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The MHC class I-related receptor, FcRn, plays an essential role in the maternofetal transfer of γ-globulin in humans

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Abstract

The transfer of maternal γ-globulin (IgG) provides the neonate with humoral immunity during early life. In humans, maternal IgG is transported across the placenta during the third trimester of pregnancy. The expression of the MHC class I-related receptor, FcRn, in the human placenta suggests that this Fc receptor might be involved in the delivery of maternal IgG, but direct evidence to support this is lacking. In the current study an *ex vivo* placental model has been used to analyze the maternofetal transfer of a recombinant, humanized (IgG1) antibody in which His435 has been mutated to alanine (H435A). *In vitro* binding studies using surface plasmon resonance indicate that the mutation ablates binding of the antibody to recombinant mouse and human FcRn. Relative to the wild-type antibody, the H435A mutant is deficient in transfer across the placenta. Significantly, the mutation does not affect binding to FcγRIII, an FcR that has been suggested in earlier studies to mediate the transfer of maternal IgG. The analyses demonstrate that binding of an IgG to FcRn is a prerequisite for transport across the perfused placenta. FcRn therefore plays a central role in the maternofetal delivery of IgG and this has implications for the use of protein engineering to improve the properties of therapeutic antibodies.

Introduction

Transfer of γ-globulin (IgG) from mother to young provides the neonate with essential humoral immunity during the first few weeks (rodents) or months (humans) of life. In rodents, the MHC class I-related receptor, FcRn (Fc receptor, n = neonatal), is known to mediate the transport of maternal IgG across the neonatal brush border (1–4) or the fetal yolk sac during gestation (4–6). More recent analyses have identified the expression of a human ortholog of FcRn in the placental syncytiotrophoblast (7–10), suggesting that this FcR may also play a role in the maternofetal transfer of IgG in humans. However, it is still an unanswered question as to which IgG-binding proteins in the placenta are involved in the transfer of maternal IgG (reviewed in 11). A corollary of this question is what are the molecular properties of an IgG that endow it with the ability to be transferred?

Analyses in β_2-microglobulin-deficient mice that do not express functional FcRn indicate that in addition to being involved in the delivery of maternal IgG to offspring, this Fc receptor is responsible for maintaining constant serum IgG levels (12–14). This is consistent with the close correlation between binding affinity of an IgG or Fc for FcRn and its serum half-life and maternofetal transfer in mice (6,15–17), and suggests that FcRn can carry out multiple roles depending on its location in the body (reviewed in 18). FcRn appears to be able to perform these apparently diverse functions by binding and transporting IgG within and across cells in either a recycling or transcytotic route (18). FcRn is therefore believed to act as a 'protective' receptor and IgG that enters FcRn-expressing cells in the absence of binding to this FcR is destined for degradation. To date, the molecular details of

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FcRn trafficking are poorly understood, although conserved dileucine- and tryptophan-based motifs in the cytoplasmic tail have been shown to regulate FcRn internalization (19,20). Despite the limited knowledge of FcRn trafficking, much is known about the interaction site of FcRn on IgG. Recombinant Fc fragments with mutations of isoleucine 253 (Ile253), histidine 310 (His310) and histidine 435 (His435) have lower binding affinity for FcRn and reduced activity in FcRn-mediated functions in mice (15,17,21). The involvement of these histidines in the IgG:FcRn interaction most likely accounts for the pH dependence of complex formation (21,22), with binding at pH 6.0 and release at pH 7.4 (1). The residues involved in binding to FcRn are located at the C_H2–C_H3 domain interface (15,17,23,24) and are distinct to those that mediate FcγR binding (reviewed in 25).

In the current study we have used an *ex vivo* placental transfer model to investigate a role for FcRn in mediating IgG transfer across the human placenta. The transfer of a recombinant, humanized (IgG1) antibody has been compared with that of a mutated variant (His435 to alanine; H435A) that does not bind to FcRn. The data indicate that binding to FcRn is essential for an IgG to be transferred across the human placenta, directly implicating this FcR in the maternofetal transfer of IgG in humans. This has relevance to the engineering of improved antibodies for therapy.

Methods

Generation of plasmid for expression of mutated human IgG1

DNA encoding the genomic region of the human IgG1 constant region (26) was a generous gift of Dr Jeff Foote (Fred Hutchinson Cancer Center, Seattle, WA). This fragment was subcloned as a *Bgl*II fragment into pGEM-T (Promega, Madison, WI) and used as a template for splicing by overlap extension (27) to generate the H435A mutant. The following mutagenic primers were used: H435Afor 5′-CTG CGT GTA GGC GTT GTG CAG-3′ and H435Aback 5′-CTG CAC AAC GCC TAC ACG CAG-3′. PCR products were digested with *Xma*I, and the resulting ~400 bp *Xma*I fragment used to replace the wild-type segment of the C_H3 domain and downstream sequences. Clones harboring the plasmid with the desired orientation of the gene fragment were identified and sequenced (Thermo Sequenase radiolabeled terminator cycle sequencing kit; USB, Cleveland, OH) to verify that there were no second site mutations. The entire human IgG1 constant region gene was then ligated as a *Bgl*II fragment into the *Bam*HI site of a modified form of αLys37 (26). This variant does not contain the human IgG1 constant region gene but instead has a unique *Bam*HI site downstream of a V_H domain gene encoding a humanized, high-affinity variant of the anti-hen egg lysozyme (HEL) V_HD1.3 domain (26). Clones with the desired orientation were identified by restriction enzyme digestion.

