic antibodies are frequently carried out in mice to evaluate pharmacokinetics and efficacy. However, the observation that mouse IgG are cleared rapidly from the human circulation suggests that mice may not always be an ideal model. The Fc receptor, FcRn, regulates the serum half-lives of IgG in mice and most likely has a similar function in humans. In the current study we have carried out an extensive analysis of the interaction of the human or mouse forms of FcRn with IgG from various species using surface plasmon resonance. We show that in contrast to mouse FcRn, human FcRn is surprisingly stringent in its binding specificity for IgG derived from different species. Human FcRn binds to human, rabbit and guinea pig IgG, but not significantly to rat, bovine, sheep or mouse IgG (with the exception of weak binding to mouse IgG2b). In contrast, mouse FcRn binds to all IgG analyzed. The lack of binding of human FcRn to mouse IgG1 has been confirmed using transfectants that have been engineered to express human FcRn on the cell surface. Our results provide a molecular explanation for the enigmatic observation that mouse IgG behave anomalously in humans. These studies have implications for the successful application of therapeutic antibodies.

Introduction

During the past several years there has been an increased interest in the development of therapeutic antibodies, primarily due to the development of genetic engineering approaches to produce IgG with improved properties (reviewed in 1). Prior to use in humans, preclinical tests are routinely carried out in animal species. For example, analyses of the pharmacokinetics of a therapeutic IgG are frequently performed in mice. Knowledge of the pharmacokinetics is essential for effective use of an antibody in a particular therapeutic/diagnostic application. An indication that mice may not be an ideal species in which to model the pharmacokinetics of an IgG for use in humans, however, is the observation that mouse IgG is cleared rapidly from the human circulation relative to its long serum persistence in mice (2,3). This rapid clearance may contribute to the disappointing performance of mouse antibodies in clinical trials, particularly when extended treatment regimens are used (4). Understanding the molecular basis for this phenomenon is the focus of the current study.

It has been suggested previously that the short half-life of mouse IgG in humans is due to differential glycosylation of IgG in mouse versus human cells, resulting in the presence of Galα1–3Gal residues in antibodies produced in murine cells (4). In humans, these residues may be recognized by a high titer of anti- Galα1–3Gal antibodies resulting in short serum persistence. However, therapeutic humanized antibodies produced in murine cells have been reported to have half-lives of 20 days, indicating that the involvement of anti-Galα1–3Gal antibodies in the rapid removal of murine IgG may be negligible (5). Thus, the reason for the anomalous pharmacokinetics of mouse IgG in humans has been a matter of controversy.

Until recently, knowledge of the mechanism by which serum IgG levels are maintained in the circulation was limited. Studies in mice indicate that the MHC class I-related receptor, FcRn (6), regulates the serum levels of IgG (7–9), in addition

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to the known role of this receptor in the transfer of IgG from mother to young (10). The receptor carries out these apparently diverse roles by binding to the Fc region of IgG, and transporting the bound IgG within and across cells (11,12). It is therefore a protective receptor, salvaging IgG molecules from degradation following uptake into FcRn-expressing cells (13). As a consequence, IgG molecules that do not bind to FcRn are not transported from mother to young (14) and have an abnormally short serum persistence (15,16). Furthermore, there is a direct correlation between binding affinity of IgG/Fc fragments for FcRn and their serum half-lives in mice (16). This knowledge has been used to engineer antibodies with predefined serum persistence (17).

Although much is now known about FcRn function in rodents (13), less is known about the role of the human homolog (18). Human FcRn is expressed in placenta (19–21) and recent studies indicate that this receptor plays an essential role in IgG transfer across the *in vitro* perfused placenta (22). As in mice (23), human FcRn has been reported to be expressed in endothelial cells, suggesting that it is also involved in serum IgG homeostasis (24). The site of binding for rodent FcRn on human and mouse IgG molecules closely overlaps, and encompasses Ile253, His310 and His435 (16,25,26). These residues are located at the C_H2–C_H3 domain interface (27) in the Fc region of IgG and are highly conserved across species (28). The less-conserved residue at position 436 (His in most mouse and rat IgG isotypes, Tyr or Phe in human IgG) also plays a role in binding to rodent FcRn (16,26,29). A recent analysis indicated that individual mutation of Ile253, Ser254, His435 and Tyr436 of human IgG1 to alanine essentially ablates binding to human FcRn (29). An essential role for His435 in FcRn-mediated transfer of human IgG1 across the *ex vivo* placenta has also been demonstrated (22). However, to our knowledge, the interaction of human FcRn with IgG from heterologous species has not been investigated. In addition, analysis of the binding characteristics of mouse/rat FcRn has been limited to rodent FcRn–rodent IgG or rodent FcRn–human IgG interactions (15,25,26,30–32).

