Comparative studies of rat IgG to further delineate the Fc:FcRn interaction site

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Recent data have indicated that the MHC class I-related receptor, FcRn, regulates the half-lives of serum IgG in addition to its known role in transferring IgG from mother to young. In the current study, the activity of rat IgG (rIgG) isotypes in FcRn-mediated functions has been analyzed. The serum half-life and maternofetal transfer in mice decreased in the order rIgG2a > rIgG1 > rIgG2c > rIgG2b. This decrease in activity correlates well with reduced binding affinity for soluble mouse FcRn, and site-directed mutagenesis of a recombinant Fc-hinge fragment has been used to investigate the molecular basis for the differences in activities of the rIgG. Analysis of the serum half-lives of the mutated Fc-hinge fragments demonstrated that, in addition to Ile253, His310, His435 and His436 that were identified in earlier studies, amino acids at positions 257, 307 and 309 play a role in building the FcRn interaction site of IgG. The study also excludes the involvement of amino acids in a fourth loop located at the CH2-CH3 domain interface that encompasses residues 386–387 in FcRn binding. Sequence differences at positions 257, 307 and 309 between rIgG most likely account for the reduced affinity of rIgG2b and IgG2c relative to rIgG1 and rIgG2a for binding to FcRn.

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1 Introduction

We have recently used site-directed mutagenesis of recombinant Fc-hinge fragments derived from mouse IgG1 to identify residues at the CH2-CH3 domain interface involved in the regulation of serum half-life and neonatal/maternofetal transfer of IgG [1–4]. Amino acid residues in both the CH2 domain (Ile253 and His310) and CH3 domain (His435) play a central role in regulating both the control of catabolism and transcytosis of the Fc fragments across the neonatal intestine and yolk sac of pregnant mice [4]. Recent data have indicated that serum persistence of IgG is regulated by the MHC class I-related receptor, FcRn [5–7], a protein that was identified earlier to be responsible for transcytosis of IgG [8]. Consistent with the involvement of FcRn in these processes, Fc-hinge fragments with individual mutations of Ile253, His310 and His435, which have short serum half-lives and low neonatal/maternofetal transmission, also bind very poorly to recombinant, soluble mouse FcRn [9].

To date, Ile253, His310 and His435 are conserved in the IgG isotypes of all mammals with the exception of mouse IgG2b and human IgG3 (allotype G3m, s, t) which have tyrosine or arginine, respectively, instead of histidine at position 435 [10, 11]. Both IgG have a shorter biological half-life than the other isotypes [12] and, in addition, a decreased intestinal transfer in neonatal mice was reported for mouse IgG2b relative to IgG2a [13]. This indicates that His435 is also involved in the regulation of serum persistence and transcytosis of native, intact IgG in both mice and humans. In contrast to His435, the role of Ile253 and His310 in the catabolism/transcytosis of native, naturally occurring IgG cannot be evaluated since these residues are invariant across all known mammalian IgG [10].

For rat IgG (rIgG) isotypes, significant differences for the serum half-life in rats [14] and interaction with rat neonatal brush border [15] have been reported, despite conservation of Ile253, His310 and His435. However, in addition to these three residues other amino acids in the spatial vicinity of Ile253 (Thr256) [16] and His435 (His433

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Abbreviations: MES: (2-[N-morpholino]ethanesulfonic acid) hydrate **SPR:** Surface plasmon resonance **TCA:** Trichloroacetic acid

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Table 1. Sequence variation of rat IgG isotypes in the region proximal to FcRn interaction site [10]

	Amino acid sequence at position[a)]		
Isotype	252-253-254-255-256-257	307-308-309-310-311	385-386-387
IgG1[b)]	Thr-Ile-Thr-Leu-Thr-Pro	Pro-Ile-Leu-His-Gln	Gly-Gln-Pro
IgG2a	Thr-Ile-Thr-Leu-Thr-Pro	Pro-Ile-*Val*-His-*Arg*	Gly-Gln-Pro
IgG2b	*Leu*-Ile-*Ser-Gln-Asn-Ala*	Pro-Ile-*Gln*-His-Gln	Gly-*His-Ile*
IgG2c	*Met*-Ile-Thr-Leu-Thr-Pro	*His*-Ile-*Gln*-His-Gln	Gly-*Glu-Leu*

a) The sequence His433-Gln434-His435-His436 is invariant for all rat IgG isotypes.
b) The amino acid residues that show differences between isotypes are indicated by italics.

