Short paper

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Localization of the site of the IgG molecule that regulates maternofetal transmission in mice

Site-directed mutagenesis of a recombinant Fc hinge fragment has recently been used to localize the site of the mouse IgG1 (mIgG1) molecule that is involved in the intestinal transfer of recombinant Fc hinge fragments in neonatal mice. This site encompasses Ile-253, His-310, Gln-311, His-433 and Asn-434, localized at the CH2-CH3 domain interface and overlapping with the staphylococcal protein A-binding and catabolic sites. In the present study, the effect of these mutations on the maternofetal transfer of Fc hinge fragments has been studied. Experiments to analyze transfer of radiolabeled Fc hinge fragments from the circulation of 15–18 day pregnant mice to fetuses *in utero* demonstrate that the mutations affect the maternofetal transmission in a way that correlates closely with the effects of the mutations on intestinal transfer and catabolism. The studies indicate that the neonatal Fc receptor, FcRn, is involved in transcytosis across both yolk sac and neonatal intestine in addition to the regulation of IgG catabolism.

1 Introduction

The route of transmission of maternal IgG in mice and rats before birth is through the yolk sac and after birth through colostral-intestinal absorption [1]. Maternofetal transmission through the yolk sac is, however, a minor route of IgG transfer and as a consequence the concentrations of serum IgG at birth are 20–30 % of those of adult mice [2]. Transintestinal transfer of IgG in rats and mice is mediated by a highly homologous neonatal Fc receptor (FcRn) localized on the neonatal brush border [3–6]. FcRn binds IgG in a pH dependent way, with binding occurring at the luminal pH (6–6.5) of the jejunum and release at the pH of the blood (7.4) [3, 4]. Interestingly, despite having a distinct function, FcRn shares homology with class I major histocompatibility complex heavy chains [6] and comprises a 45–53 kDa α chain associated with β_2-microglobulin. This class I homolog binds to amino acid residues within both CH2 (Ile-253, His-310, Gln-311) and CH3 (His-433, Asn-434) domains, located at the CH2-CH3 domain interface of IgG [7, 8]. FcRn α chain mRNA has been detected in fetal yolk sac of mouse [5, 9] and expression of a human homolog of FcRn has been reported in human placenta [10]. In addition, FcRn has been isolated as a protein from rat yolk sac [11]. This suggests that both maternofetal and intestinal transfer of IgG are mediated by the same Fc receptor. The aim of this work was to investigate whether the amino acids located at the CH2-CH3 domain interface involved in the intestinal transfer of IgG are the same as those that play a role in maternofetal transmission.

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Abbreviations: **mIgG:** Mouse gamma globulin **FcRn:** Neonatal Fc receptor

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2 Materials and methods

2.1 Expression, purification and analyses of Fc hinge fragments

Recombinant wild-type Fc hinge and mutant Fc hinge fragments derived from mIgG1 were expressed in *Escherichia coli* and purified using Ni^{2+}-NTA (nitrilotriacetic acid)-agarose as described [12]. The following mutants were used: I253A, H285A, H310A-Q311N, H433A-N434Q and H310A-Q311N/H433A-N434Q. Nomenclature of mutants used is as follows: I253A = Ile 253 to Ala, etc. The results of SDS-PAGE and circular dichroism analyses were reported previously [12].

2.2 Mouse IgG1 (mIgG1)

Purified monoclonal anti-human CD25 antibody (Abbott Lab, Needham, MA) of the IgG1 isotype was used in this study.

2.3 Radiolabeling of proteins

The Iodo-Gen (Pierce, Rockford, IL) procedure [13] was used to radiolabel proteins with Na[^{125}I] (Amersham, Arlington Heights, IL). The free iodine was removed by centrifugation on Microspin™ G-25 colums (Pharmacia, Piscataway, NJ). The final volume of radioactive protein was adjusted to 1 ml with PBS containing 1 mg/ml egg albumin (Sigma, St. Louis MO). The specific activities of the radiolabeled proteins were 10^6 to 5×10^6 cpm/μg with

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less than 10% free iodine. The radioactive proteins were stored at 4°C for not more than 1 week prior to injection into mice.