In the current study we have attempted to better understand FcRn function in humans by comparing the binding properties of human and mouse FcRn. Extensive BIAcore analyses of the binding of IgG from various species (human, mouse, rabbit, guinea pig, bovine, sheep and rat) to recombinant human and mouse FcRn have been carried out to analyze the cross-species specificity of these two Fc receptors. In addition, a novel cell binding assay in which transfectants engineered to express human FcRn on the cell surface has been used to analyze IgG–FcRn interactions with membrane-bound receptor. The binding data using the two approaches are consistent and indicate that in contrast to mouse FcRn which binds to IgG of every species analyzed, human FcRn is surprisingly stringent in its specificity. The lack of binding of human FcRn to most mouse isotypes, in particular, provides a molecular explanation as to why mouse IgG have short serum persistence in humans. This provides further support for the hypothesis that FcRn regulates serum IgG half-life in man. The marked differences in binding specificity between human and mouse FcRn have implications for the successful application of therapeutic antibodies in humans.

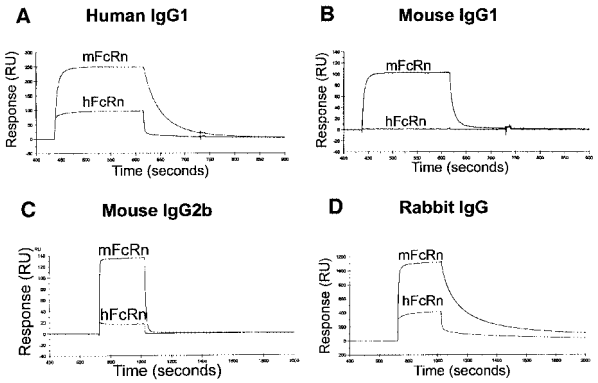

Fig. 1. Overlays of sensorgrams of 1 μM mouse or human FcRn injected over immobilized human IgG1 (784 RU) (A), mouse IgG1 (914 RU) (B), mouse IgG2b (650 RU) (C) or rabbit IgG (3023 RU) (D). All sensorgrams are representative of duplicate injections.

Methods

Sources of IgG

Recombinant human IgG1 (HuLys10) secreted by transfected myeloma cells (a generous gift of Dr Jeff Foote, Fred Hutchinson Cancer Center, Seattle) was isolated and purified as described previously (33). This is a humanized variant of the murine anti-hen egg lysozyme antibody D1.3 (33). Human IgG was isolated from a commercial 'gammaglobulin' preparation (Sandoz, East Hanover, NJ; containing IgG, and a low percentage of IgM, IgA, transferrin and dimeric IgG) by affinity purification over Protein G–Sepharose and HPLC on a TSK-3000SW column. Rabbit and guinea pig IgG were obtained from normal sera (purchased from Sigma, St Louis, MO) by affinity chromatography on Protein A–Sepharose. The subclasses of guinea pig IgG were obtained by further fractionation on DEAE–Sepharose (34). Bovine and sheep IgG were purchased from the Jackson Laboratory (Bar Harbor, MA). Rat and mouse IgG subclasses were purchased from Zymed (South San Francisco, CA). Mouse IgG2b was also isolated from mouse serum using previously described methods (35). The source of mouse IgG1 was either a mAb directed against human CD22 (RFB4) (36), against human CD25 (RFT5) or against hen egg lysozyme (D1.3) (37).

Recombinant FcRn

Recombinant mouse and human FcRn were expressed in infected High-Five cells and purified as described previously (15,22).

Surface plasmon resonance (SPR) experiments

CM5 sensor chips were coupled with IgG of different species at densities ranging from ~600 to 3000 RU, using a BIAcore 2000. In addition, for use as a reference cell, one flow cell was treated with coupling buffer only during the coupling reaction. Human or mouse FcRn was injected over the coupled flow cells at concentrations ranging from 0.17 to 2 μM at a flow rate of 10 μl/min in PBS, pH 6.0 plus 0.01% Tween 20. Each injection was carried out in duplicate and the data were processed by zero adjusting and reference cell subtraction

Table 1. Relative binding of human FcRn and mouse FcRn to IgG

Species and isotype	R_{eq} (mFcRn)[a]	R_{eq} (hFcRn)[a]	R_{eq} (hFcRn)/R_{eq} (mFcRn)
Human IgG1[b]	243.8	91.8	0.38
Mouse IgG1[c]	100.6	0.22	0.002
Mouse IgG2a	698.6	3.9	0.006
Mouse IgG2b[d]	142.5	16.8	0.13
Rabbit IgG	1117.7	409.3	0.37
Guinea pig IgG1	87.1	61.6	0.71
Guinea pig IgG2	279.7	183.5	0.66
Bovine IgG[e]	643.8	25.8	0.04
Sheep IgG[e]	304.6	8.5	0.03
Rat IgG1	224.2	3.8	0.02
Rat IgG2a	417.9	9.4	0.02
Rat IgG2b	54.1	<0[f]	ND[f]
Rat IgG2c	484.5	<0[f]	ND[f]

[a]Average of two duplicate injections.
[b]Representative of at least six experiments with different coupling densities of human IgG1 (HuLys10).
[c]Carried out with three different sources of mouse IgG1 (D1.3, RFT5 and RFB4).
[d]Carried out with two different sources of mouse IgG2b.
[e]Mixture of all isotypes for this species.
[f]Sensorgrams for 1 μM human FcRn were slightly below zero after reference cell subtraction. ND, not determined as <0.