and His436) [1, 4, 17] have also been implicated in the control of IgG catabolism/transcytosis or interaction of IgG/Fc fragments with mouse or rat FcRn, but to a lesser extent [1, 4]. This suggests that amino acids proximal to key residues may modulate the activity in FcRn-mediated functions, indicating that the differences in biological activity of rIgG may be due to sequence differences in proximity to the FcRn interaction site. In this region, rIgG isotypes differ from each other at positions 252, 254, 255, 256, 257, 307, 309 and 311 (Table 1). In addition, a loop encompassing residues 386–387 of the CH3 domain contains sequence differences between rat isotypes (Table 1), and from X-ray crystallographic analyses residues in this loop have the potential to contact FcRn [18].

The hypothesis of this study was that some of these sequence differences might account for the activity differences of rIgG in FcRn-mediated properties. The effects of these amino acid changes might be via a direct effect on the IgG:FcRn interaction or an indirect effect by modulating the properties of a proximal critical residue such as Ile253 and/or His310. This has prompted us to analyze the pharmacokinetics, maternofetal transfer, and binding to FcRn of these IgG and correlate any observed differences with sequence differences. The sequence differences have been used to design mutations of a recombinant, murine IgG1 Fc-hinge fragment, and the effects of the mutations on FcRn-mediated functions determined. These studies have been carried out in mice, since mouse and rat FcRn α-chains share 91 % amino acid identity [19] and the available data indicate that the interaction of mouse and rat IgG with rat FcRn are very similar [20]. This approach has allowed us to identify additional amino acids that affect the IgG:FcRn interaction and has implications for the engineering of therapeutic IgG having optimal properties with respect to FcRn-mediated functions. Furthermore, it demonstrates that the grafting of amino acid changes from complete rIgG to a homologous recombinant murine Fc-hinge fragment can be used to assess the role of a particular residue in the IgG:FcRn interaction. The analysis also demonstrates that the excellent correlation between serum half-life, transcytosis and binding to FcRn reported previously for recombinant Fc-hinge fragments can be extended to intact, glycosylated IgG.

2 Results

2.1 Catabolism, maternofetal transmission and affinity for FcRn of rIgG isotypes

The clearance curves for radiolabeled rIgG are shown in Fig. 1. For each rIgG the elimination curves in different mice were similar and therefore Fig. 1 shows representative curves for one mouse from within each group. The serum samples collected at 24 h post-injection were analyzed by non-reducing SDS-PAGE/autoradiography and precipitation with TCA. All rIgG ran as a single band with a molecular mass of 150 kDa and were over 90 %

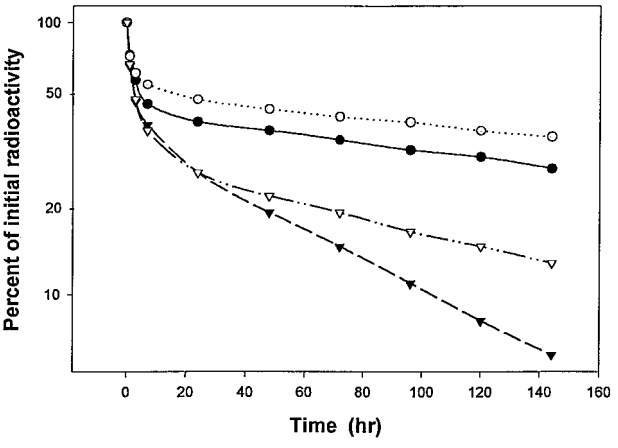

Figure 1. Clearance of rat IgG isotypes. Data for one representative mouse within each group are shown. ● IgG1; ○ IgG2a; ▼ IgG2b; ▽ IgG2c.