Generation of transfectants expressing recombinant antibodies

The DNA construct encoding the H435A mutant heavy chain was introduced into a stable transfectant of NSO cells that expresses the humanized anti-lysozyme D1.3 light (κ) chain [a generous gift of Dr J. Foote (26)] by electroporation. Transfectants were selected as described (26) and cloned by limiting dilution. Supernatants of clones were analyzed by ELISA with HEL (Sigma, St Louis, MO)-coated plates using an anti-human IgG (Fc fragment)–horseradish peroxidase conjugate (ICN, Aurora, OH) for detection. Clones secreting the highest levels of antibody were used for expression. Recombinant antibodies were purified from the culture supernatants of expanded clones using HEL–Sepharose and the methodology described by Foote and Winter (26). Dr J. Foote generously provided transfectants for the expression of the wild-type humanized anti-HEL D1.3 antibody (26). Purified proteins were analyzed by SDS–PAGE.

Recombinant FcRn

The gene encoding human FcRn α chain (7) was isolated using RT-PCR and RNA from HT-29 cells (ATCC, Rockville, MD). The gene was tailored with 5′ *Eco*RI–*Bcl*I sites and a 3′ *Sal*I site using the following primers: hFcRnback 5′-ATCA GAA TTC TGA TCA ATG GGG GTC CCG CGG CCT CAG CCC-3′ and hFcRnfor 5′-ATCA GTC GAC CAG CTC CAC CCT GAG GGG-3′. A DNA duplex encoding a polyhistidine tag was ligated into the *Sal*I site. The gene for the α chain was cloned into the *Bgl*II site of the vector pAcUW51 (PharMingen, San Diego, CA) and the gene for β_2-microglobulin (28) into the *Bam*HI site. Mouse and human FcRn were expressed and purified using the baculovirus system as described previously (21). The purity and mol. wt of proteins were checked by SDS–PAGE and HPLC.

Treatment of human IgG1 with diethylpyrocarbonate (DEPC)

Human myeloma IgG1 was kindly provided by Dr C. Medesan (Bucharest). The protein was purified by chromatography on Protein G–Sepharose and Sephacryl S-200HR columns. IgG1 was dissolved in 0.1 M acetate buffer (pH 6.0) at 2 mg/ml and was treated with 10 μl DEPC (Sigma) (10 mg/ml in EtOH). The mixture was incubated at room temperature for 30 min and the reaction stopped by addition of a 5-fold molar excess of imidazole (0.2 mg/ml). The DEPC-treated human IgG1 was immediately gel filtered on Sephadex G-25M (Pharmacia) equilibrated in PBS (pH 7.5). The first peak was passed over a Protein A–Sepharose column (Pharmacia) equilibrated in PBS, and the unbound fraction was collected, concentrated and kept sterile at 4°C for no longer than 2 weeks before use. By using this concentration of DEPC, 90% of the IgG1 lost its Protein A-binding ability which could be restored by treatment with 1.0 M hydroxylamine (29).

Analysis of binding of the wild-type and H435A antibodies to FcRn using surface plasmon resonance (SPR)

All SPR experiments were carried out using a BIAcore 2000 (BIAcore). CM5 chips were coupled with wild-type and H435A antibodies to densities of about 620 RU (equilibrium binding) or 730 RU (kinetic experiments) using amine-coupling chemistry. FcRn was not used as immobilized ligand as it can bind to both possible interaction sites on IgG1 (30–32) and this would result in valency effects. FcRn (concentration range of 0.03–1.6 μM) was injected over the flow cells at 25°C using programmed methods and the kinject command. Each injection was carried out in duplicate or triplicate and PBS (pH 6.0)

plus 0.01% v/v Tween 20 was used as running buffer. For equilibrium binding experiments, a flow rate of 10 μl/min was used. For kinetic analyses, higher flow rates of 80 μl/min were used to minimize mass transport. The off-rate of the human FcRn:IgG1 interaction was immeasurably fast (assessed using BIAevaluation 2.2.4; not shown) and for this reason, dissociation constants were estimated from equilibrium binding analyses. For these estimations, linear regression analysis in the MatLab programming language was used. However, the dissociation constant should be taken to be an apparent value as mouse/human FcRn has two possible interaction sites on IgG which are not equivalent (31,32). In addition to using FcRn as analyte, HEL was also flowed over the chip in PBS (pH 7.2) plus 0.01% Tween 20 to ensure that the wild-type and H435A antibodies did not lose activity during coupling.

Biotinylation of IgG

IgG (human IgG1, DEPC–human IgG1 or recombinant wild-type/H435A antibodies) were dissolved in 0.1 M carbonate/bicarbonate buffer (pH 8.6) at 2 mg/ml. Then 0.1 ml of EZ-Link Sulfo-NHS-biotin (Pierce, Rockford, IL) at 3 mg/ml in distilled water was added and incubated at room temperature for 1 h. The mixture was gel filtered on Sephadex G-25M (Pharmacia, Uppsala, Sweden), and the first peak containing the biotinylated protein was collected, concentrated and stored at 4°C for no longer than 1 month.

Detection of biotinylated IgG by ELISA

Ninety-six-well plates (Costar, Corning, NY) were coated with neutravidin (Sigma) (10 μg/ml in 0.1 M carbonate/bicarbonate buffer, pH 9.6). After washing and blocking steps, 200 μl of biotinylated IgG at concentrations ranging from 5 to 250 ng/ml (for standard curve) or 200 μl of samples containing biotinylated IgG obtained from the placental transfer experiment were added in triplicates at appropriate dilutions. After 1 h incubation at 37°C followed by washing, 200 μl of rabbit anti-human IgG conjugated to horseradish peroxidase (Sigma) was added to each well. After 1 h incubation at 37°C wells were washed and *o*-phenylendiamine/hydrogen peroxide in citrate phosphate buffer (pH 5.0) added. Reactions were stopped by addition of an equal volume of 0.2N sulfuric acid and the absorbance (490 nm) in each well determined.