using either BIAevaluation 3.0 or custom written software. Dissociation constants were not calculated as for some species, flow cells were coupled simultaneously with all IgG isotypes of that species. This resulted in heterogeneity of the ligand, making calculated dissociation constants meaningless. In addition, for some species FcRn is known to bind to two possible sites on IgG with different affinities (38,39) and the stoichiometry of the FcRn–IgG interaction for the majority of the IgG used in this study has not been analyzed. Thus, the binding of human FcRn relative to mouse FcRn to IgG of each species is expressed as R_{eq}(hFcRn)/R_{eq}(mFcRn) where R_{eq}(hFcRn) is the signal level (in RU) at equilibrium for 1 μM human FcRn and R_{eq}(mFcRn) is the signal level (in RU) at equilibrium for 1 μM mouse FcRn.

Generation of transfectants expressing human FcRn

The gene encoding full-length human FcRn was isolated from the intestinal epithelial cell line, Caco-2 (ATCC HTB 37), using RT-PCR and designed oligonucleotide primers. This gene was tailored with 5′ and 3′ *Eco*RI sites and cloned in frame into the *Eco*RI site of pEGFP-N1 (Clontech, Palo Alto, CA). A mutated variant of the FcRn gene was also cloned into this vector. This mutant was generated by converting Leu320-Leu321 (in the cytoplasmic tail) to Ala320Ala321 using designed oligonucleotides and splicing by overlap extension (40). The FcRn–green fluorescent protein (GFP) expression plasmids were transfected into Jurkat cells by electroporation and clones selected in RPMI containing 10% FCS, 10 mM HEPES, 1 mM L-glutamine and 1 mg/ml G418. Selected clones were screened by flow cytometry and cloned by limiting dilution.

Fluorescence microscopy

Images of transfectants expressing human FcRn–GFP fusions were acquired using a Zeiss Axiovert 100 microscope with a Hamamatsu Orca 100 camera. Epifluorescence images were acquired with a Zeiss ×100 NA 1.4 Plan Apo objective and Chroma EGFP filter set. Total internal reflection microscopy (TIRFM) images were acquired with an Olympus ×100 NA 1.65 Plan Apo objective. TIRFM was achieved by excitation with a Spectra Physics laser at 488 nm. The total internal reflection set-up was custom made following the through-objective excitation model. In TIRFM the illumination level decreases exponentially depending on the distance from the coverslip. As a result fluorescent labels (GFP) are excited only in a relatively thin layer near the coverglass that includes the membrane of a cell juxtaposed on the coverglass. Images for both mutant and wild-type cells were acquired with the same exposure time (3 s). Individual cells were placed at the center of the field of view using transmitted and epifluorescent illumination, and the focal plane scanned away from the coverslip. No subsequent processing such as contrast adjustment was carried out.

Binding of IgG to transfectants expressing human FcRn

Two types of binding assay were used. For use in all assays, cells were grown in medium containing FCS which had been depleted of endogenous IgG by passage over Protein G–Sepharose. In the flow cytometric assays, IgG were biotinylated (NHS-biotin; Pierce, Rockville, MD) and preincubated with Extravidin–phycoerythrin (PE) in an ~4:1 (IgG:Extravidin) molar ratio to 'multimerize' the IgG molecules. These complexes (containing 2 μg IgG) were then added to transfectants (2×10^6/ml in 100 μl), incubated for 30 min at 4°C in 1% BSA/PBS, pH 6.0 and washed twice with PBS, pH 6.0 or 7.2. Cells were analyzed by two-color flow cytometry and data were plotted using WinMDI version 2.8.

In additional binding assays, IgG dimers were generated and used. Human and mouse IgG1 (RFB4) were dimerized by cross-linking with succinimidyl 4-(maleimidomethyl cyclohexane) and *N*-succinimidyl S-acethylthio-acetate (Pierce) as described (41). The IgG dimers (M_r ~ 300 kDa) were purified by HPLC using a preparative TSK-3000SW column (TosoHaas, Montgomeryville, PA), and contained <10% monomers and polymers as determined by SDS–PAGE (PhastSystem; Pharmacia, Piscataway, NJ). The IgG dimers were biotinylated or radiolabeled with [^{125}I]Na using the Iodogen method (42) to a specific activity of 1 μCi/μg. Biotinylated dimer was incubated with transfectants as above, except that cells were first incubated with 2–4 μg dimer for 30 min at 4°C, washed once in PBS, pH 6.0 and then incubated with 0.4 μg Extravidin–PE for a further 30 min at 4°C. Cells were washed twice with PBS, pH 6.0 or 7.2 and analyzed by flow cytometry as above.