2.4 Maternofetal transfer

Previously described methodology [14, 15] was used. Pregnant albino Swiss mice (30–45 g) from the Center of Immunology, Bucharest, Romania and outbred severe combined immunodeficient (SCID) mice (20–25 g) from Taconic (Germantown, NY) near term (15–18 days) were fed 0.01% NaI in drinking water. One day later, mice were injected with 150 µl radiolabeled protein (2×10^7–5×10^7 cpm) in the tail vein and bled retro-orbitally with a 20 µl capillary 3 min post-injection and then 24 h later when the fetuses were delivered by cesarean section. The fetuses of a litter were pooled (discarding the placenta), washed in saline, weighed, frozen in liquid nitrogen and homogenized in ten volumes of 10% trichloroacetic acid (TCA). The suspension was centrifuged and the radioactivity of the precipitate was measured in a gamma counter. The percentage of transmission was calculated with the formula: % Transmission (%T) = (R3)/[(R1-R2) × (W × 0.072)/0.02)] where R1 = radioactivity in 20 µl maternal blood at 3 min; R2 = radioactivity in 20 µl maternal blood at 24 h; W = body weight (grams) and R3 = radioactivity of the fetuses.

The total weight and number of fetuses in a given litter varied from litter to litter and for this reason, the transmission data are presented per unit weight of fetuses rather than the amount transferred per litter (% T/g) [14]. The blood volume of pregnant mice was considered equal to 7.2% of body weight [16]. The radioactivity in the maternal blood available for transmission to the fetus was calculated by deducting the radioactivity remaining at 24 h from that introduced into the circulation and measured at 3 min. mIgG1 and recombinant Fc hinge fragments do not have the same elimination rate in the first 24 h following administration (alpha phase) [12], and therefore the percentage of transmission was corrected by multiplying it with the elimination rate of the alpha phase as follows: % Transmission (corrected) = % T/g × $[(0.693/(T_{1/2}) \times 100]$ where $T_{1/2}$ is the half-life of the alpha phase in days and was obtained from published data [12, 17].

2.5 Inhibition of maternofetal transfer

Outbred pregnant SCID mice (Taconic) were injected i.p. with 1.5 ml mIgG1 solution in PBS containing 25 mg protein.

After 3 h, the animals were injected in the tail vein with 150 µl radiolabeled wild-type Fc hinge or the H310A-Q311N/H433A-N434Q mutant (3×10^7 cpm) and bled retro-orbitally with a 44 µl heparinized capillary at 3 min and 24 h. At the latter time point the fetuses were delivered by cesarean section. The remainder of the procedure is described above.

2.6 Determination of serum IgG concentration

The concentration of serum IgG of outbred pregnant SCID mice was determined using radial immunodiffusion with Nanorid™ and Bindarid™ kits (The Binding Site, Birmingham, GB). This was carried out for both untreated mice and mice into which mIgG1 (25 mg) was injected. Precipitin ring diameters were measured electronically. Untreated SCID mice with an IgG concentration over 10 µg/ml were considered leaky and were not used for the maternofetal transfer experiments.

2.7 Polyacrylamide gel electrophoresis (PAGE) of serum samples

Serum samples were obtained from blood collected from pregnant SCID mice and fetuses at 24 h after administration of radiolabeled Fc derivative. The sera of individual fetuses of a litter were pooled before analysis. PAGE was run under nonreducing, native conditions using 7.5% PhastGels (Pharmacia). Gels were stained with Coomassie brilliant blue R-250 (Sigma) and the radioactivity was detected using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

3 Results and discussion

The transfer of radiolabeled mIgG1 and recombinant Fc hinge fragments from the circulation of 15–18 day pregnant Swiss mice to the fetuses was analyzed by measuring the protein-bound radioactivity taken up by the fetuses relative to the radioactivity present in the maternal blood during the 24 h interval used for the transfer experiment. The results are presented in Table 1.

The wild-type Fc hinge is transferred across the yolk sac of pregnant Swiss mice in a similar way to intact mIgG1 and the H285A mutant. The histidine at position 285 is located in a loop on the external surface of the CH2 domain which

Table 1. Maternofetal transmission of recombinant Fc hinge fragments in Swiss mice

mIgG1/Fc hinge fragment[a]	Percentage transmission[b] For all fetuses of one litter	Per gram of fetuses	Corrected for catabolic rate per gram of fetuses
mIgG1	2.29 ± 0.67	0.715 ± 0.316	2.46 ± 1.09
Wild-type Fc hinge	3.37 ± 0.56	0.426 ± 0.145	2.81 ± 0.96
H285A	2.76 ± 1.01	0.337 ± 0.076	2.69 ± 0.61
I253A	0.12 ± 0.05	0.016 ± 0.003	0.17 ± 0.03
H310A-Q311N	0.06 ± 0.05	0.013 ± 0.002	0.15 ± 0.02
H433A-N434Q	0.29 ± 0.05	0.100 ± 0.023	0.67 ± 0.15
H310A-Q311N/ H433A-N434Q	0.05 ± 0.02	0.024 ± 0.008	0.18 ± 0.06

a) For mIgG1 and all Fc hinge fragments, except H433A-N434Q and H310A-Q311N/H433A-N434Q, four pregnant mice were used. For H433A-N434Q and H310A-Q311N/H433A-N434Q, three mice were used.

b) For calculation of the percentage of transmission, see Sect. 2.4.