Placental transfer assays

Methods used in this study were modifications of those previously described (33,34). The use of the placental transfer model is described in detail by Redman and colleagues (35). Placentas were obtained from term vaginal or cesarean section deliveries. The use of human placentas in this study was approved by the University of Texas Southwestern Medical Center Institutional Review Board for Human Studies. Placentas were transported to the perfusion laboratory in a bath of saline within minutes of separation from the uterus. A suitable non-traumatized cotyledon was identified, and the fetal artery and vein were cannulated with umbilical vessel catheters. The catheterized cotyledon was gently perfused with perfusion media for 15–30 min to stabilize the tissue and remove retained blood. After 15 min the maternal side of the placenta was capped, sealed and three 20G blunt needles were inserted into the intervillous space on the isolated cotyledon close to the blanched area near the fetal intravillous tree. Briefly, the experimental conditions in the study were a temperature of 37°C, pH 7.4, maternal flow rate of EMEM containing 3% BSA at 17.0 ml/min and a fetal flow rate of 4.5 ml/min (34). Once the cotyledon was stabilized, 0.5 mM [^{14}C]antipyrine was added to the maternal circulation, and both the maternal and fetal circulations were open. The use of antipyrine, a freely diffusible small molecule, determines the maternofetal match in the isolated cotyledon. In a single pass of antipyrine, 45.3 ± 10.7% (average of all experiments) crossed the placental membranes. Values around 40% passage of antipyrine are indicative of a good maternal–fetal match (34). Samples from both the maternal and fetal circulation were collected every 10 min for 1 h to determine the transfer of antipyrine. Once the transfer was established, both maternal and fetal circulation were closed and IgG ligands were added to the maternal circulation (3 mg/150 ml medium), allowed to mix for 5 min, and samples collected from the maternal and fetal circulation at 5 min, and 0.5, 1, 2 and 3 h. The samples were centrifuged, and the amount of IgG in both maternal and fetal compartments measured by ELISA (for biotinylated IgG) or γ-counting after precipitation with 10% trichloracetic acid (for radioiodinated IgG). From those values, the fetal/maternal ratio (%) was calculated for each time point (34).

Inhibition of IgG transfer was carried out by measuring the transfer rate of [^{125}I]IgG1 in the presence of a 400-fold excess (1.2 g) of unlabeled human IgG (Sandoz, Basel, Switzerland). This unlabeled IgG was added 90 min after the start of the transfer experiment, i.e. time of addition of [^{125}I]IgG1, so that transfer rates in the absence and presence of inhibitor could be compared using the same placenta. The rate of [^{125}I]IgG1 transfer was calculated with the equations: $TR = [(F/M)_{90} - (F/M)_{30}]/t$ for the normal transfer and $TR = [(F/M)_{180} - (F/M)_{120}]/t$ for the inhibited transfer, where F/M is the fetal/maternal ratio (%) at 30, 90, 120 and 180 min, and t is the interval of time (in h).

Radioiodination of recombinant antibodies

Wild-type and H435A (IgG1) antibodies were radiolabeled with Na^{125}I (Amersham, Arlington Heights, IL) using the Iodogen reagent as described previously (36). Free iodine was removed by centrifugation on Microspin G-25 columns (Pharmacia, Piscataway, NJ). The specific radioactivities of the radiolabeled IgG were ~10^7 c.p.m./μg, with <5% free iodine. The radiolabeled IgG were stored at 4°C for no more than 1 week before use.

Pharmacokinetic analysis

Pharmacokinetics of radiolabeled IgG were determined in Swiss mice (Harlan Sprague-Dawley Laboratory, Indianapolis, IN) as described previously (36).

Isolation of human NK cells

Human NK cells were obtained from human blood by isolation of mononuclear cells on Ficoll-Hypaque (Sigma) followed by removal of monocytes, T and B lymphocytes by negative selection using anti-CD14, -CD3 and -CD19 monoclonal

mouse antibodies (Caltag, Burlingame, CA), and capture with magnetic beads (Dynal, Oslo, Norway). Alternatively, cells were panned on tissue culture flasks (Becton Dickinson, Bedford, MA) to remove adherent cells and subsequently on anti-CD3- and -CD19-coated plates to remove T and B cells (37). The purity of the NK population was verified by flow cytometric analysis using an anti-CD16 antibody (Caltag) and a FITC–goat anti-mouse antibody (Kirkegaard & Perry, Gaithersburg, MD).

Binding of wild-type/H435A antibodies to FcγRIII bearing cells

Human NK (FcγRIII$^+$) cells (2×10^6 cells/0.5 ml RPMI 1640 medium with 5% FCS and 15 mM sodium azide) were treated with different concentrations of radiolabeled wild-type or H435A antibodies. After incubation at 4°C for 1 h, cells were washed twice with cold medium and the bound radioactivity determined for each concentration added. The specificity of the binding of wild-type and H435A antibodies to NK cells was demonstrated by the inhibition of their binding by a 4-fold excess of anti-CD16 (FcγRIII) (Caltag) antibody, 3G8 (38), or a 500-fold excess of unlabeled human myeloma IgG1. For inhibition experiments, 20 μg of [^{125}I]wild-type or [^{125}I]H435A antibodies were added per 10^7 cells.