For the analysis of binding of radiolabeled dimers, cells (1×10^7/ml) were incubated at 4°C for 1 h with varying amounts of radioligands and for each concentration, 100 μl aliquots (in triplicate) were centrifuged in 200 μl Fisher plastic tubes over an 1:1 oil mixture dibutylphthalate/phthalic acid bis(2-ethylhexylester) (Sigma). The test tubes were frozen at –80°C, tips containing the sedimented cells cut off and the radioactivity measured in a γ-counter (Pharmacia). The binding of the radioligands was carried out at pH 6.0 and

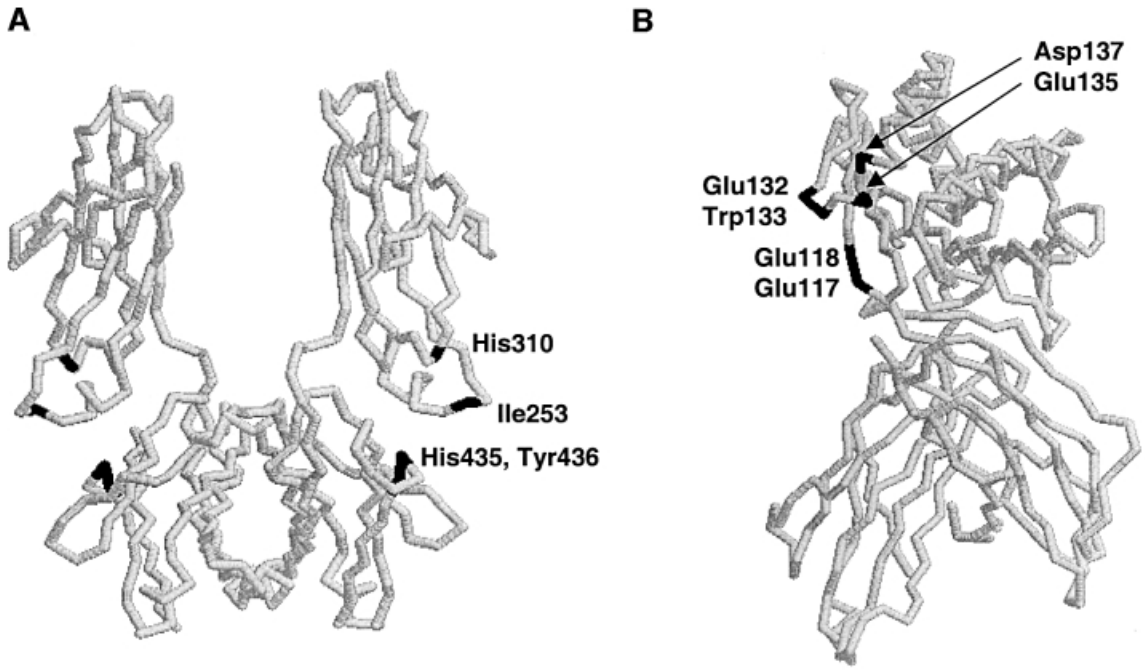

Fig. 2. (A) Structure (α-carbon trace) of the Fc region of human IgG1 (27) with the location of the residues shown to be involved in mouse, rat or human FcRn binding indicated (22,25,26,29,30). (B) Structure α-carbon trace of rat FcRn (55) showing the residues involved in the rat FcRn–rat Fc complex (26). (Drawn using RASMOL, courtesy of Roger Sayle, Bioinformatics Research Institute, University of Edinburgh, UK.)

7.5. The specific binding of the ligands was calculated by subtracting the amount bound at pH 7.5 from that bound at pH 6.0. The specificity of the binding was demonstrated by using a 500-fold excess of unlabeled human IgG dimer.

Analysis of biotinylation of IgG

Biotinylated, monomeric IgG were coated onto wells of 96-well plates in varying concentrations and detected with an Extravidin–horseradish peroxidase conjugate (Sigma).

Binding of IgG dimers to FcγRII

Daudi cells (FcγRII$^+$) at 2×10^6 cells/ml were treated with various concentrations of radiolabeled human and mouse IgG dimers in RPMI 1640 medium containing 10% FCS at pH 7.4 for 1 h at 4°C. The cell-bound radioactivity was determined using centrifugation through oil as described above. The specificity of the binding was determined by using a 500-fold excess of unlabeled human or mouse IgG dimer.

Results

Initial studies using soluble recombinant human or mouse FcRn using SPR indicated that both species of FcRn bind to human IgG1 (Fig. 1). Binding to other human IgG isotypes was not analyzed here as recent studies indicate that the human FcRn–IgG interaction is similar for all subclasses (43) (our unpublished data). Consistent with our earlier data (22), the affinity of the human FcRn–human IgG1 interaction is significantly lower than that of mouse FcRn–human IgG1 complex formation (Fig. 1 and Table 1). However, in contrast to mouse FcRn, human FcRn shows negligible binding to mouse IgG1 (Fig. 1 and Table 1). Analyses of the binding of human FcRn to other mouse IgG isotypes demonstrated essentially no interaction with mouse IgG2a and a weak interaction with mouse IgG2b (Fig. 1 and Table 1). Analysis of sequence differences between human IgG1 and the murine IgG isotypes in the vicinity of the FcRn interaction site (16,25,26,44,45) (Fig. 2) suggested that variation of position 436 (histidine in mouse IgG1, 2a; tyrosine in human IgG1, mouse IgG2b) (28) might explain the differential binding. This prompted an analysis of the interaction of human and mouse FcRn with IgG from different species for which position 436 is either histidine (all rat isotypes), tyrosine (sheep, rabbit, bovine) or valine (guinea pig) (28). The binding data are summarized in Table 1 by expressing the activity of human FcRn as the equilibrium signal level (R_{eq}, in RU) for 1 μM human FcRn divided by the equilibrium signal level for 1 μM mouse FcRn. From these data it is clear that the majority of IgG are not bound at significant levels by human FcRn. In contrast, mouse FcRn binds well to every IgG tested. For several reasons dissociation constants for each interaction were not calculated in these studies. First, in several cases, mixed isotypes were used as immobilized ligand and this results in ligand heterogeneity. Second, IgG has two possible interaction sites for FcRn (38,39) and the stoichiometry of the FcRn–IgG interaction is not known for most of the IgG isotypes analyzed. However, the semi-quantitative data shown in Table 1 clearly demonstrate that human FcRn is highly stringent in its binding specificity, whereas mouse FcRn cross-reacts with all IgG tested.

