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Transcytosis and Catabolism of Antibody

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Abstract

This review describes the evolution of our knowledge of the transmission of immunoglobulin G (IgG) from mother to infant and the factors which regulate the persistence of IgG in the circulation. These apparently unrelated processes involve the same Fc receptor, FcRn (n = neonatal). FcRn appears to carry out these diverse roles by binding to IgG and then either transporting the bound IgG across cells (transcytosis) or recycling its cargo back to the cell surface (control of catabolism). IgG that is taken up by cells in the absence of binding to FcRn undergoes degradation. Thus, FcRn is the "protective" receptor that serves to maintain IgG homeostasis and deliver IgGs across cellular barriers.

Key Words

Transcytosis
Catabolism
Antibody
Immunoglobulin G
Fc receptor
Neonatal Fc receptor

The Structure and Effector Functions of Immunoglobulin G

The five immunoglobulin classes (G, M, A, D, and E) differ in structure and function. Immunoglobulin G (IgG) is the major class of immunoglobulin, with its serum level (about 10 mg/mL) being three times higher than that of all other immunoglobulins taken together. The IgG molecule consists of two heavy chains (H) and two light chains (L) linked together by interchain disulfide bonds (Fig. 1). Each heavy chain is built from one variable (VH) and three constant (CH1, CH2, and CH3) domains. The VH domain, together with the light chain variable (VL) domain, are highly variable in sequence from one antibody to the next and constitute the antigen binding site. In contrast, for a given IgG subclass the CH2 and CH3 domains are almost invariant and are responsible for the effector functions, such as complement fixation and binding to Fc recep-

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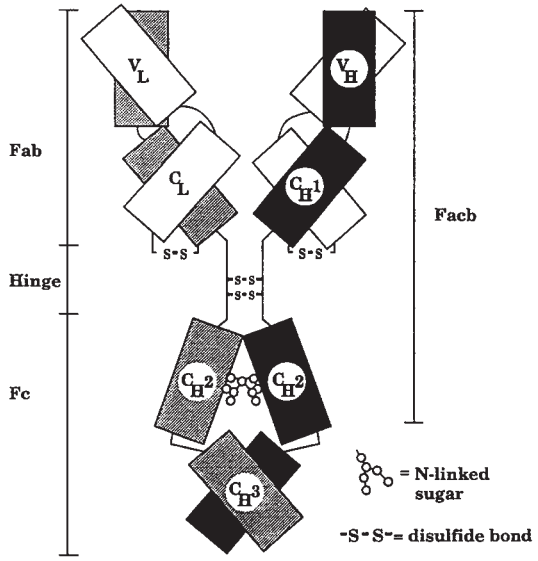

Fig. 1. Schematic representation of the domain structure of the IgG molecule.

tors. The hinge region provides a flexible linker between the Fc region and the Fab arms, and is variable in sequence and length for different IgG subclasses. This flexible region of the IgG molecule is susceptible to digestion by proteases such as papain which cleave the molecule into Fab and Fc fragments. The IgG molecule is invariably glycosylated on the CH2 domain, whereas less frequently glycosylation has been observed in the variable regions *(1)* (Fig. 1).

The efffector functions of the Fc region of an IgG antibody can be divided into two categories *(2)*:

1. Effector functions that operate following the binding of antibody to antigen. The antigen-bound antibody destroys antigen by recruiting Fc-binding molecules of the complement cascade (complement mediated cytotoxicity) or Fc-receptor (FcR) bearing cells (phagocytosis and antibody dependent cell mediated cytotoxicity).
2. Effector functions that operate independently of antigen binding. The Fc region is responsible for the transfer of an IgG across cellular barriers (transcytosis) and regulates serum persistence (control of catabolism).

Transcytosis of IgG

The transfer of IgG from mother to young provides the newborn with humoral immunity during the early part of its life. Despite the observation many years ago that this process occurred, the molecular mechanisms by which this transfer is brought about have only begun to be unraveled during the past 10–15 yr. As the majority of work has been carried out for rodents and humans, the transfer of maternal IgG in these species will be the focus of this review. For maternal IgG to be transferred, several cellular barriers have to be crossed by the IgG molecules: from maternal blood across the placenta (humans) or yolk sac (rodents) to the fetus; From maternal blood into milk and then across the neonatal intestine following ingestion (rodents). These processes necessitate that IgG is transferred intact across a variety of cell types including trophoblast and intestinal epithelial cells. This process of transport is called transcytosis. In addition, IgG transcytosis occurs in several processes in adults, including delivery of IgG from blood to liver *(3,4)*, across adult intestinal epithelial cells *(5)*, from blood to milk in the lactating mammary gland *(6)*, and in the regulation of serum IgG homeostasis (see below).

Almost 40 yr ago Brambell proposed that the receptors involved in the transfer of maternal IgG were saturable and able to bind to the Fc region of IgG *(7)*. Furthermore, he suggested that a similar receptor was involved in both the transmission of maternal IgG and the regulation of serum IgG levels. The saturable nature of the putative receptor was confirmed by Waldmann and colleagues several years

later *(8,9)*, but it was not until the mid-1990s that direct evidence linking the regulation of IgG catabolism and transmission was obtained *(10–12)*. The Fc receptor involved in the passage of IgG across the neonatal intestine was also shown to bind to IgG at acidic pH, whereas binding at neutral pH could not be detected *(13–16)*. This led to the hypothesis that in the neonatal gut (pH 6.0), binding of IgG to the Fc receptor on the cell surface could occur *(13,14)*. This would then be followed by endocytic uptake of the receptor bound IgG into acidic intracellular vesicles, transport across the cells and release of IgG upon fusion of the vesicle with the basolateral cell surface at near neutral pH. However, for transport across the yolk sac or placental trophoblast, a modification to this mechanism needs to be invoked *(17)*. IgG binds to the Fc receptor following fluid phase uptake into endocytic vesicles rather than at the cell surface which is near neutral pH. Consistent with these hypotheses, expression of the Fc receptor was detected on the membrane of neonatal brush border *(15,18)*, whereas for rodent yolk sac only intracellular expression could be detected *(17)*.

