Mapping the site on human IgG for binding of the MHC class I-related receptor, FcRn

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The analysis of the pharmacokinetics of wild-type and mutated Fc fragments derived from human IgG1 indicates that Ile253, His310 and His435 play a central role in regulating serum half-life in mice. Reduced serum half-life of the recombinant, mutated fragments correlates with decreased binding to the MHC class I-related neonatal Fc receptor, FcRn. In addition, the analysis of an Fc fragment in which His435 is mutated to Arg435 demonstrates that the sequence difference at this position between human IgG1 (His435) and IgG3 (Arg435) most likely accounts for the shorter serum half-life of IgG3 relative to IgG1. In contrast to His310 and His435, the data indicate that His433 does not play a role in regulating the serum half-life of human IgG1. Thus, the interaction site of mouse FcRn on human and mouse IgG1 involves the same conserved amino acids located at the CH2-CH3 domain interface of the IgG molecule. The sequence similarities between mouse and human FcRn suggest that these studies have direct relevance to understanding the factors that govern the pharmacokinetics of therapeutic IgG.

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1 Introduction

Site-directed mutagenesis has been used to identify the amino acids at the CH2-CH3 domain interface of mouse IgG1 that are involved in the regulation of its serum half-life and neonatal/maternofetal transfer [1–4]. Residues in both the CH2 (Ile253 and His310) and CH3 domain (His435) play a central role in these processes. The neonatal Fc receptor (FcRn) is responsible for IgG transfer across the neonatal intestine or maternofetal barrier [5–11], and recent studies indicate that it also plays a role in maintaining the serum levels of IgG [12–14]. FcRn most likely functions in these roles by binding and trafficking IgG both within and across cells as a recycling or transcytotic receptor (reviewed in [15]). Consistent with this, Fc-hinge fragments with mutation of Ile253, His310 and His435 which have short persistence in the circulation and poor neonatal/maternofetal transmission also bind with very low affinity, if detectable, to recombinant soluble mouse FcRn [4, 16].

An understanding of the molecular nature of the IgG:FcRn interaction in humans is of importance for the successful application of therapeutic antibodies. Ile253, His310 and His435 are conserved in human IgG isotypes with the exception of IgG3 (allotype G3m,s^-,t^-) which has arginine instead of histidine at position 435 [17]. IgG3 has a shorter biological half-life than the other isotypes [18–20], suggesting that amino acid differences at position 435 might be responsible for this decreased serum persistence. However, in an earlier study describing the analysis of the interaction of chimeric (human Fc, IgG4-derived) IgG with rat FcRn it was concluded that His435 did not play a role in FcRn binding [21]. This suggests that the interaction of human and mouse IgG1 with rodent FcRn might differ with respect to the involvement of His435, and also that Arg435 of IgG3 might not be responsible for the shorter half-life of this isotype. In addition, differences in the pH dependence of binding of mouse IgG2a *vs.* mouse IgG2b to rat FcRn, which differ

[I 19644]

Abbreviations: CD: Circular dichroism **Fc-hinge:** Recombinant Fc fragment of human IgG1 **Fc-papain:** Fc fragment obtained by papain digestion of human IgG **FcRn:** Neonatal Fc receptor **WT:** Wild type **Fcr:** Fractional catabolic rate **AUC:** Area under curve **SPR:** Surface plasmon resonance

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at position 433 by a His (IgG2a) to Lys (IgG2b) change, led to the suggestion that His433 is involved in FcRn:Fc binding [21], contrasting with data from our studies indicating that His433 of mouse IgG1 does not contribute to the interaction [4].

The conservation of Ile253, His310 and His433 in human IgG [17] precludes an analysis of effects of differences at these positions on binding to FcRn, and, by extension, on serum half-life. In the current study we have therefore investigated the involvement of these three residues and His435 of human IgG1 in FcRn-mediated processes by generating recombinant, mutated human Fc-hinge fragments. Ethical reasons prohibit the use of humans for pharmacokinetic studies, and for this reason mice have been used for the functional analysis of these molecules. Mice have been shown previously to be a suitable model for the analysis of the pharmacokinetics of human IgG [22, 23], although differences for the serum persistence of IgG4 in these two species have been noted [23]. Furthermore, the similarities at the amino acid level between mouse and human FcRn [24] suggest that these studies have direct relevance to humans.