Binding of wild-type/H435A antibodies to FcγRI- and II-bearing cells

Human U937 (FcγRI$^+$) and Daudi (FcγRII$^+$) cells (2×10^6 cells/0.5 ml RPMI 1640 medium with 5% FCS and 15 mM sodium azide) were treated with different concentrations of radiolabeled wild-type or H435A antibodies in either monomeric form (U937) or IgG:lysozyme immune complexes (Daudi cells). After incubation at 4°C for 1 h, cells were washed twice with cold medium and the bound radioactivity determined for each concentration added. The specificity of the binding was demonstrated by using a 500-fold excess of unlabeled human myeloma IgG1 (for U937 cells) or heat-aggregated IgG1 (for Daudi cells). The amounts of [^{125}I]wild-type and [^{125}I]H435A antibodies added in inhibition experiments were 1.5 μg/10^7 cells (for U937 cells) and 5 μg/10^7 cells (for Daudi cells).

Results

Interaction of recombinant antibodies with FcRn

The location of the H435A mutation on human IgG1 is shown in Fig. 1. Recombinant human FcRn was expressed and purified from baculovirus-infected insect cells using Ni^{2+}-NTA-agarose followed by size exclusion chromatography. The interaction of the wild-type and H435A antibodies (human IgG1-derived) with mouse and human FcRn was analyzed using SPR. H435A binds almost undetectably to human FcRn (Fig. 2A), whereas the wild-type IgG1 binds with an apparent dissociation constant (K_D) of 2.35 μM (Fig. 2B). Similar results were obtained when mouse FcRn was used as analyte, except that the mouse FcRn–IgG1 interaction is of significantly higher affinity (apparent K_D=0.265 μM) (Fig. 2C and D). The stoichiometry of the mouse/human FcRn interaction with human IgG1 is 2FcRn:1IgG1 with two non-equivalent interaction sites (31,32) and in sedimentation equilibrium studies of the mouse FcRn:mouse IgG1 interaction the affinities of the two sites differ by ~50-fold (<130 nM and 6 μM) (31). Under the conditions of the SPR experiments, therefore, the higher-affinity interaction would predominate and this is consistent with the linear Scatchard plots (Fig. 2B and D). In addition, the experiments were carried out with immobilized antibodies and FcRn in solution to avoid avidity effects of immobilized FcRn binding to both possible interaction sites on IgG1. However, when FcRn is membrane bound these avidity effects would be expected to enhance the FcRn:IgG1 interaction. Consistent with this, higher avidities are seen for FcRn:IgG interactions when FcRn is immobilized on the sensor chip (21–23). The SPR data clearly demonstrate that the H435A mutation ablates binding of the recombinant IgG to human and mouse FcRn. Significantly, this mutation does not result in misfolding of the antibody as it retains binding to FcγRs (below) and the antigen, HEL (data not shown).

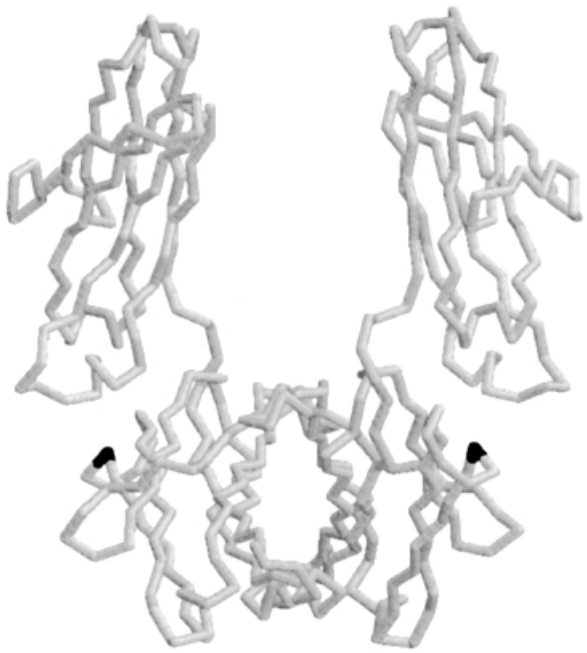

Fig. 1. Location of the H435A mutation (black) on the α-carbon trace of the human IgG1 Fc region (55). The figure was drawn using the RASMOL program (Roger Sayle, Bioinformatics Research Institute, University of Edinburgh, UK).

Table 1. Pharmacokinetics of wild-type and H435A antibodies in mice

Antibody	No. of animals	β-phase half life (h)	Fractional catabolic rate (day^{-1})[a]	AUC (h^{-1})
Wild-type	7	217.9 ± 21.0	0.19 ± 0.01	4028 ± 203
H435A	7	25.8 ± 2.0	1.16 ± 0.04	750 ± 25

[a]$\ln2/T_{1/2}$, where $T_{1/2}$ was calculated for the time interval 0–6 days post-injection.