Major progress was made in the understanding of IgG transcytosis with the isolation of the gene encoding the "neonatal" Fc receptor *(19)*. This Fc receptor was designated FcRn (n for neonatal) and, unexpectedly, was found to be related to MHC Class I molecules. Similar to MHC Class I molecules, it is a heterodimer of a 45–50 kDa alpha chain associated with the invariant 12 kDa polypeptide, beta2-microglobulin (β2m). FcRn was shown to be highly expressed in the neonatal gut *(19)*. Consistent with its suggested role in the transmission of maternal IgG in mice, animals deficient in β2m due to homozygous deletion of the β2m gene are defective in this IgG transfer *(20)*. In addition, more recent analyses with recombinant FcRn confirmed earlier cell binding studies demonstrating the pH dependence of the IgG-FcRn interaction *(21,22)*. Although FcRn expression was reported to be high in neonatal brush border, using Northern blotting some expression was also detected in adult tissues such as heart and kidney *(19)*. This was interpreted to be due to cross-reactivity between FcRn and the ubiquitously expressed MHC class I molecules. However, more recent studies have demonstrated that FcRn expression is not limited to the neonatal period but extends throughout adult life *(10)* and discussed further below), although the available data suggests that the expression levels in adult tissues are lower than in the neonatal gut.

Several in vitro systems for the analysis of FcRn function have recently been described *(23–26)*. Transfectants of polarized cells (Madin-Darby canine kidney and inner medullary collecting duct) using expression constructs encoding FcRn have been made, and as monolayers these cells are active in the transcytosis of IgG *(24,25)*. Using these transfectants, in addition to transfectants expressing a FcγRIIb-FcRn chimera *(27)*, dileucine and tryptophan based motifs in the cytosolic tail of FcRn have been shown to play a role in internalization and transcytosis *(27,28)*. In addition, the trophoblast cell line BeWo *(23)*, the adult intestinal epithelial cell line T84 *(29)*, and a placental endothelial cell line *(26)* have been shown to express endogenous FcRn and these cells are active in the transcytosis of IgG. These systems should be useful tools to further investigate FcRn trafficking.

Catabolism of IgG

Similar to any plasma protein present in a living organism, IgG can be considered to be located in three distinct compartments: In the vascular system, the extravascular tissue/fluids

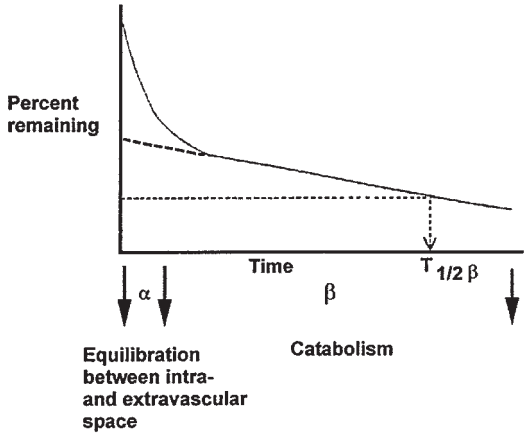

Fig. 2. Schematic curve showing the biphasic clearance of IgG from the circulation and the calculation of the β phase half-life.

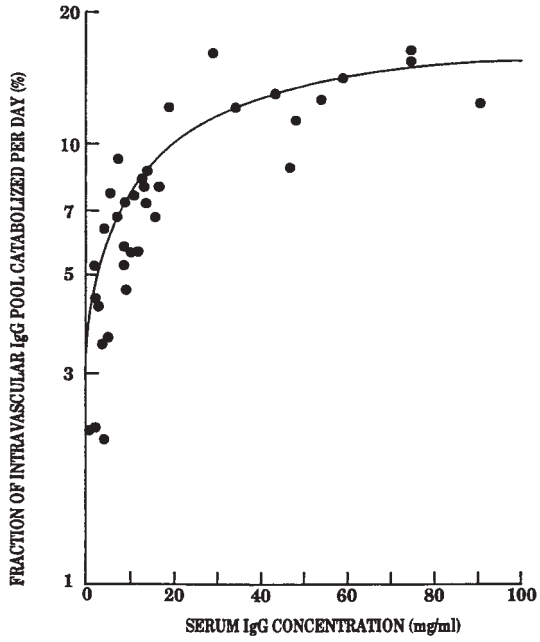

Fig. 3. The relationship between the fractional catabolic rate and the serum IgG concentration in patients. Data points represent experimentally determined values and the solid line indicates the values predicted from the $T_{1/2} = 0.69c/a(c-b)$ equation.

and the lymphatics. The proteins continuously circulate from the vascular system into the extravascular tissue/ fluids and from there through the lymphatic system and back into the vascular compartment. Following intravascular delivery of IgG, its disappearance from the circulation can be divided into a short period of distribution in the circulation (few minutes), a longer phase of equilibration into the extravascular compartment, followed by return to the circulation through the lymphatics (one day) and slow removal from the circulation. This slow removal is due to the metabolic/catabolic destruction of IgG and usually takes several days (Fig. 2). A clearance curve of elimination of an IgG from the circulation can therefore be divided into two phases: The alpha (fast) and beta (slow) phases for which the corresponding half lives can be calculated.