A cell binding assay was also developed to allow a possible correlation between IgG interactions with soluble FcRn in SPR

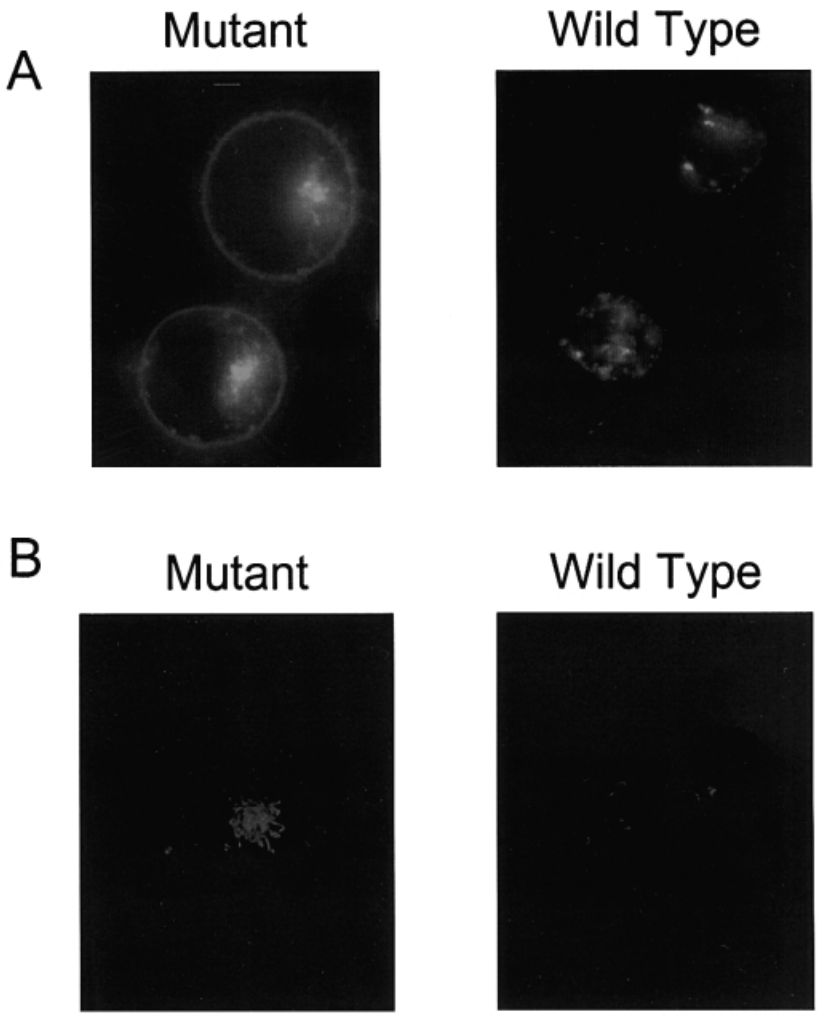

Fig. 3. Expression of mutated or wild-type human FcRn tagged with GFP in transfected Jurkat cells. (A) Epifluorescence images. (B) TIRFM images to visualize only membrane associated fluorescence. All images are representative of multiple cells analyzed.

and membrane-bound FcRn to be investigated. Jurkat cell transfectants which express a mutated, human FcRn–GFP fusion on the cell surface were generated. The mutation of FcRn involves conversion of the dileucine motif (residues 320/321) in the cytosolic tail to di-alanine, and this motif is known to regulate receptor internalization (46,47). Relative to transfectants expressing wild-type FcRn–GFP ('WT') which have undetectable levels of surface FcRn, microscopic analyses using both epifluorescence and TIRFM demonstrate that the mutated FcRn ('MUT') is present on the cell surface of transfectants (Fig. 3). Both transfectants express similar levels of total FcRn–GFP (Fig. 4), excluding the possibility that the differential surface expression was due to variations in protein levels.