Table 2. Maternofetal transmission of recombinant mouse Fc hinge fragments in SCID mice

Fc hinge fragment	No. of animals	Percent of transmission per gram of fetuses
Wild-type Fc hinge	5	1.700 ± 0.270
Wild-type Fc hinge in mice injected with mIgG1[a)]	3	0.955 ± 0.099
H310A-Q311N/ H433A-N434Q mutant	3	0.060 ± 0.020
H310A-Q311N/ H433A-N434Q mutant in mice injected with mIgG1[a)]	3	0.019 ± 0.005

a) SCID mice injected with 25 mg mIgG1 3 h before the administration of the wild-type or mutant Fc hinge fragment.

is distal to the CH2-CH3 domain interface [18]. The transfer rate of aglycosylated wild-type Fc hinge (2.81 %) is similar to that of the glycosylated mIgG1 (2.46 %), indicating that for this isotype, glycosylation does not affect maternofetal transmission. This suggests that the conclusions concerning the localization of the site involved in maternofetal transmission drawn from experiments using aglycosylated Fc fragments can be extended to glycosylated IgG.

The other Fc hinge mutants showed considerably lower transmission, indicating that the mutations at the CH2-CH3 domain interface affect maternofetal transfer. Mutations in the CH2 domain (I253A and H310A-Q311N) have a more marked effect than those in the CH3 domain (*e.g.* H433A-N434Q) (Table 1) but this does not exclude a role for other conserved amino acid residues in the CH3 domain in building the FcRn interaction site.

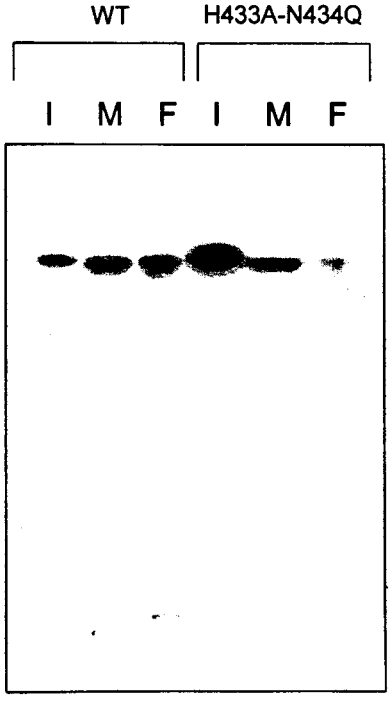

Figure 1. 7.5 % PAGE (native) followed by PhosphorImager analyses of the sera from pregnant mice and fetuses (I, Fc hinge prior to injection; M, serum of pregnant mouse; F, pooled sera of fetuses of one litter).

Fc hinge fragments with mutations at the CH2-CH3 domain interface are cleared from the serum with shorter alpha and beta-phase half-lives than the wild-type Fc hinge or H285A mutant [12, 17] and it is therefore possible that the lower transmission of these mutants might be due to rapid elimination from the circulation of the pregnant mice, fetuses, or both. To investigate this possibility, the percentages of transmission (per gram of fetuses) were corrected for the elimination rates (alpha phase) of the corresponding mutants. For the wild-type and mutant Fc hinge fragments, the corrected values presented in Table 1 correlate with the uncorrected values with a Pearson's correlation coefficient of 0.99 ($p = 0.001$), clearly indicating that the catabolic rate of these Fc hinge fragments does not significantly influence the values obtained for maternofetal transmission.

These data were confirmed and extended using pregnant outbred SCID mice devoid of serum IgG. As a consequence of the absence of serum IgG, the competition for binding to yolk sac FcRn by endogenous IgG would be predicted to be minimal and the transfer of injected IgG might be more efficient. Analysis of maternofetal transfer of the wild-type Fc hinge and the H310A-Q311N/H433A-N434Q mutant (Table 2) confirms that even in the absence of competition by endogenous IgG, residues at the CH2-CH3 domain interface play a major role in the transfer of Fc hinge fragments. Furthermore, wild-type Fc hinge is transferred with higher efficiency in SCID mice (1.7 %) than in Swiss mice (0.43 %). That this difference is indeed due to lack of competition was demonstrated by injecting mIgG1 into pregnant SCID mice and measuring the transmission of radiolabeled wild-type Fc hinge or H310A-Q311N/H433A-N434Q mutant to fetuses. The results presented in Table 2 show that when the serum concentration is raised to 2–3 mg/ml, a 44 % and 68 % inhibition of the