The beta-phase represents the loss of IgG from the circulation due to its catabolism and for this reason it is frequently called the biological half-life of IgG, $T_{1/2}$ *(9)*. In addition, other pharmacokinetic parameters such as fractional catabolic rate ($k = \ln 2/T_{1/2}$) can be determined, and this parameter inversely correlates with serum half-life.

The Concentration-Catabolism Effect

The serum level of IgG is determined by two opposing factors: The rates of synthesis and catabolism. If the synthetic rate increases, the serum IgG level rises and the catabolic process accelerates. The serum IgG concentration rises to a new level before the increased catabolic rate matches the synthetic rate. The fractional catabolic rate of IgG (between 3–17% per day for different species) is directly proportional to its serum concentration. Thus, IgG catabolism is accelerated in patients with increased IgG levels and conversely, is reduced in patients with low serum IgG concentration *(9)* (Fig. 3). This correlation, called the con-

centration-catabolism effect, also holds true in mice and rabbits but not in guinea pigs *(9)*. The concentration-catabolism effect is also observed for serum albumin but not for the other immunoglobulin classes *(30)*.

The Mechanism of IgG Catabolism

Originally, there were two distinct hypotheses put forward to explain the regulation of serum IgG homeostasis and other plasma proteins:

The selective hypotheses state that the catabolism of plasma proteins (including IgG) is a stepwise process, with the elimination mechanism selectively removing only molecules that have undergone structural or conformational changes. The appearance of the modified protein molecules in the blood can be time-dependent ("aged" proteins) or time independent (modified stochastically).

The random hypothesis which is now generally accepted states that the protein molecules perish as they were made i.e., in an unmodified form. The main experimental argument for the random character of breakdown is that the decrease of plasma radioactivity after intravenous injection of radiolabeled IgG follows first order kinetics.

The Selective Models

The selective hypotheses are based on the observation that the catabolic rate of a protein depends on the molecular integrity being influenced even by minor in vitro changes in conformation or composition. Thus, protein molecules having undergone modifications induced by low pH *(31,32)*, desialylation *(33)*, gain and loss of certain groups (e.g., by deamidation) *(34)* or in vivo aging *(35)* are catabolized faster than their normal counterparts. These observations suggested that a plasma protein (e.g., IgG) which becomes modified in a variety of possible ways in vivo is rapidly broken down. Thus, plasma IgG catabolism can be considered as a selective process in which only modified IgG molecules are prone to elimination. One selective hypothesis *(36,37)* suggests that the altered molecules are the result of a time-dependent process of repetitive alterations of the Fc region of IgG molecules during their transcapillary movement from the intravascular to interstitial compartment via endothelial cells. Since only a few percent of IgG are catabolized per day (e.g., 5% in humans) *(9)* the proportion of the modified IgG in the circulation should, in this model, approximate this percentage. It was therefore postulated that the so called "cytophilic" IgG, which binds to various cell types and is present in the circulation in a percentage similar to that of IgG catabolized per day, may represent the modified IgG. Cytophilic IgG isolated from the rabbit serum contained higher amounts of neutral hexoses and had a 10-fold higher affinity for FcR on macrophages than the initial IgG *(38)*. These altered IgG molecules were proposed to be recognized either by cytophilic FcR *(39,40)* or by preformed physiologic autoantibodies and eliminated as immune complexes via opsonic FcR *(41)* (Fig. 4). However, although the half-life of this cytophilic IgG was shorter (133 h) than that of initial IgG (153 h), the relatively small difference does not support the hypothesis that the cytophilic IgG is selectively catabolized *(38)*.

An alternative hypothesis proposed a unifying mechanism for the selective catabolism of all plasma glycoproteins that had been deglycosylated *(42)*. This hypothesis was proposed to apply to IgG (but not to albumin) and invoked limited desialylation of plasma glycoproteins as the mechanism for the generation of altered proteins. The altered glycoproteins were subsequently recognized by lectin-like receptors on the liver cells, endocytosed and destroyed *(42)* (Fig. 4). It was suggested that

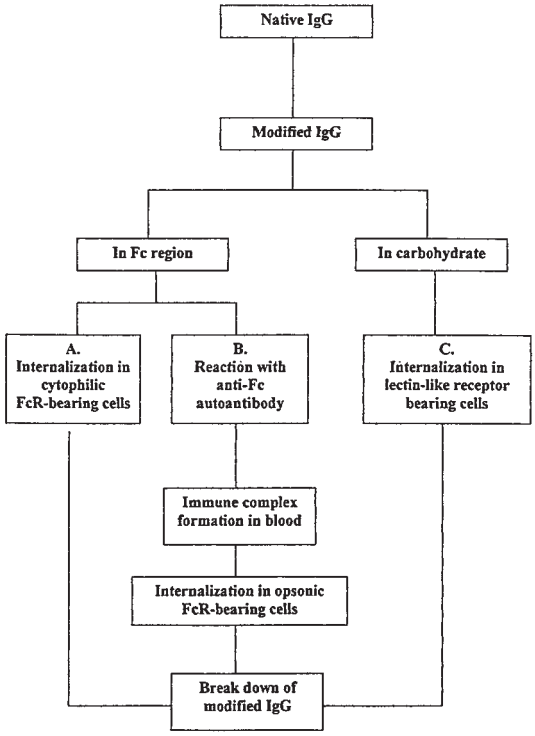

Fig. 4. The selective models of IgG catabolism: **A.** Elimination of modified IgG by cytophilic FcγRI-bearing catabolic cells. **B.** Elimination of modified IgG by autoantibody and opsonic FcγRII-bearing catabolic cells. **C.** Elimination of desialylated IgG by lectin-like receptors-bearing catabolic cells.