Table 2. Properties of rat IgG

Rat IgG isotype	Half-life (h)[a] α phase	β phase	Maternofetal transmission (% per gram of fetuses)	Affinity (K_d) for binding to FcRn[b] (nM)
IgG1	22.1 ± 1.0[c]	223.2 ± 17.4	2.05 ± 0.88	210
	(6)	(6)	(6)	
IgG2a	31.8 ± 3.2	234.7 ± 28.0	3.19 ± 1.50	140
	(6)	(6)	(6)	
IgG2b	14.7 ± 2.1	57.2 ± 8.1	0.69 ± 0.28	1067
	(6)	(6)	(6)	
IgG2c[d]	15.1 ± 0.6	101.5 ± 8.1	1.53 ± 0.28	380
	(5)	(5)	(4)	

a) For mouse IgG1 the following values were obtained: Half-life, α phase: 23.3 ± 0.9 h (eight animals), β phase: 237.8 ± 40.2 h (eight animals); maternofetal transmission: 3.39 ± 0.77 % (four animals).
b) Apparent affinities were calculated by running duplicate experiments at 80 μl/min (rIgG1, rIgG2a, rIgG2c) or 40 μl/min (rIgG2b), with the exception of rIgG1 for which a single experiment was run at 80 μl/min, but duplicate analyses at 40 μl/min yielded a K_d of 230 nM. On- and off-rates for each were determined using BIAevaluation software and the values for k_{on} and k_{off} averaged. Dissociation constants were calculated by $K_d = k_{off}/k_{on}$. Standard errors for k_{on} and k_{off} determinations from individual sensorgrams were < 3.5 % (rIgG1, rIgG2a, rIgG2b) or < 5.6 % (rIgG2c).
c) The number of animals are shown in parentheses.
d) From ICN Pharmaceuticals; all other IgG were obtained from Zymed laboratories.

insoluble in TCA (data not shown). This indicates that the rIgG persist in the serum as intact molecules and are neither associated with other serum proteins nor proteolytically degraded in the intravascular space. The pharmacokinetic parameters of the rIgG isotypes are shown in Table 2 where the α phase represents the equilibration time between the intra- and extravascular space, and the β phase represents the elimination of the equilibrated protein from the intravascular space and therefore can be used to determine the biological half-lives of the injected proteins. The data clearly demonstrate that rIgG1 and rIgG2a have similar half-lives in the circulation (ca. 230 h), which are insignificantly different from that of mouse IgG1 used as homologous control. Although the half-life of rIgG2a is slightly longer than that of rIgG1, the difference is not significant due to the approximate 10 % SD that is seen in the half-life measurements. IgG2c has an intermediate biological half-life (ca. 100 h) whilst rIgG2b has the shortest serum persistence (ca. 60 h). The half-lives of rIgG2b and rIgG2c are significantly lower than that of rIgG1 or rIgG2a ($p \leq 0.01$). The IgG concentration in the sera of mice used in pharmacokinetic studies was in the range of 1–4 mg/ml, but in the same group of mice injected with the same isotype the small differences in the biological half-life did not correlate with the IgG concentration.

The maternofetal transmission of the IgG was studied by measuring the protein-bound radioactivity taken up by fetuses of one litter relative to the radioactivity present in the maternal blood during the 24-h interval used for the transfer experiment. The results presented in Table 2 are in agreement with the pharmacokinetic data showing that both rIgG1 and rIgG2a are transcytosed more efficiently than rIgG2b. The transmission of rIgG2b and rIgG2c was significantly lower than that of rIgG1 or rIgG2a ($p \leq 0.01$).

All rIgG were transferred into the fetuses as intact molecules, demonstrated by the unchanged molecular mass of the radiolabeled IgG in the serum of pregnant mice 24 h after injection and the low percentage of radiolabeled TCA-soluble material (under 10 %). However, it is interesting to note that the TCA-soluble fraction in fetuses was higher for rIgG2b (54.5 ± 15.8 %) and rIgG2c (34.6 ± 5.9 %) than for rIgG1 (21.1 ± 1.8 %) and rIgG2a (16.0 ± 2.8 %), and this is consistent with the lower α phase half-life of both rIgG2b and rIgG2c *vs.* rIgG1 and rIgG2a (Table 2). In the few cases when the TCA-insoluble fraction was measured in the serum of fetuses injected with rIgG isotypes the values were over 90 % irrespective of the isotype injected, indicating that the proteolysis observed in the fetuses occurred in the extravascular space.