The surface expression of FcRn allows the MUT cells to be used in direct binding assays with labeled IgG. However, when labeled, monomeric IgG were used in various forms, undetectable binding was observed. This is consistent with the relatively low affinity and fast dissociation of human FcRn for/from cognate ligand (~2 μM) (22). To compensate for the low affinity of the FcRn–IgG interaction, the avidity of the interaction was increased by incubating biotinylated IgG with Extravidin (labeled with PE) to generate multimeric complexes. Figure 4 shows binding analyses using flow cytometry of biotinylated, multimerized human IgG1 and mouse IgG1 to the WT and MUT transfectants. Binding analyses were carried out at pH 6.0 with washes at either pH 6.0 or 7.2, as FcRn interacts with IgG at slightly acidic but not near neutral pH (15,30). This pH dependence is necessary for the binding and recycling/transcytotic function of FcRn (13). For human IgG1 a minor amount of binding to WT cells can be consistently detected at pH 6.0 (Fig. 4 and data not shown). This binding cannot be detected at pH 7.2 (data not shown), suggesting specificity for FcRn. The minor amount of IgG binding at pH 6.0 indicates that for the WT FcRn transfectants, there may be a limited amount of surface FcRn expression which is below the level of detection by immunofluorescence (Fig. 3). The data with transfectants expressing MUT FcRn confirm the high level of surface expression of this FcRn variant (Figs 3 and 4); for human IgG1 significant amounts of binding are

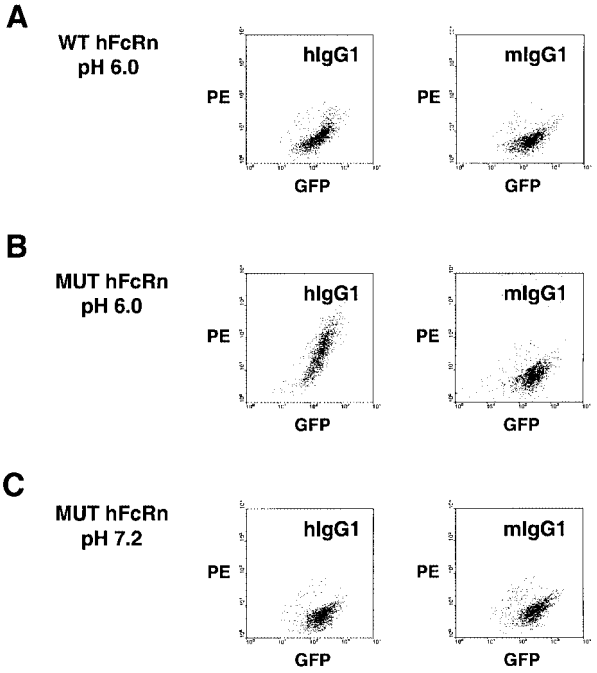

Fig. 4. Flow cytometric analysis of binding of biotinylated human and mouse IgG1 (hIgG1 and mIgG1 respectively) to transfected Jurkat cells expressing either intracellular wild-type FcRn (WT hFcRn) or mutated FcRn on the surface (MUT hFcRn). Biotinylated IgG were preincubated with Extravidin–PE to allow multimerization of IgG molecules and detection in flow cytometry. (A) Binding of PE-labeled hIgG1 or mIgG1 to WT hFcRn transfectants at pH 6.0. (B) Binding of PE-labeled hIgG1 or mIgG1 to MUT hFcRn transfectants at pH 6.0. (C) Binding of PE-labeled hIgG1 or mIgG1 to MUT hFcRn transfectants followed by washes at pH 7.2. Two-dimensional dot-plots are shown to indicate level of FcRn expression (GFP) and staining by PE-labeled IgG (PE). Data are representative of a total of three experiments with either biotinylated IgG or biotinylated IgG dimers.

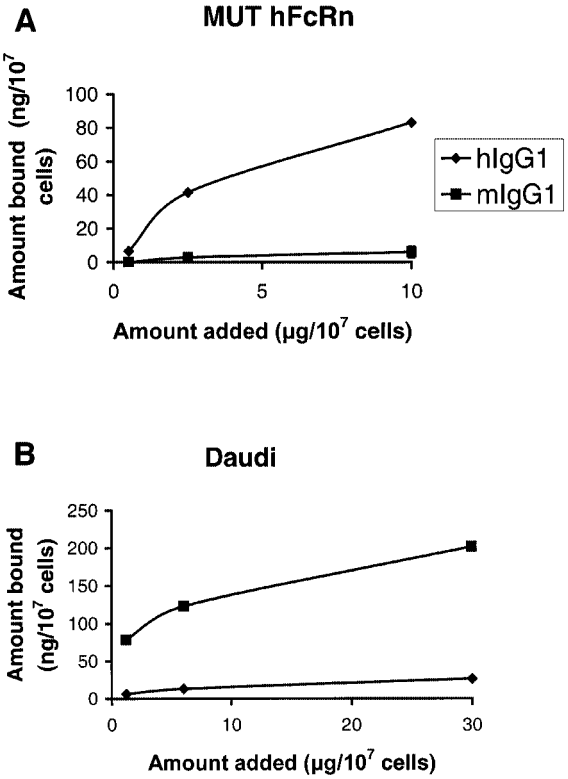

Fig. 5. Binding of radiolabeled human and mouse IgG1 dimers (hIgG1 and mIgG1 respectively) to Jurkat transfectants expressing mutated human FcRn (MUT hFcRn; A) or to Daudi cells (B). For (A), data are representative of five independent experiments. For (B), averages of three experiments are shown. For both (A) and (B), error bars are smaller than the symbols and are therefore generally not visible.

seen at pH 6.0 but not at pH 7.2. In contrast, and consistent with the SPR data, insignificant binding is seen for biotinylated mouse IgG1 (Fig. 4). The activity and biotinylation of the mouse IgG1 were analyzed using SPR (with mouse FcRn) and ELISA respectively, and excluded the possibility that either the mouse IgG1 is not active or has not been biotinylated.