the appearance in the bloodstream of the protein molecules with altered carbohydrate, and consequently prone to catabolism, might be the result of some time-independent desialylation which occurred stochastically. For some glycoproteins, the in vitro removal of sialic acid groups yields desialylated forms which have faster catabolic rates than native ones *(33,42)*. However, not all the desialylated counterparts of glycoproteins have a faster breakdown (for example asialotransferin and asialoIgG) *(42,43)*, suggesting that the catabolism of IgG is not regulated by its carbohydrate moiety. The shorter half-life of some deglycosylated IgGs *(44)* may be the result of some modification in the conformation of the Fc region induced by the absence of the carbohydrate and not to the lack of carbohydrate *per se* (discussed further below).

As an alternative to deglycosylation, IgG exposed to a high concentration of glucose in vitro or obtained from sera of hyperglycemic diabetics *(40)* had altered cytophilic properties, most likely due to glucosylation. Vlassara and colleagues suggested that glucosylation of proteins could act as a specific signal for recognition and degradation *(45)*. Consistent with this, the rate of vascular clearance of the glucosylated IgG was found to be significantly higher than that of unmodified IgG *(46)*. A potential mechanism for the removal of glucosylated proteins (including albumin) involves their binding to specific high-affinity receptors present in kidneys and this may be responsible for the removal of senescent proteins from the circulation *(45)*. However, this is most likely a minor route of degradation.

The Random Model

The random hypothesis assumes that breakdown of a plasma protein is a random event, with (IgG) molecules having the same probability of destruction independent of their age. This currently accepted mechanism of regulation of serum IgG levels was essentially proposed by Brambell and colleagues *(47)* as a theoretical model. The model postulates that circulating IgG is taken up by the cells that are involved in IgG breakdown. Following uptake, the IgG may bind to "protective" receptors which salvage them from degradation. The Fc receptor bound molecules are returned intact into the circulation. Conversely, IgG molecules that do not bind to the protective receptors are destined for breakdown. Assum-

ing that the protective receptors are saturable, this model explains how constant serum IgG levels can be maintained: As the serum IgG levels rise, the protective receptors become saturated and more IgG is destined for degradation following uptake. In contrast, if the serum
IgG levels decrease then a greater proportion of the IgG is salvaged. This can be expressed quantitatively by the equation $T_{1/2} = 0.69c/a(c–b)$ where c is the concentration of IgG in serum, a is the proportion salvaged by the receptor and b is the minimum concentration of IgG that will saturate the receptors. By solving this equation the predicted half-life for each IgG concentration in mice and humans was compared with the experimentally determined half lives *(47)*. The theoretical curve is consistent with the observed data (Fig. 3), providing support for the concentration-catabolism effect and the Brambell hypothesis. Although the Brambell model predicts that IgG breakdown is a random process which is independent of the age of the IgG molecule, this model does not exclude the possibility that conformationally altered IgGs do not bind to the protective receptors and are broken down in addition to the stochastic destruction of normal IgG molecules *(48)*.

The random hypothesis of Brambell and colleagues *(47)* was put forward in the absence of any knowledge of the nature of the protective receptors that might be involved. In fact, only recently have the molecular details been unraveled. The updated model invokes the neonatal Fc receptor (FcRn) as the Fc receptor which binds and salvages IgG from degradation. This receptor binds IgG at slightly acidic pH (6.0–6.5) and releases it at pH 7.0–7.4 *(13,14,16,49)* (see Transcytosis of IgG). The IgG molecules are taken up by fluid phase pinocytosis and traffic into the acidic intracellular vesicles (endosomes) where they

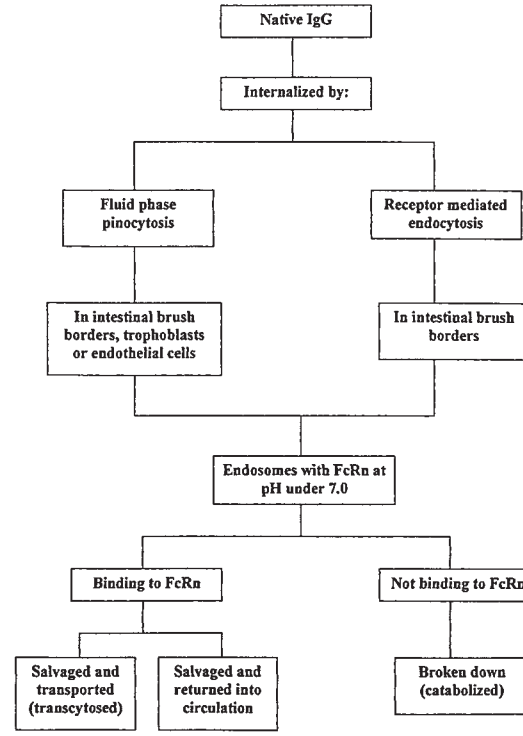

Fig. 5. The random model of IgG catabolism.