The relative affinities of the rIgG for binding to immobilized FcRn were analyzed by surface plasmon resonance (SPR). As others have reported [20], the association and

dissociation curves are biphasic when FcRn is immobilized on the chip. These binding curves fit well to interaction models involving two classes of non-interacting binding sites which has led to the suggestion that FcRn dimerizes to form the high-affinity binding receptor for IgG [20], whereas the monomeric form has a lower affinity [20, 21]. Due to this complexity, and for the purposes of the current study where relative affinities were of primary importance, we analyzed the dissociation curves during the initial part of the dissociation phase where the rapid dissociation (low affinity) was dominant. Furthermore, analysis of this part of the curve for which the FcRn on the chip was almost saturated minimized the effects of rebinding on the off-rate determination, with the exception of the analysis of rIgG2b for which saturation of the surface was impossible to achieve at the concentrations of rIgG2b used. Due to the inherent limitations of kinetic analyses using SPR [22] and the complexity of the interaction model [20], the kinetic constants and calculated dissociation constants may not be the absolute values, but give a good representation of the relative values. For these reasons, they are referred to as apparent K_d rather than *the* K_d and are shown in Table 2. From this analysis, the affinities decrease in the order rIgG2a > rIgG1 > rIgG2c > rIgG2b. In this respect, the apparent on-rate of rIgG2b is about tenfold lower than that of the other isotypes and this is the primary reason for the lower affinity. However, the apparent off-rate for this IgG may be an underestimate due to the fact that the immobilized FcRn was only about 30 % saturated when the dissociation phase was started (not shown); the low affinity of the rIgG2b : FcRn interaction precluded saturation of the FcRn binding sites on the sensor chip under the experimental conditions used.

The relative affinities of the rIgG isotypes for binding to recombinant mouse FcRn were further investigated by measuring the ability of various rIgG to inhibit the binding of radiolabeled FcRn to mouse IgG1-Sepharose as described previously [4]. Using this assay, the affinities decreased in the order rIgG2a > rIgG1 > rIgG2c > rIgG2b (data not shown), consistent with the SPR measurements. Statistically significant correlation coefficients (Pearson) were found between the biological half-life, maternofetal transmission and affinity for FcRn (Fig. 2). This is in agreement with previously published data for wild-type and mutated mouse Fc-hinge fragments derived from murine IgG1 [4], demonstrating that this correlation is also valid for native, intact IgG.

2.2 Catabolism of mouse Fc-hinge derivatives

The data obtained for the rIgG prompted us to use comparative sequence analyses of residues in the vicinity of the FcRn interaction site to design mutations of a recombinant murine (IgG1-derived) Fc-hinge fragment and to analyze the effects of these mutations on catabolism. Murine IgG1 shares a high degree of sequence identity with all rIgG isotypes [10]. The following mutations of mIgG1 residues were made to assess the effects of sequence differences in the different rat isotypes: Leu255, Thr256, Pro257 to Gln255, Asn256, Ala257 (L255Q/T256N/P257A) for rIgG2b; Pro257 to Ala257 (P257A) for rIgG2b; Pro307 to His307 (P307H) for rIgG2c; Pro307, Met309 to His307, Gln309 (P307H/M309Q) for rIgG2c; Gln386, Pro387 to Glu386, Leu387 (Q386E/P387L) for rIgG2c; Gln386, Pro387 to His386, Ile387 (Q386H/P387I) for rIgG2b. The locations of these amino acids on the three-dimensional structure of the highly homologous human Fc fragment [23] are shown in Fig. 3. Although from the sequences in Table 1, positions 252 and 254 are possible candidates for mutagenesis, our earlier studies indicated that Thr252 and Thr254 of

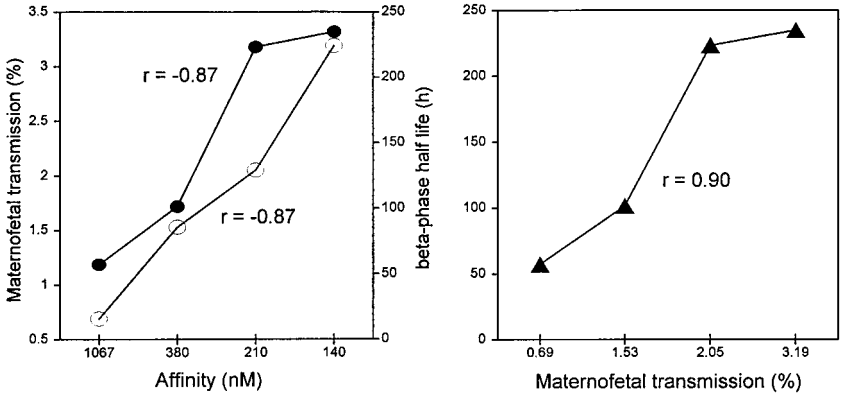

Figure 2. Correlation between serum half-life, maternofetal transfer and affinity for FcRn of rat IgG. ○ maternofetal transmission *vs.* affinity; ● beta phase half-life *vs.* affinity; ▲ beta phase half-life *vs.* maternofetal transmission.