2 Results and discussion

The clearance curves of radiolabeled human IgG1 and IgG3 are shown in Fig.1. The pharmacokinetic data derived from the elimination curves are presented in Table 1. The data demonstrate that IgG1 has a longer half-life (both α- and β-phases) than IgG3, and this is consistent with the values reported by others in mice [22, 23] and in human volunteers [18–20]. The fractional catabolic rates (Fcr) and the area under the curves (AUC) reflect the calculated values for the half-lives of these two human IgG isotypes. Consistent with the pharmacokinetics, the relative affinity of mouse FcRn for IgG1 was higher than that for IgG3 in FcRn binding assays (Table 1).

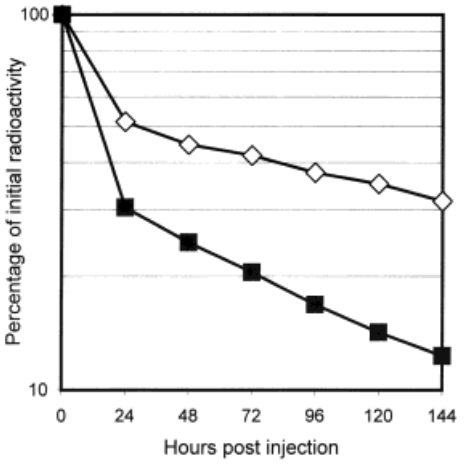

Figure 1. Elimination curves of human IgG1 and IgG3 in mice. ◇ = IgG1; ■ = IgG3.

The good correlation between the half-life and affinity for FcRn of these two IgG indicates that the shorter half-life of IgG3 in humans (7 days) relative to that of the other IgG isotypes (around 20 days) [18–20] may be due to differences in the amino acid residues at position 435 (Arg in IgG3 and His in IgG1). Although it is possible that the sequence difference at residue 436 between human IgG1 (Tyr) and IgG3 (Phe) is responsible for the difference in half-life, this is made less likely for two reasons. First, in mice His436 plays a minor, but significant, role in regulating serum half-life whereas the role of His435 is more marked [4]. Second, the higher variability of this amino acid across species [17] is consistent with a minor role for this residue. Therefore, expression plasmids encoding wild-type (WT) human IgG1 Fc-hinge and the mutants H435A (His435 to Ala) and H435R (His435 to Arg) were constructed. Contradictory results have been reported for the role of His433 in the rodent FcRn:Fc/IgG interaction [4, 21] and for this reason a plasmid to express the mutant H433A (His433 to Ala) was generated to investigate the function of this residue in the

Table 1. Pharmacokinetics in mice and relative affinity for mouse FcRn of human IgG1 and IgG3

IgG isotype	No. of animals	Half-life (h) α-phase	Half-life (h) β-phase	Fcr[a] (day^{-1})	AUC[b] (h^{-1})	Relative affinity[c] (%)
IgG1	3	24.7 ± 1.1	178.3 ± 21.6	0.16 ± 0.01	10570 ± 1298	100
IgG3	4	15.0 ± 1.4	107.1 ± 12.7	0.26 ± 0.02	5383 ± 645	35.8 ± 5.9

a) Fcr, fractional catabolic rate (= $\ln 2/T_{1/2}$) where $T_{1/2}$ was calculated for the time interval 0–6 days post-injection.
b) AUC, area under the curve.
c) Measured as percent of binding of ^{125}I-labeled IgG1 or IgG3 to mouse FcRn-Sepharose. The value for IgG1 was considered to be 100 %.