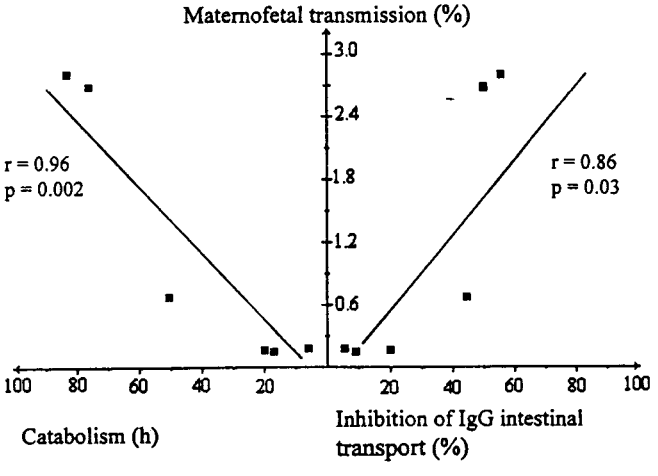

Figure 2. Correlation between the percentage of maternofetal transmission, the inhibition of neonatal transintestinal transport of IgG and catabolism (beta phase half-life) of wild-type/mutant Fc hinge fragments (r = Pearson's correlation coefficient: p = probability).

transmission of wild-type Fc hinge and the H310A-Q311N/H433A-N434Q mutant, respectively, was observed.

PAGE followed by PhosphorImager analysis of serum samples of both maternal and fetal origin indicate that the radiolabeled Fc hinge derivatives persist in the blood of both pregnant mice and fetuses as intact molecules that are not covalently associated with other serum proteins (Fig. 1). These data are in agreement with previously published results on nonpregnant mice [12]. For both the wild-type Fc hinge fragment and the less efficiently transmitted mutant H433A-N434Q, PAGE analyses of sera from pregnant mothers and fetuses yield the same pattern as that of the protein prior to injection. For poorly transmitted mutants such as I253A, H310A-Q311N and H310A-Q311N/H433A-N434Q, such PAGE/PhosphorImager analysis of fetal serum samples was not possible since the concentration of these fragments in the sera of fetuses was below the detectable level. However, for all Fc hinge fragments, less than 10 % of the fetal radioactivity was TCA soluble, indicating that the proteins were almost quantitatively TCA precipitable after transfer to the fetal circulation.

The data obtained in Swiss and SCID mice clearly show that the site of the mIgG1 molecule that is involved in binding to yolk sac FcRn is located at the CH2-CH3 domain interface and overlaps with the sites involved in intestinal transfer [7] and the control of catabolism [12] (Fig. 2). The correlation between maternofetal transmission and inhibition of neonatal intestinal transfer of mIgG by the wild-type and mutant Fc hinge fragments [7] or beta-phase half-life [12] of these Fc derivatives are excellent (Pearson's coefficients of 0.86 and 0.96, respectively), suggesting that FcRn is involved in all three processes. This possibility is also suggested by previous results demonstrating high levels of FcRn α chain mRNA in both intestinal brush border and yolk sac [5, 9]. In addition, mice that lack FcRn have a decreased intestinal transmission of IgG [19] and abnormally short serum half-lives of mIgG1/wild-type Fc hinge [9, 20]. However, the cellular location of the FcR involved may differ so that pH-dependent binding of IgG might occur on the cell surface, as in intestinal transfer [2, 3] or in the endosomal system of yolk sac cells, as in maternofetal transfer [11]. For both maternofetal transfer and the control of IgG catabolism, endosomally located FcRn/IgG interactions might be a shared feature; further experiments are in progress to test this hypothesis. Finally, the CH2-CH3 domain interface residues analyzed in this study are highly conserved in human IgG isotypes [21], suggesting that this study has relevance to the transfer of passive immunity across the human placenta.

4 Concluding remarks

This study, involving the use of wild-type and mutant Fc hinge fragments, has resulted in the identification of amino acid residues of mIgG1 that regulate maternofetal transfer in mice. These residues are located in both the CH2 (Ile-253, His-310, Gln-311) and CH3 (His-433, Asn-434) domains and are positioned at the CH2-CH3 domain interface [18]. The effects of mutations of these residues on maternofetal transfer correlate closely with the effects on both intestinal transfer [7] and control of catabolism [12]. Taken together with earlier data [7, 9, 20], the findings of this study implicate a common role for the Fc receptor, FcRn, in these three processes.

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