are bound by FcRn. IgG molecules in excess of FcRn (or unable to bind to these receptors due to some structural defect) are destined for lysosomal degradation. The IgG molecules which are bound to FcRn are recycled back to the membrane where they are released into the circulation due to the slightly basic pH of the blood (Fig. 5). This assumes that the cells involved in catabolism of IgG should be in close contact with the blood, suggesting that the endothelial lining of the microvasculature could be the catabolic compartment. Paradoxically, both salvage and destruction of IgG therefore occurs in the same cells *(50)*. In support of the involvement of endothelial cells, FcRn is expressed in both mouse *(51)* and human *(52)* endothelial cells. Furthermore, analysis of the distribution of radiolabeled IgG

in mice indicates that endothelial cells in skin, muscle, and liver microvasculature are the primary sites of IgG catabolism *(51)*. These observations confirm that IgG catabolism occurs at diffuse body sites *(50)*.

Interestingly, an analogy exists between the catabolism of IgG and serum albumin. Albumin is subject to the concentration-catabolism effect *(30)* and its catabolism occurs at diffuse sites in close relationship with the vasculature *(53)*. Albumin receptors are also expressed in endothelial cells *(54,55)*. In agreement with this observation, blockade of the reticuloendothelial system with carbon particles decreases the half-life of both rat serum albumin and IgG from 74 h to 54 h and from 130 h to 96 h respectively *(56)*. This behavior suggests that for both albumin and IgG, the primary site of catabolism is the endothelial cells.

Identifying the Fc Receptor that Regulates Serum IgG Levels in Mice

Brambell and colleagues *(47)* also postulated that although IgG transcytosis and catabolism occur in different cell types, both involve the same Fc receptor (*see* Transcytosis). This receptor is now known to be FcRn. The first experimental indication that this Fc receptor is involved in both transcytosis and the regulation of catabolism was obtained from the mapping of the site of IgG that regulates these two processes.

The delineation of the amino acid residues involved in regulating the catabolism and transcytosis of IgG was initiated by the observation that rabbit or mouse IgG complexed with Staphylococcal protein A (SpA) or its 7kDa fragment B is rapidly eliminated from the circulation *(57)*. Fragment B of SpA binds to the CH2-CH3 domain interface, and the interaction site encompasses three regions *(58)*. The amino acid residues in these regions are well conserved in the IgG molecule across many species *(59)*: 252–254 (Thr-Ile-Ser or Met-Ile-Ser), 308–312 (Val-Leu-His-Gln-Asp) and 433–436 (His-Asn-His-Tyr or His-Asn-His-His). To directly test the concept that the SpA-binding sites overlaps with the site(s) of IgG involved in catabolism and transcytosis, the amino acids that are both conserved in IgG sequences and involved in SpA binding were changed by in vitro mutagenesis *(60–63)*. These mutations were made on recombinant Fc fragments and their location is shown in Fig. 6. The resulting mutants were tested for transport through the neonatal intestine or yolk sac, serum half-life and affinity for binding to recombinant mouse FcRn. The results demonstrated that amino acid residues from both the CH2 (Ile253, His310) and CH3 (His435) domains are essential for both transcytosis and the regulation of catabolism of IgG (Table 1). The X-ray solution of Fc/FcRn cocrystals at 4.5–6 Å resolution is consistent with the results obtained by site directed mutagenesis, indicating that Ile253, His310, His435, and His436 are in proximity to the FcRn interaction site *(64)*. However, the resolution of this structure did not allow the precise atomic contacts at the interaction site to be defined. The presence of highly conserved histidine residues at positions 310 and 435 provides an explanation for the strict pH dependence (binding at pH 6.0–6.5 and release at pH 7.0–7.5) of the FcRn-IgG (or Fc) interaction that was observed in binding studies *(21,22,61)*.

In the studies using recombinant aglycosylated Fc fragments, no significant differences were found between the activity of the recombinant fragment and that of a Fc fragment obtained by papain digestion of a monoclonal IgG1 *(60,61)*. This indicates that the absence of carbohydrate on the recombinant Fc fragment does not influence its catabolism or transcytosis. However, expression and analysis of an aglycosylated human Fc fragment indicate that for full activity in FcRn-mediated