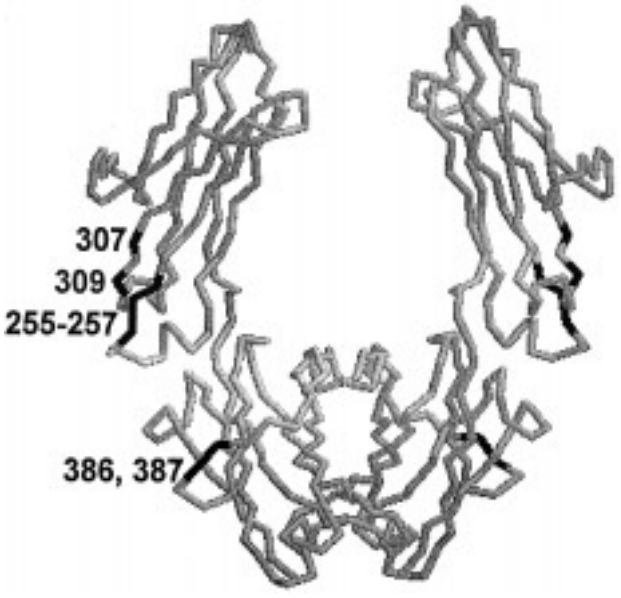

Figure 3. Location of the amino acids targeted for mutagenesis in the current study on the human IgG1 Fc structure [23] that is highly homologous to mouse and rat IgG [10].

the mouse IgG1-derived Fc-hinge fragment could be mutated to alanine and serine, respectively, without affecting FcRn binding affinity or serum half-life [16], and these were therefore not mutated in the current study. In addition, serine is a common residue at position 254 in many mammalian IgG [10].

The wild-type and mutated Fc-hinge fragments were expressed and purified from recombinant *E. coli* cells. Analysis using reducing and nonreducing SDS-PAGE indicate that the wild-type and mutant Fc-hinge fragments are expressed as a mixture of noncovalently linked and disulfide-linked homodimers (not shown). Using HPLC on TSK3000SW columns, the proteins emerged as a single peak with a retention time corresponding to a molecular mass of 55 kDa (not shown). Radiolabeled proteins were injected into mice and the serum radioactivity monitored over 6 days. For each recombinant protein, the plasma samples collected at the 24-h time point from a mouse within each group was subjected to HPLC on TSK3000SW. For all the Fc-hinge fragments, 90 % of the radioactivity was eluted as a major peak that has a retention time identical to that of the corresponding material prior to injection (data not shown). This indicates that the Fc-hinge derivatives persist in plasma as intact homodimers and are not associated with other serum proteins. The pharmacokinetic parameters of the Fc-hinge derivatives are shown in Table 3. L255Q/T256N/P257A and P257A have half-lives which are significantly shorter than that of the wild-type Fc-hinge fragment, indicating that the sequence differences at these positions in rIgG2b compared with other rIgG most likely account for the differences in pharmacokinetics, transcytosis and FcRn binding of IgG2b compared with the other rat isotypes. The properties of proline suggested that, in particular, A257 in IgG2b might be primarily responsible for the differences. Consistent with this concept, the P257A mutant has properties that are insignificantly different from those of the triple mutation.

P307H/M309Q has a shorter half-life than that of the wild-type fragment, suggesting that sequence differences at these positions between rIgG2c and rIgG1/rIgG2a are the cause of the lower serum persistence of IgG2c. The half-life of Q386H/P387I (IgG2b) is not significantly different from that of the wild-type unmutated Fc-

Table 3. β half-lives of mouse Fc-hinge derivatives with mutations corresponding to amino acid residues of rat IgG isotypes

Fc-hinge derivative[a]	Corresponding rat IgG isotype	Number of animals	β phase half-life (h)[b]
P257A[c]	2b	9	66.3 ± 10.5[d]
L255Q/T256N/P257A	2b	14	67.0 ± 14.0[d]
P307H	2c	7	135.1 ± 22.0
P307H/M309Q	2c	4	101.4 ± 12.4[d]
Q386E/P387L	2c	6	171.2 ± 12.8
Q386H/P387I	2b	7	135.4 ± 16.8

a) Wild-type Fc-hinge has a β phase half-life of 147.3 ± 22.7 h (10 animals).
b) β phase measured from 24–144 h.
c) Nomenclature P257A = Pro257 to Ala, etc.
d) $p \leq 0.01$ *vs.* wild-type; all other values are not significantly different ($p > 0.05$) from wild type.

hinge fragment, whereas unexpectedly, the half-life of Q386E/P387L (IgG2c) is longer but the increase is not significant ($p > 0.05$).