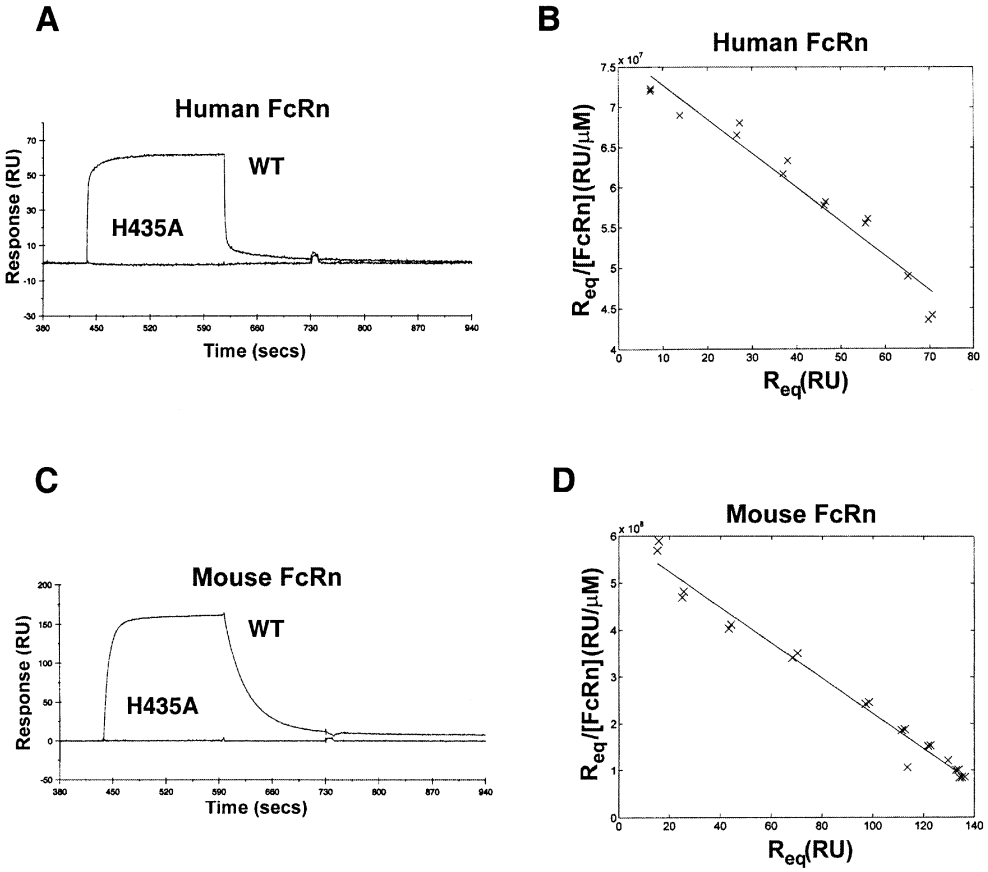

Fig. 2. Biosensor analysis of FcRn interactions with wild-type and H435A antibodies. (A) Binding of human FcRn to wild-type and H435A antibodies. (B) Scatchard analysis of binding of human FcRn to wild-type IgG1. (C) Binding of mouse FcRn to wild-type and H435A antibodies. (D) Scatchard analysis of binding of mouse FcRn to wild-type IgG1. CM5 chips were coupled with wild-type and H435A antibodies to densities of 730 RU (A and C) or 620 RU (B and D). Either 0.8 μM human FcRn (A), 0.7 μM mouse FcRn (C) or varying concentrations (0.03–1.6 μM) of mouse or human FcRn (B and D) in PBS (pH 6.0) plus 0.01% Tween 20 (v/v) were injected over the flow cells at a flow rate of 80 (A and C) or 10 (B and D) μl/min.

Ex vivo placental transfer

The demonstration that H435A binds almost undetectably to human FcRn in SPR experiments indicated that it would be a useful tool to investigate the role of FcRn in the maternofetal transfer of IgG in an *ex vivo* human placental model. This *in vitro* model has been shown to be useful for the analysis of the transfer of antibodies across the placenta by others (39,40). In initial studies, in order to analyze the reliability of the placental transport model, IgG1 (from myeloma) and DEPC-treated IgG1 were used. DEPC treatment has been shown previously to result in modification of histidines (29) and, due to the role of histidines in FcRn:IgG binding (17,22), would be predicted to ablate this interaction. Consistent with this prediction, SPR experiments indicated that the DEPC-treated IgG1 did not bind detectably to FcRn (data not shown). Pilot experiments showed that due to the variability in placentas, it was essential to compare the transfer of IgG1 and DEPC–IgG1 in the same experiment. These proteins were therefore either radioiodinated or biotinylated and the transfer quantitated by γ-counting (for radioiodinated IgG) or ELISA (for biotinylated IgG). Figure 3 shows that DEPC treatment significantly reduces the transfer of IgG1 and this reduction is independent of the way in which the antibodies are labeled. Subsequently, the activities of the wild-type and H435A antibodies were directly compared in the placental transfer assay. The H435A mutation reduces the transfer of the recombinant antibody to levels similar to those observed for the DEPC treated IgG1 (Fig. 4). The specificity of the transport was demonstrated by measuring the transfer rate of [^{125}I]IgG1 in the absence and presence of an excess of unlabeled IgG in the maternal circulation. The transfer rate of [^{125}I]IgG1 was decreased from 0.41 to 0.04%/h by excess unlabeled IgG, representing an inhibition of 90.3% (Fig. 4C).

Correlation between placental transfer and serum half-life in mice

The effect of the H435A mutation on the serum pharmacokinetics of the H435A antibody in mice was analyzed. The β-phase half-life of this mutated antibody is short (25.8 h) relative to that for the wild-type antibody (217.9 h) (Table 1 and Fig. 5). This indicates a direct correlation between the transfer of the mutated IgG across the human placenta

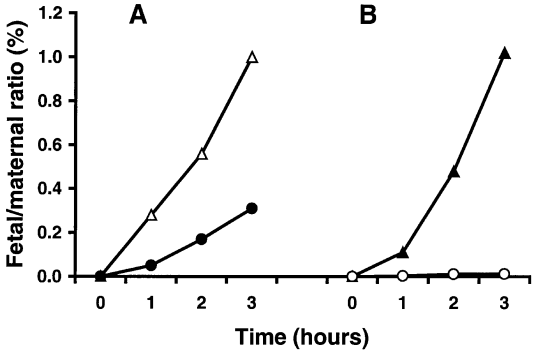

Fig. 3. Transport of human IgG1 (△, biotinylated, ▲, radiolabeled) or DEPC-treated IgG1 (○, biotinylated, ●, radiolabeled) across the *ex vivo* human placenta. After isolation and stabilization of perfusion through a non-traumatized cotyledon, human IgG1 or DEPC-treated IgG1 were injected into the maternal compartment. Samples from both maternal and fetal compartments were collected at 5 min, and 1, 2 and 3 h. Panels (A) and (B) show data from two experiments in which the labeling of the antibodies was interchanged. For all data points, SD < 0.04.