These studies were extended to the analysis of the binding of human or mouse IgG1 dimers (41) to the transfectants (Fig. 5). To assess the dose dependency and specificity of binding, the dimers were radiolabeled and added in varying amounts to the transfectants. The radiolabeled (^{125}I) human IgG1 dimer bound to MUT transfectants in a dose-dependent way, whereas essentially background levels of binding of radiolabeled mouse IgG1 dimer were seen (Fig. 5). Similar results were obtained using flow cytometry and biotinylated human and mouse IgG1 dimers (using Extravidin–PE for detection; data not shown). The binding of radiolabeled human IgG1 dimer (at 0.5 µg/10^7 cells) could be inhibited by >90% by the addition of a 500-fold excess of unlabeled human IgG1 (data not shown). To assess the integrity of the Fc region of the radiolabeled mouse IgG1 dimer, binding to FcγRII on the surface of Daudi cells was analyzed. This Fc receptor is known to bind to residues in the C_H2 domain of IgG (48,49). The mouse IgG1 dimer binds to significantly higher levels than the human IgG1 dimer, demonstrating that it has retained functional activity. The binding of both human and mouse IgG1 dimers to Daudi cells are equally well inhibited by a 500-fold excess of unlabeled human or mouse IgG dimers (>90% inhibition; data not shown), indicating specificity of binding.

Discussion

In the current study we have carried out an extensive analysis of the binding of IgG from a range of species to soluble recombinant mouse and human FcRn using SPR (BIAcore) experiments and a cell binding assay. It is well established that FcRn is a receptor that salvages IgG from degradation and regulates the serum half-life of IgG in mice (7–9). Recent data indicate that the human homolog of mouse FcRn serves a similar function in humans (22,24). Our analyses demonstrate that mouse FcRn is promiscuous in binding specificity and binds to all IgG species analyzed, i.e. human, mouse, rabbit, guinea pig, bovine, sheep and rat IgG. In contrast, human FcRn is surprisingly stringent, and only interacts well with human, rabbit and guinea pig IgG. Of particular interest,

Table 2. Residues known (26) or predicted to be involved in FcRn–IgG interactions

Rat FcRn[a]	Rat IgG	Human FcRn[b]	Human IgG[b]	Mouse FcRn[b]	Mouse IgG[b]
Glu117	His310	Glu117	His310	Glu117	His310
Glu118	Gln311[c]	Glu118	Gln311	Glu118	Gln311
Glu132	His435	Asp132	His435[d]	Glu132	His435[e]
Trp133	Ile253	Trp133	Ile253	Trp133	Ile253
Asp137	His436	Leu137	Tyr436[f]	Glu137	His436[g]

[a]Glu135 is not shown, but makes interactions with backbone amide nitrogens of rat IgG2a residues 253 and 254 (26).
[b]Predicted from the X-ray crystallographic structure of rat FcRn complexed with rat IgG2a (26). Note that for human FcRn the two amino acid deletion (residues 85,86) is ignored in the numbering which corresponds to the homology alignment of rodent and human FcRn.
[c]In all rat isotypes, except in rat IgG2a where residue 311 is Arg (28).
[d]In all human isotypes, except in an IgG3 allotype where residue 435 is Arg (28).
[e]In all mouse isotypes, except in IgG2b where residue 435 is Tyr (28).
[f]In all human isotypes, except in an IgG3 allotype where residue 436 is Phe (28).
[g]In all mouse isotypes, except in IgG2b where residue 436 is Tyr (28).

human FcRn does not bind detectably to mouse IgG1 and IgG2a, and shows a very weak interaction with mouse IgG2b. The high selectivity of the human FcRn–IgG interaction therefore gives a molecular explanation for the enigmatic observation that mouse IgG are cleared rapidly from the human circulation (2,3). Despite this observation many years ago, to date a satisfactory explanation had been lacking (5). Significantly, the current analyses further implicate a role for FcRn in regulating the pharmacokinetics of serum IgG in humans.