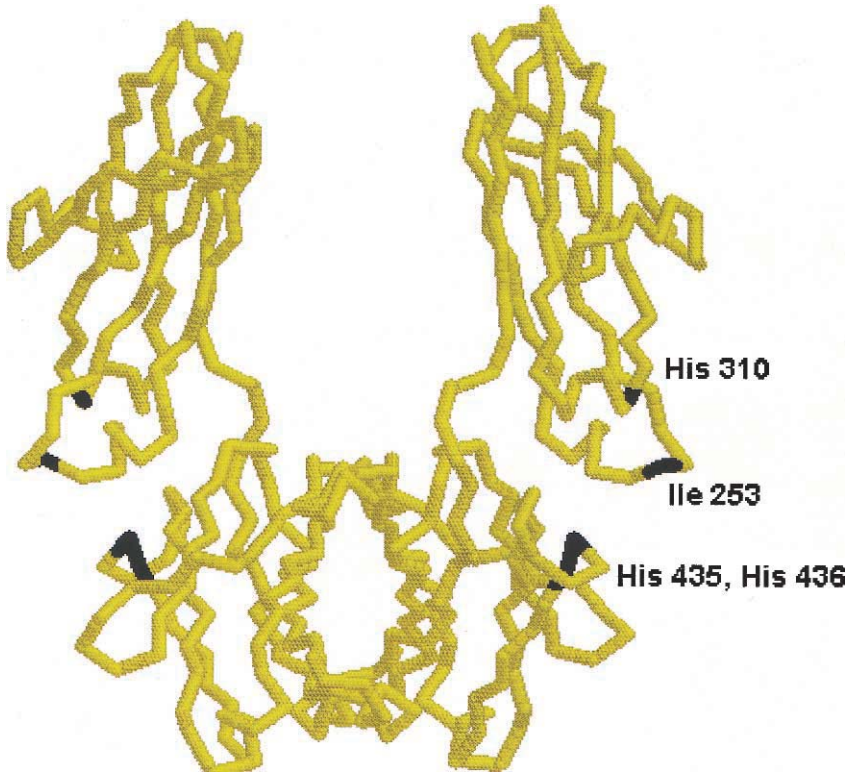

Fig. 6. Alpha carbon trace of the human Fc (IgG1) region. Residues shown to be involved in binding to FcRn are shown in black. The figure was drawn using RASMOL (Roger Sayle, Bioinformatics Research Institute, University of Edinburgh, UK).

Table 1. Activities of wild-type and mutated Fc fragments in catabolism, transcytosis, and binding to mouse FcRn *(63)*[*]

		Transcytosis		
Fc-derivative	Catabolism	Intestine	Yolk sac	Affinity for FcRn
Wild-Type	100	100	100	100
Ile253>Ala[**]	22.0	10.2	22.2	18.0
His285>Ala[#]	89.4	97.9	83.3	97.5
His310>Ala	14.1	12.2	16.7	12.5
His433>Ala	96.5	71.4	77.8	75.0
Asn434>Ala	92.4	83.7	77.8	84.5
His435>Ala	14.6	24.5	11.1	5.0
His436>Ala	40.9	51.0	83.3	20.2

[*]The activities are expressed as a percentage of the activity of the wild-type.

[**]Isoleucine 253 mutated to alanine.

[#]The histidine is located in a loop on the external surface of the CH2 domain which is distal to the CH2-CH3 domain interface *(58)*.

Table 2. Pearson correlation coefficient between catabolism, transcytosis, and affinity for FcRn *(63)*

Activities	Correlation coefficient
Catabolism vs Intestinal transmission	0.945
Catabolism vs Materno-fetal transmission	0.870
Catabolism vs Affinity for FcRn	0.780
Intestinal transmission vs Materno-fetal transmission	0.893
Intestinal transmission vs Affinity for FcRn	0.953
Maternofetal transmission vs Affinity for FcRn	0.836

functions, the human fragment needs to be glycosylated *(65)*. Thus, there appears to be differences in the requirements for glycosylation across species (discussed further below).

The close correlation between the effect of a given mutation of the Fc fragment on catabolism, transcytosis across the neonatal intestine/yolk sac and affinity for FcRn supports the concept that FcRn is involved in all these processes (Table 2). The role of FcRn in the regulation of IgG catabolism was clearly demonstrated by measuring the half-life of mouse IgG1 and IgG1-derived recombinant Fc fragments in mice that do not express functional FcRn due to homozygous deletion of the gene encoding β2-microglobulin (β2m; *[10]*). The half lives of IgG1 and the wild-type Fc fragment were considerably decreased in β2m knockout mice relative to wild-type mice. In contrast, IgA and a mutated Fc fragment which are both unable to bind to FcRn have the same half lives in both normal and β2m deficient mice (Table 3). These observations were independently confirmed by several other groups *(11,12,66)*. In addition, the serum IgG concentration in β2m deficient animals is 30 times lower than in normal mice. This decreased concentration is the consequence of the high rate of IgG catabolism in β2m deficient mice rather than a lower IgG synthetic rate (Table 3).

The IgG molecule has two possible interaction sites for FcRn per Fc region. This raises the question as to whether both sites on an IgG molecule are necessary for activity in FcRn-mediated functions. A heterodimer of an Fc fragment comprising one wild-type polypeptide and a mutated polypeptide with a defective FcRn binding site was cleared from the circulation with a half-life which is more than twofold less than that of the wild-type Fc homodimer *(67)*. This heterodimer was also transferred across the neonatal intestine with significantly reduced efficiency *(60)*. Despite the need for two functional FcRn interaction sites per Fc or IgG molecule, recent data indicate that these sites are not equivalent *(68–70)*. Thus, there appears to be asymmetry in the Fc region with respect to FcRn binding, with one low affinity site and one high affinity site. The degree of asymmetry may vary across species, and could be brought about by two possible mechanisms: First, binding of FcRn to one "side" of the Fc may induce a conformational rearrangement which alters the affinity of the second site. Second, FcRn binding to one site of the Fc molecule may sterically hinder binding to the second site.

The excellent correlation between serum persistence and affinity for FcRn of Fc fragments (Table 2) suggests that an increase in affinity of an Fc fragment or IgG would result in a longer serum persistence and increased transcytosis. Using phage display technology,

Table 3. Half lives of IgG1, IgA, and Fc fragments in wild-type and β2-microglobulin knockout (β2m–/–) mice *(10)*

Mice	Half-life (h) of: IgG1	IgA	Wild-type Fc	Mutant Fc*	IgG1 conc. (mg/mL)	IgG1 synthesis (mg/day/mouse)
β2m+/+	97.7	25.0	76.9	13.7	1.39	0.308
β2m–/–	17.6	24.0	12.6	14.8	0.05	0.313

*mutated Fc fragment that does not bind to FcRn

a mutated Fc fragment with a 3.5-fold higher affinity for FcRn than the parent wild-type Fc was isolated. This fragment has Thr252, Thr254, and Thr256 mutated to Leu, Ser, and Phe, respectively and the mutations result in an increase in serum half-life from 93 to 153 h *(71)*. These studies indicate that it should be possible to engineer antibodies with the desired serum persistence e.g., short for radioimaging or long for therapeutic purposes.