In addition to the above mutations, Met309 and Gln311 of the murine IgG1 Fc-hinge fragment were also mutated to Val309 and Arg311 to assess whether these residues might be responsible for the slightly increased activity of rIgG2a relative to the other rIgG. However, the serum half-life of this mutant (M309V/Q311R) was not significantly different from that of the wild-type unmutated Fc-hinge fragment (not shown).

3 Discussion

This report describes the analysis of the serum half-lives and maternofetal transfer of rIgG and this information, taken together with sequence data, has been used to identify additional amino acid residues that are involved in the IgG/Fc:FcRn interaction. Verification of the contribution of a particular rIgG residue to the FcRn interaction site has been carried out by mutating the corresponding residue in a recombinant Fc-hinge fragment derived from the highly homologous murine IgG1 isotype. This recombinant Fc-hinge fragment can be expressed in *E. coli* and therefore provides us with a rapid route to assess the role of a particular residue in regulating serum IgG levels.

Our data concerning the catabolism of rIgG isotypes are in contrast to those reported by Bazin [14]. Rat IgG2b is the isotype with the shortest half-life in our experiments (3–4 days), whereas Bazin [14] reported the longest half-life for this isotype (15 days) and the reciprocal for rIgG2a. The apparent discrepancy is most likely due to the use of unpurified ascitic fluid as a source of rIgG, whereas in the current study commercially available purified rIgG have been used. Alternatively, the differences may be due to the use of rats in the study of Bazin and mice in our study, although this is made unlikely by the indications that the rat IgG:rat FcRn and mouse IgG:rat FcRn interactions are similar [20]. Furthermore, the data for the binding affinities of rIgG for FcRn in the current study are partially consistent with the results on the binding of rIgG to detergent-isolated FcRn from neonatal rat brush borders [15]. Thus, in agreement with our data, Peppard and colleagues [15] have shown that rIgG2b has the lowest affinity for brush border FcRn but, contrary to our findings for rIgG1, the affinity of rIgG1 for brush border FcRn was found to be as low as that of rIgG2b [15].

The apparent affinities determined from kinetic analyses using SPR for the rIgG:FcRn interactions are lower than those determined previously for the murine IgG1-derived Fc-hinge fragment [9, 16]. This is unexpected due to the longer half-life of, for example, rIgG1 relative to the Fc-hinge fragment. There are several possible reasons, related to the technicalities of SPR, which might explain these apparent discrepancies. First, in our SPR studies the observed on-rates of the IgG:FcRn are about tenfold lower than those of the Fc-hinge fragment, and our analyses indicate that the binding of rIgG to immobilized FcRn, with the possible exception of rIgG2b, is significantly mass transport limited. Consistent with this, the on-rates of rIgG binding to immobilized FcRn are both flow rate and ligand density dependent (not shown). Although alterations in ligand density could affect the configuration of the interaction [20] and as a result affect the kinetics, this is not so for variations in flow rate. Second, for the wild-type Fc-hinge fragment the higher coupling density of FcRn on the flow cells that were used in our earlier experiments [9, 16] suggest that the reported higher affinity might also be accounted for by an increased amount of the high-affinity FcRn dimer recently described by Bjorkman and colleagues [20, 21]. In this respect, our apparent affinities for the rIgG:FcRn interactions correlate with the lower affinity mode of binding reported using equilibrium methods when rIgG are immobilized [20], with the exception of rIgG2b which apparently could not be coupled to the sensor chip in this earlier analysis. The possibility that the lower affinities of rIgG for murine FcRn is due to the use of a heterologous system in the binding studies is made unlikely by our observation that the affinities of binding of murine IgG1, rIgG1 and rIgG2a all fall within the same range (Table 2, and not shown).