The high off-rate of the human FcRn–IgG interaction reported previously in SPR studies (22) together with the undetectable binding of monomeric human IgG1 to transfectants expressing surface FcRn (MUT transfectants) raises the question as to how FcRn carries out its role *in vivo*. FcRn functions to maintain circulating levels of monomeric IgG or to transport monomeric IgG molecules across cellular barriers (reviewed in 13). This suggests that wild-type, membrane-bound FcRn is able to form higher-order aggregates (e.g. dimers) (50) that increase the avidity of the FcRn–IgG interaction. Consistent with a role for avidity effects, in SPR experiments immobilized human FcRn has a significantly higher affinity/avidity for human IgG than the affinity of the FcRn interaction with immobilized IgG (22,43). Furthermore, studies with rodent and human FcRn indicate that FcRn can bind to two possible sites on IgG, albeit with different intrinsic affinities (38,39,43). A possible reason for the need to multimerize IgG to detect binding to MUT FcRn transfectants is therefore that mutation of the cytoplasmic dileucine motif to dialanine results in defects in aggregation of the membrane exposed FcRn. Alternatively, the density of FcRn on the cell surface may be lower than that when it is endosomally located. Both these possibilities would be predicted to result in a decreased efficiency of FcRn binding to the putative receptor interaction sites on both 'sides' of an IgG molecule to form 1:2 IgG:FcRn complexes. Despite this, the MUT transfectants provide a valuable cell binding assay and give rise to data that is consistent with our SPR analyses.

The homology shared between human and mouse FcRn (18,51), together with the observation that IgG residues such as His435 play a central role in the IgG:FcRn interaction of both rodent and human FcRn (16,22,25,26,29), led to the earlier conclusion that these interactions are very similar across species. In the current study we have carried out an analysis of the interactions of human and mouse FcRn with human, mouse, rabbit, guinea pig, bovine, sheep and rat IgG which significantly extends our knowledge of human/mouse FcRn interactions to IgG of multiple species. Previously, analyses of FcRn binding to IgG had been restricted to homologous IgG with the exception of mouse FcRn/rat or human IgG and rat FcRn/human or mouse IgG (15,25,26,30–32,53). The current analyses indicate that unexpectedly, human and mouse FcRn behave quite differently with respect to their cross-species binding behavior.

The distinct cross-species behavior of human/mouse FcRn raises questions concerning the molecular basis for this phenomenon. Although the key residues that constitute the FcRn interaction site on IgG are conserved across species, there are some variations at 'secondary' IgG residues such as His436 (28). Rat FcRn residues which have been shown in both functional and structural studies (Glu117, Glu118, Glu132, Trp133, Glu135 and Asp137) to be involved in the rat FcRn–IgG interaction (26,32), are well conserved across species with the exception of Asp137 (6,18,51,52) (Table 2). The location of these residues on the three-dimensional structure of rat FcRn is shown in Fig. 2. In human FcRn residue 137 is replaced by Leu and Glu132 is conservatively replaced by Asp (18) (Table 2). In the rat FcRn–rat IgG complex (26), Asp137 makes a salt bridge with His436 (Table 2). The replacement of this amino acid with Leu in human FcRn would preclude formation of this salt bridge and would also be energetically unfavorable due to the presumed juxtaposition of a hydrophobic residue next to His436 (54). Although this could explain the lack of binding of human FcRn to mouse IgG1, IgG2a and all rat IgG, it cannot be implicated in the inability of human FcRn to bind to bovine and sheep IgG which, like human IgG, have Tyr at position 436. We (16,31) and others (29) have shown that in addition to the key IgG residues involved in binding to FcRn (residues 253, 310, 435 and 436), there are other residues at positions 254, 255, 257, 288, 296, 307, 309, 315, 415 and 433 that may play more minor (indirect) roles in the IgG–FcRn interaction. Analysis of the variability of these amino acids across species does not give a consistent explanation for the lack of binding

of human FcRn to mouse, rat, sheep and bovine IgG. Thus, there is no obvious explanation for our observations concerning the high specificity of human FcRn. However, it is possible that the differences in cross-species behavior of mouse versus human FcRn could be due to the less exposed position of Trp133, a residue that contacts Ile253 in the rat FcRn–Fc complex (26), and/or deletion of residues 85 and 86 (located in the α_1 domain) in human FcRn (43).

In addition to giving an explanation for the observation that mouse IgG are cleared rapidly from the human circulation which may in turn explain their limited efficacy in human therapy (4), our findings have an additional consequence of practical significance: they raise questions concerning the validity of using mice as a model to predict the pharmacokinetic behavior of a therapeutic antibody in humans. Mutation of His435 to alanine ablates the binding of human IgG1 to both mouse and human FcRn, in addition to decreasing activity of the IgG1 in FcRn-mediated functions such as transcytosis (22,25,29). This suggests that FcRn–IgG interactions are, in some respects, very similar across species. However, we show here that there are also significant differences in the specificity of the interactions, with human FcRn demonstrating high selectivity for binding to IgG of a subset of species analyzed. The lack of interaction of human FcRn with most mouse and all rat IgG indicates that mice cannot be reliably used to extrapolate half-life data of rodent antibodies to humans. Although rodent IgG are now less frequently used in therapy, our observations indicate that a genetically manipulated antibody may behave differently in mouse and man. To predict the half-life of a therapeutic antibody in humans, preclinical studies in mice should therefore be complemented by binding studies with human FcRn. The high stringency of the human FcRn–IgG interaction described in this study has obvious implications for the effective use of genetically engineered antibodies in diagnosis and therapy.

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Abbreviations

GFP	green fluorescent protein
SPR	surface plasmon resonance
PE	phycoerythrin
TIRFM	total internal reflection microscopy

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