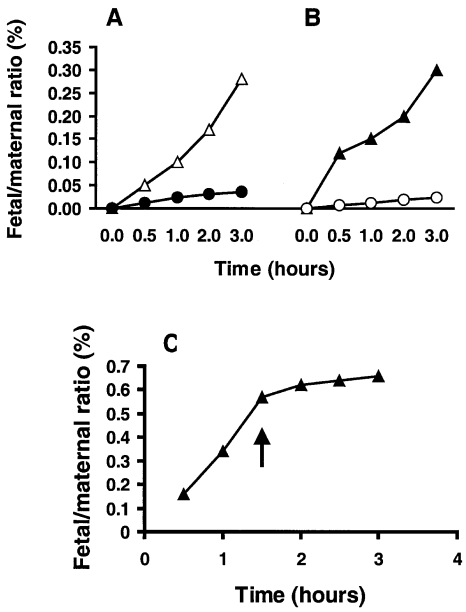

Fig. 4. Transport of wild-type (△, biotinylated, ▲, radiolabeled) and H435A (○, biotinylated, ●, radiolabeled) antibodies across the *ex vivo* human placenta. After isolation and stabilization of perfusion through a non-traumatized cotyledon, antibodies were injected into the maternal compartment. Samples from both maternal and fetal compartments were collected at 5 and 30 min, and 1, 2 and 3 h. Panels (A) and (B) show data from two experiments in which the labeling of the antibodies was interchanged. For all data points, SD < 0.02. (C) Inhibition of transport of human IgG1 by a 400-fold excess of unlabeled antibody. After isolation and stabilization of perfusion through a non-traumatized cotyledon, 3 mg of [^{125}I]IgG1 was added to the maternal circuit. This was followed by addition of 1.2 g unlabeled hIgG at 90 min (arrow). Samples were harvested at 5, 30, 60, 90, 120, 150 and 180 min for both circuits.

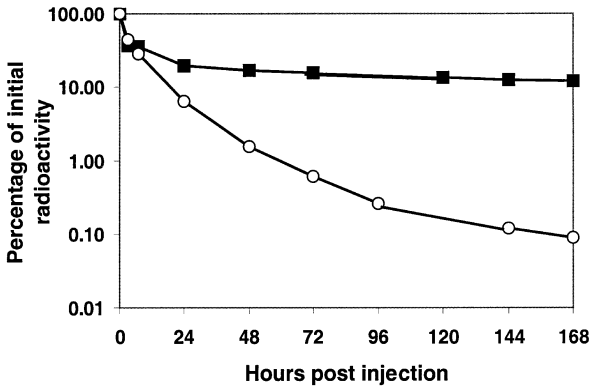

Fig. 5. Clearance curves of [^{125}I]wild-type (■) and [^{125}I]H435A (○) antibodies in mice. Data from one representative mouse is shown for each antibody.

and its serum half-life in mice. The other pharmacokinetic parameters (FCR and AUC) for these antibodies are in agreement with the values obtained for the β-phase half-lives.

Interaction with FcγRIII

The Fc region is involved not only in binding to FcRn but also to FcγRIII, and this FcγR has been suggested to be involved in the placental transfer of IgG (41). Although residues that are of importance for FcγRIII binding (25,42) are distal to the FcRn interaction site (6,17,22,24), it was essential to exclude the possibility that the H435A mutation reduced IgG transfer by affecting binding to FcγRIII. The binding of (monomeric) wild-type or H435A antibodies to FcγRIII on human NK cells was therefore analyzed and there was no significant difference in the binding of the two antibodies (Fig. 6A). Significant decreases in the amount of bound wild-type (95%) and H435A (78%) antibodies were observed in the presence of the anti-FcγRIII antibody, 3G8 (Fig. 6B). Similar levels of inhibition were observed in the presence of an excess of unlabeled human myeloma IgG1 (Fig. 6B).

Interaction with FcγRI and II

FcγRI and II have been reported to be distributed in human placenta on Hofbauer and endothelial cells, and not in syncytiotrophoblasts, suggesting that they do not play a role in the transfer of maternal IgG (41). However, to exclude the possibility that the effect of the H435A mutation on placental transfer was due to an alteration in binding to either or both of these Fc receptors, the binding of monomeric (to FcγRI$^+$ U937 cells) or dimeric (to FcγRII$^+$ Daudi cells) wild-type or H435A antibodies was also analyzed. The results presented in Fig. 7 show there are no significant differences in the binding of the two antibodies to these cells. Significant decreases in the amount of bound wild-type and H435A antibodies were observed in the presence of an excess of unlabeled myeloma IgG1 in native (95–98% for U937 cells) or aggregated (82–87% for Daudi cells) form (Fig. 7C).