The Role of Spatial Conformation

The IgG residues that are critical for FcRn binding are located on three spatially close loops that are distal in amino acid sequence (Fig. 6). This suggests that the relative position of the residues might be dependent on the conformation of the beta-strands that support them and also on the disposition of the CH2 and CH3 domains relative to each other. Experimental evidence indicates that there is considerable flexibility in this region of the IgG molecule *(72,73)*. Site directed mutagenesis studies support the concept that the FcRn interaction site is sensitive to conformational alterations which affect binding to this Fc receptor: First, the detrimental effect of mutation of proline to alanine at position 257 on the serum half-life of a recombinant Fc fragment is most likely due to perturbation of the conformation of the loop encompassing Ile253 *(74)*. This same sequence difference is probably responsible for the shorter serum half-life of rat IgG2b (57 h) relative to rat IgG1 (223 h) *(74)*. Second, mutation of Glu333 to alanine in a recombinant Fc fragment results in a significant decrease in serum persistence of the mutant (43 h) relative to the wild-type Fc fragment (89 h) (unpublished data). This amino acid is located in the beta-strand of the CH2 domain that is in the "interior" of this domain, and consequently inaccesible for direct interaction with FcRn *(58)*. However, this strand links the CH2 to CH3 domain, suggesting that the mutation might affect the relative disposition of the two Fc domains. Third, removal of the hinge region from the Fc fragment results in a shorter serum half-life (48.7 vs 79.8 h) *(75)*. The role of the hinge region appears to be to constrain the CH2 domains such that the configuration of FcRn interaction site at the CH2-CH3 interface is optimally conformed, rather than through a direct FcRn-hinge interaction. In fact, replacement of the hinge sequence with a "synthetic" hinge containing a distinct sequence generates an Fc fragment that has a half-life similar to that of the wild-type fragment *(75)*. Fourth, analyses of recombinant chimeric IgGs in which the CH2 and CH3 domains of human IgG2 and IgG3 were shuffled with the corresponding domains of IgG3 and IgG4 indicate that the FcRn-IgG interaction can be modulated by conformational

Table 4. Pharmacokinetics and relative affinity for FcRn of wild type and mutated human Fc fragments *(65)*

	Fragment Half-life (h)	Relative affinity (%)*
Wild-type	62.2	100
Ile253>Ala**	25.3	21.6
His310>Ala	19.2	7.2
His433>Ala	62.8	110.2
His435>Ala	21.7	7.5
His435>Arg	43.1	78.9

*Expressed as a percentage of the affinity of wild-type Fc
**Isoleucine 253 mutated to alanine

effects of amino acids distal to the CH2-CH3 domain interface *(76)*.

The Amino Acid Residues of Human IgG that are Critical for FcRn Binding

For the successful application of therapeutic (IgG) antibodies in humans it is important to know if the crucial amino acid residues Ile253, His310, and His435, which are conserved in human IgG, play the same role in catabolism and transcytosis of IgG in both mouse and man. In this context, human IgG3 with arginine instead of histidine at position 435 has a shorter half-life (7 d) than the other isotypes (21 d) *(77)*, indicating that position 435 is involved in FcRn-mediated functions in man. More recent studies have involved the analysis of recombinant human Fc fragments with mutations at positions 253, 310, and 435 in pharmacokinetic and FcRn interaction studies. Ethical reasons preclude the use of humans for pharmacokinetic analyses, but mice appear to be a suitable model for the study of human IgGs *(78,79)*. The results shown in Table 4 demonstrate the essential role of Ile253, His310, and His435. In addition, replacement of His435 by arginine in a recombinant Fc fragment (IgG1 derived) results in a reduced serum half-life in mice, consistent with the shorter half-life of IgG3 than IgG1 in both humans *(77)* and mice *(78,79)*. His433, in contrast to His435 and His310, does not appear to be involved in binding to FcRn. Thus, taken together the data indicate that the interaction sites for FcRn on human and mouse IgG1 closely overlap.

Although the absence of carbohydrate has no impact on the catabolism and transcytosis of mouse Fc, aglycosylated human Fc has a significantly shorter half-life (62.2 h) than that of the papain fragment obtained from a myeloma IgG1 (153.4 h) *(65)*. Thus, there appears to be differences in the requirements for glycosylation across species. Comparison of the X-ray structures of human and mouse IgG Fc regions *(58,80)* have shown that the CH2 attached carbohydrate can form contacts i.e. a "bridge" between the two CH2 domains. However, although this is observed in human IgG1 *(58)*, oligosaccharides on the CH2 domains of mouse IgG2a do not contact each other *(80)*. Thus, the contribution of CH2 attached oligosaccharides to interdomain contacts appears to depend on the species.