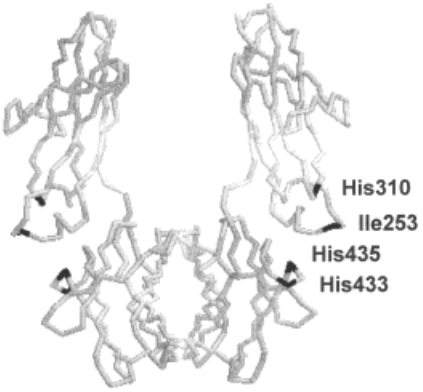

Figure 2. Structure of the Fc region of human IgG1 [34] with residues targeted for mutation shown in black. The figure was drawn using the RASMOL program (Roger Sayle, Bioinformatics Research Institute, University of Edingburgh, GB).

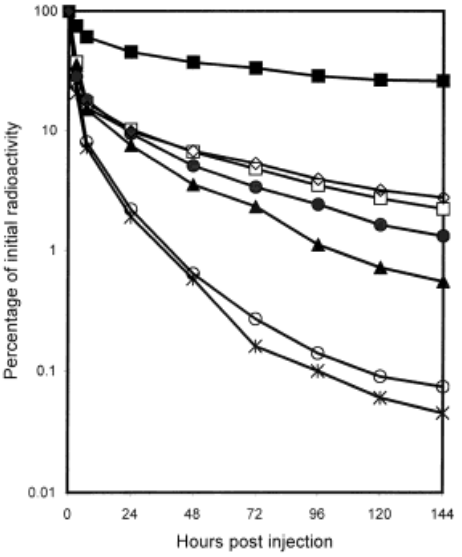

Figure 3. Elimination curves of recombinant human Fc-hinge derivatives and Fc-papain fragment in mice. □ = WT Fc hinge; ▲ = I253A; ○ = H310A; ◇ = H433A; ● = H435R; * = H435A; ■ = Fc-papain.

human Fc:mouse FcRn interaction. In addition, Ile253 and His310 have been shown to play a role in regulating the serum half-life of murine IgG1 [1, 4], and the conservation of these residues across species (including humans) prompted us to generate the human IgG1-derived mutants I253A (Ile253 to Ala) and H310A (His310 to Ala) as Fc-hinge fragments. Furthermore, in an earlier study simultaneous mutation of residues 252–254 and 309–311 of human Fc (IgG4-derived) containing human constant regions resulted in IgG with reduced affinity for rat FcRn [21], but the effects of mutations of individual residues within these triads were not delinated.

The mutated residues in the current study are located at the CH2-CH3 domain interface of human IgG1-derived Fc (Fig. 2). Analysis using reducing and non-reducing SDS-PAGE indicated that the WT and mutants are expressed predominantly as disulfide-linked homodimers (data not shown), as reported previously for mouse IgG1-derived Fc-hinge fragments [1]. Radiolabeled Fc-hinge fragments were injected into mice and the radioactivity of the blood monitored for a period of 6 days. For each recombinant Fc-hinge fragment a serum sample collected at the 24-h time point from one mouse within each group was analyzed by non-reducing SDS-PAGE and autoradiography. For all Fc-hinge fragments more than 90 % of the radioactivity was associated with a protein of 50 kDa, indicating that the proteins persisted in the blood as intact homodimers.

The clearance curves for the Fc-hinge fragments are shown in Fig. 3. In addition, a glycosylated Fc fragment produced by papain digestion of a human IgG mixture containing IgG1, IgG2 and IgG4 isotypes (Fc-papain) was analyzed (Fig. 3). To assess the binding affinities of the Fc fragments for murine FcRn, a FcRn binding assay was used (Table 2). The pharmacokinetic parameters of the recombinant Fc-hinge fragments and Fc-papain are shown in Table 2. The data clearly show that alanine substitution at positions 253, 310 and 435 results in lower relative affinities for mouse FcRn and significant decreases in both α- and β-phase half-lives, and other pharmacokinetic parameters such as Fcr and AUC. Substitution of His435 by Arg has a less marked effect on both serum half-life and relative affinity for FcRn. Taken together with the data in Table 1 this indicates that the difference in half-life between human IgG1 and IgG3 is due to this amino acid difference.