Discussion

In the current study we have used an *ex vivo* placental transfer assay to investigate a possible role for FcRn in

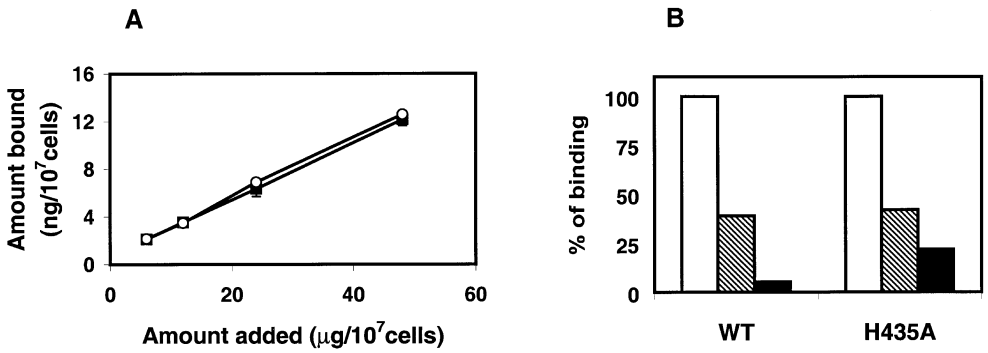

Fig. 6. Interaction of antibodies with FcγRIII on human NK cells. (A) Binding of monomeric wild-type (■) and H435A (○) antibodies to NK cells (FcγRIII$^+$). (B) Inhibition of binding by either 4-fold excess of the anti-FcγRIII antibody, 3G8 (filled) or 500-fold excess of unlabeled human IgG1 (hatched). Open symbols indicate binding levels in the absence of inhibition. Data are averages of duplicate samples and representative of three independent experiments.

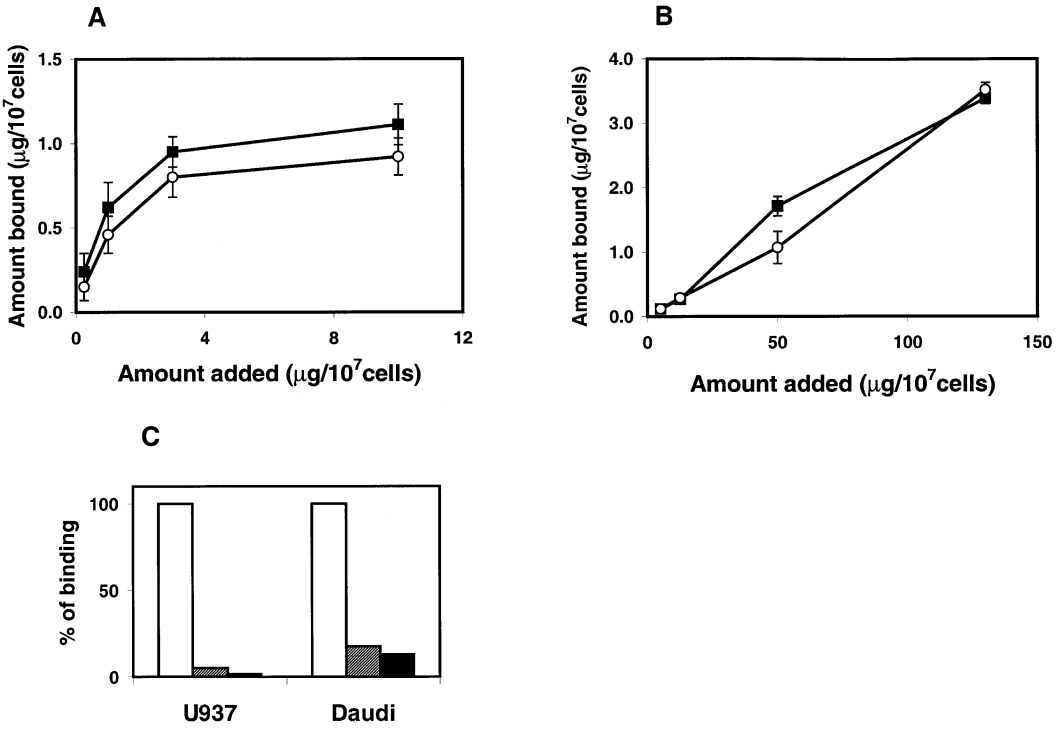

Fig. 7. Interaction of antibodies with FcγRI and II on human cells. (A) Binding of monomeric wild-type (■) and H435A (○) antibodies to U937 cells (FcγRI$^+$). (B) Binding of dimeric wild-type (■) and H435A (○) antibodies to Daudi cells (FcγRII$^+$). (C) Inhibition of wild-type (hatched) or H435A (filled) binding to U937 or Daudi cells by a 500-fold excess of unlabeled human IgG1. Open bars indicate binding levels in the absence of inhibition. Data are averages of duplicates and representative of three independent experiments.

mediating transfer of IgG across the human placenta. This has been carried out by comparing the transfer of a mutated human IgG1 molecule (H435A) with that of the wild-type, unmutated IgG1. The mutation results in loss of binding of the antibody to both human and mouse FcRn, whereas binding to FcγRI, FcγRII and FcγRIII is retained. The H435A mutant is transferred at background levels across the placenta, indicating that binding to FcRn is a prerequisite for an antibody to cross the maternofetal barrier. This provides direct evidence to support a role for FcRn in the transcytosis of IgG across the human placenta and is consistent with the observation that this FcR is expressed in placental syncytiotrophoblasts (7–10). In addition, the short serum half-life of the H435A mutant in mice indicates that pharmacokinetic analyses can be used as an indicator of activity in human FcRn-mediated functions, and this is of obvious relevance for the engineering and pre-clinical analysis of antibodies of potentially therapeutic value.