For all of the rIgG an excellent correlation between serum half-life, maternofetal transfer and affinity for FcRn is observed, and this is in agreement with our previous data obtained with recombinant wild-type and mutated Fc-hinge fragments [2, 4]. This extends the correlation between FcRn-mediated functions to glycosylated, intact IgG. Previously we have reported that the β phase half-life of mIgG1 and mIgG1-derived Fc-hinge are insignificantly different [1], whereas the data in Tables 2 and 3 would appear to argue against it. However, we have observed seasonal variations in serum half-lives of IgG/Fc-hinge fragments for reasons that are not clear. To exclude aberrant results due to this, in all our pharmacokinetics experiments of analyses of rIgG or mutated Fc-hinge fragments appropriate controls were run (mIgG1 for rIgG; wild-type Fc-hinge for mutated Fc-hinge fragments).

For rIgG2b, the studies show that the sequence difference at position 257 is most likely responsible for its shorter half-life and lower maternofetal transmission rel-

ative to other rIgG. This isotype has alanine at position 257 instead of the more usual proline and this may affect the conformation of the loop supporting Ile253 that plays a central role in the IgG:FcRn interaction. In addition, our recent data concerning the affinity improvement of a murine Fc fragment for FcRn indicate that position 256 may make contact [16], suggesting that Pro257 might have a more direct role in the IgG:FcRn interaction. Analysis of P307H/M309Q and P307H, mutations that were prompted by inspection of the rIgG2c sequence, suggest that glutamine at 309 reduces the serum half-life of the recombinant Fc-hinge fragment, whereas a proline to histidine change as a single mutation at position 307 has no effect. However, although not present in human IgG, Gln309 is present in three of four mouse IgG isotypes and two of these have serum half-lives similar to that of mIgG1. Thus, Gln appears to be permissive at this position, and the most obvious interpretation of the data for the P307H and P307H/M309Q mutants is that the combination of Gln309 and His307 is responsible for the lower binding affinity of rIgG2c for FcRn [4]. This is reminiscent of an earlier study in which mutation of two residues in the murine CH3 domain (His433 and Asn434) resulted in an Fc-hinge fragment with a shorter serum half-life than the wild-type fragment, whereas mutation of these two residues individually had no effect [4].

The data that derive from the analysis of mutants corresponding to rIgG2b (Q386H/P387I) and rIgG2c (Q386E/P387L) show that the nature of the residues at position 386–387 does not significantly affect the IgG:FcRn interaction, indicating that amino acids in this loop do not contact FcRn.

In summary, the studies have further extended the analysis of the murine FcRn interaction to rIgG and demonstrate an excellent correlation between serum half-life, maternofetal transfer and binding to FcRn similar to that observed previously for recombinant Fc-hinge fragments [4]. This analysis has resulted in the identification of additional residues that are involved in the IgG:FcRn interaction, and extend earlier analyses to exclude the involvement of amino acids in a fourth loop located at the CH2-CH3 domain interface that encompasses residues 386 and 387. Finally, these studies have relevance to using protein engineering to improve antibodies for therapeutic applications.

4 Materials and methods

4.1 Generation of mutated Fc-hinge fragments derived from mouse IgG1 (mIgG1)

Mutations were made using designed mutagenic oligonucleotides and either PCR mutagenesis or site-directed mutagenesis as previously described for other Fc-hinge mutants [1].

4.2 Expression and purification of the recombinant proteins

For the expression of recombinant proteins, *E. coli* BMH71-18 was used as host [1]. The recombinant proteins were expressed with C-terminal polyhistidine tags. Wild-type and mutated Fc-hinge fragments were purified using Ni^{2+}-NTA-agarose as described previously [1].

4.3 Immunoglobulins

Rat IgG1, IgG2a, IgG2b (Zymed Laboratories, San Francisco, CA) and IgG2c (ICN Pharmaceuticals, Cosa Mesa, CA) isotypes were used without further purification since by HPLC the percentage of monomeric IgG was over 90 % in all preparations. No IgG2b was detected in rIgG1, rIgG2a or rIgG2c using radial immunodiffusion (NanoRid kits, The Binding Site, Birmingham, GB). Mouse monoclonal IgG1 antibody specific for the human CD22 marker (RFB4) was prepared by Abbott Biotechnology (Needham Heights, MA). This antibody does neither cross-react with murine tissues nor peripheral blood cells [24] and was used as a control.