In an earlier study using surface plasmon resonance (SPR) [21], the affinity and pH dependence of binding of chimeric human IgG4 and a mutated variant (H435R) to rat FcRn were reported to be the same within the limits of experimental error. However, in the current study an analysis of hIgG4 has not been carried out, and it is possible that His435 of IgG4, unlike that of IgG1, is not involved in FcRn binding. Consistent with this is the observation that the half-lives of human IgG3 and IgG4, which differ at this residue by an Arg (IgG3) to His (IgG4) substitution, are similar in mice [23]. In addition, data reported in a recent study of mouse-human IgG with shuffled constant region exons [25] indicate there may be other regions distinct from those identified to date that regulate the serum half-life of human IgG.

Table 2. Pharmacokinetics in mice and relative affinity for mouse FcRn of human Fc-hinge fragments

Fragment	No. of animals	Half life (h)		Fcr[a] (day^{-1})	AUC[b] (h^{-1})	Relative affinity[c] (%)
		α-phase	β-phase			
WT	16	9.1 ± 0.3	62.2 ± 6.0	0.49 ± 0.03	1404 ± 210	100
I253A	5	7.2 ± 0.7	25.3 ± 3.8	0.94 ± 0.11	613 ± 74	21.6 ± 3.0
H310A	5	5.2 ± 0.4	19.2 ± 2.2	1.25 ± 0.02	400 ± 31	7.2 ± 4.6
H433A	5	9.4 ± 0.8	62.8 ± 3.3	0.47 ± 0.03	1426 ± 161	110.2 ± 9.8
H435A	5	5.2 ± 0.4	21.7 ± 1.5	1.29 ± 0.11	357 ± 25	7.5 ± 0.7
H435R	5	8.9 ± 0.1	43.1 ± 3.3	0.58 ± 0.02	986 ± 36	59.5 ± 12.5
Fc-papain	3	23.0 ± 2.5	153.4 ± 5.4	0.18 ± 0.01	9057 ± 858	78.9 ± 20.0

a) Fcr, fractional catabolic rate (= $\ln 2/T_{1/2}$) where $T_{1/2}$ was calculated for the time interval 0–6 days postinjection.
b) AUC, area under the curve.
c) Measured as percent of binding of ^{125}I-labeled fragment to mouse FcRn-Sepharose. The value for wild type was considered to be 100 %.

A role for His433 of both human Fc (IgG1) and mouse Fc (IgG1) in FcRn-mediated functions has been excluded in this and our earlier studies [4], whereas in an SPR analysis [21] it was concluded that His433 is involved in mediating the difference in pH dependence between mouse IgG2a (His433) and mouse IgG2b (Lys433). However, other sequence differences involving histidines exist between these two mouse isotypes and it therefore is possible that these modulate the pH dependence of the FcRn:IgG interaction. For example, IgG2a has histidine at position 435 whereas IgG2b has tyrosine, and together with our data implicating His435 in binding to FcRn ([4], this study), this may be an alternative explanation for the earlier results [21].

The half-lives for WT and mutated Fc-hinge fragments correlate very well with their relative affinities for FcRn (Pearson correlation coefficient = 0.98 with $p = 0.004$), suggesting that the persistence in the circulation of these proteins is related to their ability to interact with this FcR. However, the half-life of recombinant WT Fc-hinge is significantly shorter than that of the glycosylated Fc-papain fragment. The shorter half-life of the WT Fc-hinge is not due to its lower affinity relative to Fc-papain for FcRn since both proteins have a similar binding capacity for FcRn-Sepharose (Table 2). To date we have consistently observed a good correlation between FcRn affinity and serum half-life for Fc fragments or IgG [4, 16, 26]. Therefore, to investigate the reason for this apparent discrepancy, denaturation circular dichroism (CD) and pepsin digestion analyses were carried out. Denaturation CD indicated that both recombinant WT Fc-hinge and Fc-papain have similar denaturation temperatures (~ 75 °C) under the conditions of the assay (data not shown). However, analysis of the sensitivity of the fragments to pepsin digestion demonstrated that the recombinant Fc-hinge fragment is digested about three times more rapidly than Fc-papain (Fig. 4). This difference in pepsin sensitivity might reflect a conformational alteration that, although not affecting FcRn binding, does have an effect on the resistance of the protein to proteolytic digestion. Consistent with these observations, Tao and Morrison [27] observed that aglycosylated human IgG1 and IgG3 are more sensitive to proteolysis than their glycosylated counterparts. This difference in pepsin sensitivity might account for the shorter serum persis-