In an earlier *ex vivo* placental study in which the transfer of all four human IgG isotypes were compared, the fetal/maternal ratios for the four subclasses were reported to decrease in the order IgG4 > IgG1 > IgG3 > IgG2, with the

transmission of human IgG3 being ~65–69% of that of human IgG1 (43). However, in these studies direct evidence to invoke a role for FcRn in IgG transfer was not obtained. The lower level of transport of human IgG3 (allotype G3m s^-, t^-) (with arginine instead of histidine at position 435) is concordant with its shorter serum half-life in both humans [7 versus 21 days (44)] and mice [3 versus 6 days (45) or 4.4 versus 7.4 days (46)]. Despite these differences in functional activity in humans for IgG1 and IgG3, recent studies have demonstrated that the affinities of different human IgG subclasses (including allotypic variants) for human FcRn do not differ significantly (23) (E. S. Ward and C. G. Radu, unpublished data). In contrast, our analyses in mice indicate that human IgG3 has a lower affinity for mouse FcRn than human IgG1 (46). Consistent with this, the serum half-life of IgG1 is longer than that of human IgG3 when determined in mice (45,46). In the current study, the H435A mutation ablates binding to both human and mouse FcRn (Fig. 2), whereas replacement of this residue by Arg in IgG3 has differential effects on the interaction with FcRn from these two species (23,46) (E. S. Ward and C. G. Radu, unpublished data). Although X-ray crystallographic studies have demonstrated that human and rodent FcRn are very similar (23), there is some sequence variation of FcRn residues that are involved in binding to IgG. These sequence differences may have particular impact on the binding to IgG that vary at positions 435–436, as this region interacts in the vicinity of residue 137 (rodent) or the equivalent residue 135 (human) of FcRn which is not conserved across species (Leu135 in human and Glu/Asp137 in rodent) (23). Thus, although mice are believed to be a good model for FcRn function in humans (46,47) exceptions may exist, particularly for antibodies that vary in the region encompassing residues 435–436. This underscores the importance of comparing binding of a (mutated) IgG to both rodent and human FcRn prior to drawing conclusions about activity in FcRn-mediated functions across species.

Contractor and colleagues have observed that transport of IgG across the placenta is not an efficient process, with the majority of internalized IgG being broken down intracellularly (48). Similarly, we observed that less than half of the radiolabel transferred across the placenta was precipitable with trichloroacetic acid (data not shown). This suggests that the salvage of IgG from (lysosomal) degradation by FcRn is not an efficient process during transport. This IgG degradation is also reminiscent of the situation for the transcytosis of IgG across the rodent yolk sac (49) and neonatal intestine (50) where a substantial amount of breakdown occurs. The catabolism of IgG would appear to offer no advantage during maternofetal transfer, and is most likely a trade-off between FcRn acting as an IgG homeostat at some sites in the body and as an IgG transporter at others (discussed in 18).

A number of studies have described the use of human term trophoblast cells (51) or choriocarcinoma-derived trophoblast cells (52) which, as confluent monolayers, show polarized transcytosis of IgG. Although the use of these models for the study of the transcellular transport of IgG is very attractive, this may not be representative of the transplacental transfer of molecules due to the absence of some components such as the intravillous space and fetal endothelium that constitute the normal histological barrier together with the syncytiotrophoblasts. In addition, in studies in which the transcytosis of human IgG1 and DEPC-treated IgG1 were analyzed using the trophoblast cell lines JEG-3 and BeWo, no significant differences were observed between IgG1 versus DEPC-IgG1 (unpublished observations). Thus, although more cumbersome, the *ex vivo* placental perfusion model may be more physiologically relevant.

Several IgG binding proteins/receptors have been suggested to be involved in the transfer of maternal IgG to the fetus during the third trimester of pregnancy: FcRn, FcγRIII, annexin II and placental alkaline phosphatase (PLAP). All of these proteins have been detected in human placenta and bind IgG (reviewed in 11,41). However, analysis of binding affinity for monomeric IgG or expression patterns indicate that annexin II and PLAP, respectively, are unlikely to be involved (11). Although a role for FcγRIII in IgG transport cannot be excluded, the observation that it does not bind to IgG2 or IgG4 (53,54) which are both transported (43) excludes the possibility that it is the sole transporter. This is consistent with our data demonstrating that the H435A mutation, which does not affect binding to FcγRIII, ablates maternofetal transfer. The expression patterns of FcγRI and II (41) make it unlikely that these Fc receptors are responsible for maternofetal IgG transport and the lack of effect of the H435A mutation on binding to these receptors supports their lack of involvement. Taken together with the current analyses, FcRn therefore appears to be the most likely candidate for the receptor that transports IgG across the placenta.

In conclusion, our studies demonstrate that binding to FcRn is essential for the transplacental passage of an IgG. The correlation between FcRn binding and placental transfer indicates that it may be possible to engineer antibodies that are maternofetally transferred with different efficiencies, in an analogous way to that used to increase the serum persistence of recombinant Fc fragments (16). Finally, the use of the *ex vivo* human placental assay provides a valuable human-based model for the preclinical evaluation of therapeutic antibodies and may be particularly useful as a predictor of serum persistence.

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Abbreviations

DEPC	diethylpyrocarbonate
EMEM	Eagles minimal essential medium
FcR	Fc receptor
FcRn	neonatal Fc receptor
HEL	hen egg lysozyme
IgG	γ-globulin
PLAP	placental alkaline phosphatase
SPR	surface plasmon resonance

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