4.4 Radiolabeling of proteins

The proteins were radiolabeled with Na ^{125}I using the Iodo-Gen reagent as described in detail previously [1]. The specific activity of the radiolabeled proteins was approximately 10^6 cpm/μg with less than 5 % free iodine as determined by precipitation with TCA.

4.5 Determination of the IgG concentration

The concentration of serum IgG was detected in mice using radial immunodiffusion and BindaRid kits (The Binding Site). Precipitin ring diameters were measured electronically.

4.6 SDS-PAGE and HPLC

SDS-PAGE and HPLC (TSK 3000SW column; TosoHaas, Montgomeryville, PA) analyses were conducted as described previously [1]. A Pharmacia PhastSystem was used followed by autoradiography to analyze the size of the radiolabeled proteins as they persisted in the serum.

4.7 Pharmacokinetic analysis

Half-lives of radioactive rIgG were determined in SWISS outbred mice (Charles River, Wilmington, MA) as previously described [1]. The sera obtained at 24 h after injection of

rIgG were analyzed by SDS-PAGE and the molecular mass and homogeneity of radiolabeled rIgG analyzed. The sera collected at 24 h after injection of Fc-hinge derivatives were also analyzed by HPLC as previously described [1]. The percent of protein-bound radioactivity in all sera was measured by precipitation with TCA.

4.8 Recombinant FcRn

Recombinant soluble mouse FcRn was expressed and purified using the baculovirus system as described previously [9]. The molecular mass of the protein determined by gel filtration on Sephacryl S-100HR was 50 kDa and the purity was over 95 %.

4.9 SPR analyses of the interactions of rIgG with FcRn

The interactions of the rIgG with FcRn were investigated using SPR and a BIAcore 2000. It has become apparent that the interaction of IgG with FcRn is complex and involves two classes of non-interacting binding sites when FcRn is immobilized on the chip [20, 21]. Since immobilization of FcRn, rather than IgG immobilization, results in a geometry that more closely resembles the physiological situation (with FcRn being membrane bound), we have used this geometry in the current study. FcRn was immobilized to the flow cells of a CM5 chip using standard amine coupling to a density of 1410 resonance units. Rat IgG were flowed over the chip in 50 mM MES pH 6.0, 150 mM NaCl plus 0.01 % Tween-20 at flow rates of 40 μl/min (rIgG2b) or 80 μl/min (other rIgG isotypes) at concentrations ranging from 1 μM to 250 nM. Analysis of the data obtained using higher coupling densities and slower flow rates indicated that the binding was mass transport limited (not shown), and for this reason the above experimental conditions were used. A lower flow rate was used for rIgG2b than for the other isotypes as it was difficult to reach saturation of the chip at higher flow rates without using excessive amounts of material, due to the low affinity of the rIgG2b : FcRn interaction. Flow cells were regenerated between each cycle by injecting 50 mM MES pH 7.5, 150 mM NaCl plus 0.01 % Tween-20. This resulted in complete dissociation of any remaining IgG bound to FcRn due to the known marked pH dependence of this interaction [9, 17]. Data from background chips (flow cells treated with buffer only during amine coupling cycle) were subtracted from all sensorgrams prior to kinetic analyses using BIAevaluation 2.1 software. Due to the known limitations of kinetic measurements using the BIAcore [22] and the complexity of the IgG : FcRn interaction [20], the on- and off-rates will be termed apparent rather than being considered as absolute values. The apparent on- and off-rates were determined by fitting the curves to single exponentials; for this the early sections of dissociation plots and linear sections, following transformation, of association plots were used for analysis. Dissociation constants (K_d) were determined from these apparent on- and off-rates, and since data for all rIgG were processed in a similar way, these are good representations of the relative affinities but possibly do not represent the absolute K_d.

4.10 Maternofetal transmission

Previously described methodology was used with pregnant outbred SWISS mice (Charles River) near term (16–18 days) [3, 4]. The percentage of transmission was calculated with the formula: Transmission (%) = 100 Rf/3.6 Wp Wf (R3 – R24) where R3 and R24 = the radioactivity in 20 μl of maternal blood at 3 min and 24 h after injection; Rf = TCA-insoluble radioactivity of fetuses of one litter; Wp = weight of pregnant mice (in grams) and Wf = weight of fetuses (in grams).

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