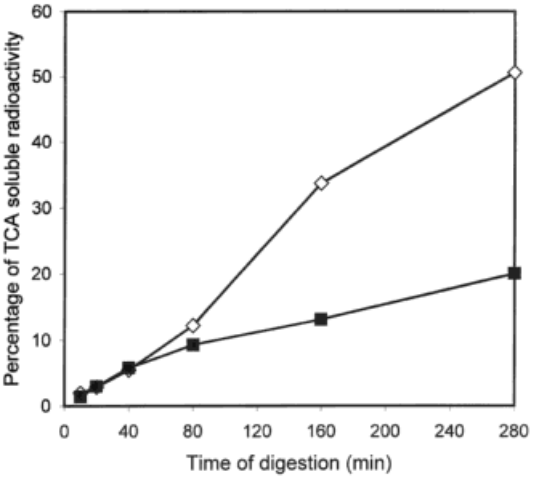

Figure 4. Kinetics of pepsin digestion of human Fc-fragments. ◇ = WT Fc-hinge; ■ = Fc-papain.

tence of the WT Fc-hinge relative to Fc-papain due to increased susceptibility to proteolytic activity in the cells involved in IgG homeostasis [15].

3 Concluding remarks

We have demonstrated that in both mouse [4] and human Fc-hinge fragments the same three amino acids at the CH2-CH3 domain interface (Ile253, His310 and His435) play a central role in the control of serum half-life. The data for the H435R mutation suggest that the differences between the serum persistence of human IgG1 and IgG3 is most likely due to the substitution of His435 (IgG1) by Arg (IgG3). In contrast to these three residues, His433 is not involved in interacting with murine FcRn. The similarities between human and mouse FcRn [24] indicate that these studies have implications for the modulation of the serum half-life of therapeutic antibodies.

4 Materials and methods

4.1 Generation of plasmids for the expression of WT and mutated Fc-hinge fragments derived from human IgG1

The gene encoding the hinge and Fc regions derived from human IgG1 was isolated by PCR from the plasmid pHγ360E [28] (a generous gift of Dr. Donald Capra) using the following primers: FcHUIG1BACK, 5' ATC ACC ATG GCC GCA GAG CCC AAA TCT TGT GAC 5' and FcHUIG1FOR, 5' ATC AGG TGA CCT TAC CCG GAG ACA GGG AGA G 3'. NcoI and BstEII restriction sites, respectively, are indicated by underlining.

The gene was then ligated into VαpelBHis as a NcoI-BstEII fragment as described previously [1] and clones harboring the correct sequence identified. Mutations were made using splicing by overlap extension [29] and the following mutagenesis primers: I253ABACK, 5' ACC CTC ATG GCC TCC CGG ACC 3'; I253AFOR, 5' GGT CCG GGA GGC CAT GAG GGT'; H310ABACK, 5' ACC GTC CTG GCC CAG GAC TGG 3'; H310AFOR, 5' CCA GTC CTG GGC CAG GAC GGT 3'; H433ABACK, 5' GAG GCT CTG GCC AAC CAC TAC 3'; H433AFOR, 5' GTA GTG GTT GGC CAG AGC CTC 3'; 5' H435ABACK, 5' CTG CAC AAC GCC TAC ACG CAG 3'; H435AFOR, 5' CTG CGT GTA GGC GTT GTG CAG 3'; H435RBACK, 5' CTG CAC AAC CGC TAC ACG CAG 3'; H435RFOR, 5' CTG CGT GTA GCG GTT GTG CAG 3'.