Due to the effects of aglycosylation, fully glycosylated recombinant human IgGs (wild-type and a mutated IgG containing His435 mutated to alanine; H435A) were also expressed in glycosylated form in mammalian cells *(81)*. The H435A mutant has a very short half-life in mice (25.8 h vs 217.9 h for WT),

does not bind detectably to mouse nor human FcRn and is transported at background levels across the ex vivo placenta (11% of the wild-type transfer at 3 h). The Fc region is involved not only in binding to FcRn but also to FcγRIII, a receptor that has been suggested to be involved in the placental transfer of human IgGs *(82)*. Although the amino acid residues that are of importance for FcγRIII binding are distal to the FcRn interaction site *(83)* it was essential to exclude the possibility that the H435A mutation reduced the transplacental transfer of IgG by affecting its binding to FcγRIII. Analyses of the binding of WT and H435A antibodies to FcγRIII on human natural killer (NK) cells indicated no effect of the mutation on the interaction of an IgG with this Fc receptor *(81)*. Thus, binding of an IgG to FcRn is a prerequisite for transport across the placenta, and this has implications for the engineering of antibodies for therapy. Furthermore, the similar effects of mutation of His435 to alanine for both mouse and human IgG1 indicate that the FcRn interaction site overlaps.

Transcytosis vs Catabolism

Despite the involvement of a common receptor, transcytosis and the regulation of serum IgG levels may have specific differences which in turn could be due to variations in the types of cells involved in the processes. For example, the cells involved in FcRn-mediated processes may have differences in the rates of fluid phase pinocytosis, expression levels of the receptor and/or ratios of salvaged : destroyed IgG, as suggested by Brambell *(7)*. Furthermore, the extent of recycling vs transcytosis may vary depending on the cell type and the availability of adaptor proteins and so on. For example, in the lactating mammary gland both recycling and transcytosis of IgG appears to occur *(6)*. Paradoxically this results in IgG molecules with high affinity for FcRn being delivered less efficiently into the milk than those IgG molecules of lower affinity. Thus, in this case there is an inverse correlation between IgG transcytosis and affinity for FcRn. The extent of recycling vs transcytosis in the regulation of serum IgG levels and other FcRn-mediated processes is as yet unknown. However, if transcytosis dominates in maintaining constant serum IgG levels, then this must occur bidirectionally in the endothelial cells that are believed to be the site of regulation *(51,52)*.

Following uptake into cells, the ratio of salvaged : destroyed IgG depends not only on the expression level/affinity of FcRn but also on the competition between the binding of IgG to FcRn and its intracellular degradation *prior to salvage by interaction with FcRn*. The pathways of intracellular degradation of internalized proteins depend on the specific cell types (e.g., endothelial or epithelial) and their physiological state. Lysosomal breakdown of ingested proteins appears to be slow and non-selective *(84)*. In contrast, ingested proteins can also exit the endosomes and enter the cytosol where they subsequently undergo rapid digestion by the ATP-dependent ubiquitin/proteasome system *(85)*. The proteasomal pathway of proteolysis has been identified in a wide variety of cell types, consistent with the widespread distribution of ubiquitin *(86)*. For ubiquitination, the N-terminus of the ingested protein is recognized by an N-end reading enzyme that facilitates the ligation of the protein to ubiquitin followed by its degradation *(87)*. Intracellular proteins with a long half-life have N-terminus stabilizing amino acids (e.g., Ser,Ala,Gly) whereas those with a short half-life have destabilizing residues (e.g., Leu,Phe,Tyr) *(87)*. N-terminal recognition followed by ubiquitination can result in removal of the "invading" foreign plasma proteins *(88)* if they avoid lysosomal degradation by entering the cytosol. It is interesting to observe that

from 37 light and heavy immunoglobulin chains the majority (over 90%) have destabilizing amino acids at their N-termini, indicating that if delivered into the cytosol IgG molecules are good candidates for selective and rapid digestion through the ubiquitin/proteasome system. Intracellular degradation of IgG was indeed observed during its transport across the human placenta *(81,89)*, rodent yolk sac *(90)* and neonatal intestine *(91)*. This indicates that the transport of IgG across various cellular barriers is not a very efficient process, with greater than 50% of the internalized IgG being broken down. However, whether this breakdown involves proteasomal activity in addition to lysosomal breakdown is as yet unknown.

It is well-known that IgG isotypes of various species have different susceptibilities to the action of proteolytic enzymes. Thus, mouse IgG2b is more sensitive to proteases than IgG2a *(92)* and human IgG3 is more readily digested than the other three isotypes *(93)*. Although the half-life of mouse IgG2b is considerably shorter than that of IgG2a (46.5 vs 178 h), the maternofetal transmission of these two isotypes are almost identical (1.6 vs 1.7 % transmission per gram of fetuses) (C. Medesan, personal communication). This difference in behavior in two processes that both involve FcRn may be due to their different susceptibilities to the lysosomal and, possibly, nonlysosomal proteolytic systems of placenta/yolk sac and endothelial cells. In addition, there may be differences between the proteolytic apparatus of these various types of cells.

Similarly, human IgG3 has an affinity for human FcRn similar to that of IgG1 *(94)* and our unpublished data) but its half-life is three times shorter than that of IgG1 *(77)* and the transmission through the placenta is only slightly decreased (65% of the IgG1) *(95)*. Moreover, during IgG transport from mother to infant the IgG molecules have to traverse two cellular barriers, namely the trophoblast/yolk sac/intestinal cells and the endothelial cells of the microvasculature of the fetus(es) or neonatal intestine. The expression of FcRn in the endothelial cells isolated from the vasculature of human placenta *(26,96)* suggests that during transplacental transport the IgG molecules are exposed to two types of FcRn expressing cells which may have differences in FcRn expression levels *(10)* and proteolytic efficiencies. Thus, although the correlation between transcytosis and the regulation of

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serum half-life appears to generally hold, there are apparently exceptions that may warrant

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