4.2 Expression and purification of the recombinant proteins

For the expression of recombinant protein, *Escherichia coli* BMH 71-18 was used as host [1]. WT and mutated Fc-hinge fragments were purified using Ni^{2+}-NTA-agarose as described previously [1].

4.3 Immunoglobulins

Human IgG1 (lambda) and IgG3 (kappa) were purchased from Sigma (St. Louis, MO). The percentage of monomeric IgG was over 90 % as determined by HPLC on a TSK3000SW column. The Fc fragment (Fc-papain) was obtained by digestion of human IgG (Sigma) with papain at pH 7 and purified by protein A-Sepharose affinity chromatography and HPLC on a TSK3000SW column.

4.4 Binding to mouse FcRn-Sepharose

Quadruplicate columns of 0.5 ml mouse FcRn-Sepharose (0.4 mg FcRn/ml gel) were loaded with four different amounts (1 to 50 μg) of radiolabeled protein (either Fc-hinge or human IgG). After incubation at room temperature for 15 min the columns were eluted with 2 × 10 mL 0.1 M phosphate buffer (pH 6.2) containing 0.145 M NaCl and 1 mg/ml BSA and then 10 ml of the same buffer but at pH 7.5. The radioactivity eluted at pH 6.2 and 7.5 was measured in a gamma-counter (LKB-Wallac, Stockholm, Sweden). The ratio of bound (eluted at pH 7.5)/free (eluted at pH 6.2) was calculated for all four concentrations and the percentage of binding of each protein relative to binding of the WT Fc-hinge (for Fc or Fc-hinge fragments) or human IgG1 (for IgG3) was calculated.

4.5 Pharmacokinetic analyses

Proteins were radiolabeled with $Na^{125}I$ (Amersham, Arlington Heights, IL) using the Iodo-Gen method [30] to a specific activity of approximately 5×10^6 cpm/μg with less than 5 % free iodine. SWISS mice (Charles River, Wilmington, MA) were given 0.1 % Lugol solution (Sigma) in drinking water 1 day prior to injection and throughout the following 6 days. The radiolabeled proteins were injected i. v. through the tail vein in a volume not larger than 150 μl and with a radioactive load of 1×10^7–5×10^7 cpm. The mice were bled with 25-μl capillary tubes from the retroorbital sinus at time points of 0.05, 3, 7, 24, 48, 72, 96, 120 and 144 h following injection. The radioactivity of the collected blood was measured in a gamma-counter. The results were expressed relative to the amount of blood radioactivity at 0.05 h post-injection.

The pharmacokinetic parameters were determined using a non-compartmental model with the PKCAL program [31]. The half-lives were calculated using 0–24 h (α-phase) and 24–144 h (β-phase) intervals of time following injection. The Fcr and AUC were calculated using the data obtained for the 0–6 day time interval. In some cases the sera obtained at 24 h after injection were analyzed using SDS-PAGE (Phast-System, Pharmacia, Sweden) followed by autoradiography

to determine the molecular mass and homogeneity of the injected radioactive protein.

4.6 CD analyses

Fc-hinge (320 μg/ml) and Fc-papain (200 μg/ml) were dialyzed into 10 mM sodium phosphate buffer, pH 7.4 and CD analyses were carried out as described previously [32].

4.7 Limited proteolysis of Fc fragments

Limited pepsin digestion was carried out essentially as described previously [33]. In brief, radiolabeled WT Fc-hinge or Fc-papain (10^5 cpm/mg) were incubated at 500 μg/ml in 0.1 M acetate buffer, pH 4 with 0.1 % pepsin at 37 °C. Aliquots (10 μl) were removed at varying intervals of time and mixed with 250 μl PBS (pH 7.5) containing 5 mg/ml BSA. Trichloroacetic acid (TCA) was added to a final concentration of 5 % (v/v) and the TCA-soluble fraction measured in a gamma-counter. The kinetics of digestion were calculated as the percentage of radioactivity in the TCA-soluble fraction as a function of time (average of triplicates with SD below 5 %). Susceptibility to pepsin digestion was expressed as the slopes of the curves.

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