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H435-CONTAINING IgG3 ALLOTYPES ARE TRANSPORTED EFFICIENTLY ACROSS THE HUMAN PLACENTA: IMPLICATIONS FOR ALLOANTIBODY-MEDIATED DISEASES OF THE NEWBORN

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SUMMARY

Background
The neonatal receptor (FcRn) extends the half-life of human immunoglobulin G (IgG) and transports it across the placenta, providing the newborn with humoral immunity. Of the four subclasses, IgG3 stands out with strong effector functions, short half-life (7 vs 21 days for other subclasses) and poor placental transport. We recently described how a single amino acid polymorphism at position 435 in IgG3, is sufficient to explain the short half-life of R435-containing IgG3, and demonstrated that H435-IgG3 has a normal half-life of 21 days. Here, we investigated whether the R435 also explains the relatively poor placental transport of IgG3.

Study design and methods
Sera were collected from paired mothers and newborns at birth. Study included six mothers expressing R435-IgG diagnosed with FNAIT and treated with IVIg (containing H435-IgG3, also known as G3m16 or G3m(s,t) allotype), as well as 33 paired samples of both G3m16− and G3m16+ mothers. Placental IgG transport was estimated by comparing cord and maternal concentrations of IgG-subclass and G3m16 allotype.

Results
The placental transport of naturally occurring H435-IgG3 allotypes was significantly more efficient than that of other R435-IgG3 allotypes, and was comparable to IgG1 transport.

Conclusion
We demonstrate that the poor maternal-fetal transport of IgG3 is only true for most individuals of western populations where the G3m16 is not common. In G3m16+ individuals, expressing H435-containing IgG3, IgG3 transport is similar to IgG1, which may give rise to enhanced complications in pregnancy-associated allo-immune disease in ethnic communities where this naturally occurring H435 containing IgG3 allotype is more frequent.
INTRODUCTION

Immunoglobulins are the hallmark of the adaptive humoral immunity, of which IgG is the most abundant. IgG comprises of four subclasses, IgG1-4, named after their decreasing abundance in serum. These subclasses have highly different affinities to myeloid IgG-Fcγ receptors (FcγR) and C1q. The general affinity and capacity to activate both effector function types follow the general rule that IgG3>IgG1>>IgG2>IgG4.1-3 While immune responses against protein antigens are characterized by both IgG1 and IgG3 responses, IgG1 is often considered to be more clinically relevant in autoimmune and alloimmune-mediated diseases due to its higher serum levels, although an important pathological role has been reported for IgG3 antibodies as well.4,5 The low serum concentration of IgG3 can only partially be explained by less class switching to IgG3, the main reason being the short half-life of 7 days, compared to 21 days for the other subclasses.

The long half-life of IgG1, IgG2, and IgG4, is caused by their recycling by the neonatal IgG-Fc Receptor (FcRn), an MHC-class I like glycoprotein. FcRn is ubiquitously expressed, but is most abundant in cells such as the vascular endothelium, epithelium, syncytiotrophoblasts of the human placenta and myeloid cells. FcRn resides mostly intracellularly, and binds IgG only after fusion with pinocytotic vesicles, and only in an acidic environment of pH<6.5, when solvent-exposed histidine residues can become protonated. It then transports its cargo back to the cell surface, releasing its cargo at neutral pH. In case of polarized cells, this can be either the same or the opposite surface, the net result being either recycling (endothelial and immune cells in blood) or transcytosis (placenta). We recently described how the short half-life of IgG3 is caused by its decreased pH-dependent binding to FcRn, as one of the key histidines for FcRn-mediated binding, the H435 in the CH3 of the IgG-Fc tail, has been replaced by an arginine in IgG3 that is constitutively positively charged within cells, even at neutral pH. However, numerous allotypes of IgG3 exists, including G3m(s,t) (WHO nomenclature G3m16*) which has a histidine at position 435 and consequently has a long half-life in humans similar to the other IgG subclasses.6 The G3m16 allotype is rare in Europe (e.g. 0.9% in Dutch, 0.4% in Finns, but more prevalent in Asian ethnic groups (e.g. 28% in Japan).7-10

In alloimmune mediated diseases, e.g. in Haemolytic Diseases of the Foetus and the New-born (HDFN) and Foetal/Neonatal Alloimmune Thrombocytopenia (FNAIT), pathogenic maternal IgG are formed against antigens expressed on foetal red blood cells or platelets, causing anaemia or thrombocytopenia, respectively, in the foetus during pregnancy and in the neonate after birth. For this to occur, the pathogenic antibodies, derived from the immunized mother, cross the placenta. The only clinically relevant antibodies in this setting are IgG antibodies, as other isotypes are only transported in trace amounts.11 Despite its low serum concentration, IgG3 is considered clinically relevant, in particular in HDFN when anti-D responses are
found. However, the relevance of the IgG3 response is controversial because IgG1 is more frequently the associated antibody found in FNAIT and HDFN (except AB0, where IgG2 responses predominate), but also because it is generally accepted that IgG3 is not efficiently transported across the placenta.13-15 Recently, we found H435-containing IgG3 has a half-life comparable to that of IgG1, suggesting that individuals expressing these IgG3 allotypes (G3m15 and G3m16 allotypes, previously known as G3m(s) and G3m(s,t), respectively) may be more prone to stronger IgG3 mediated auto- and alloimmune reactions.4,6 We therefore tested the hypothesis that H435-containing IgG3 antibodies are as efficiently transported across the placenta as IgG1.

MATERIALS AND METHODS

Serum samples
Matched serum samples from mothers and cord or peripheral blood of the newborn were collected immediately after delivery. Six mothers diagnosed with FNAIT had been treated with IVIg transfusions during pregnancy, the rest of the pregnancies were healthy. A total of 33 Dutch mothers (including IVIg-treated mothers 6 with FNAIT) and four healthy G3m(s,t) positive Chinese mothers were included in the study. Informed consent was obtained from the mothers, and the collection was approved by the Ethics Committee of the Leiden University Medical Centre and Guangzhou Blood Centre.

Monoclonal antibodies
The monoclonal 1.5A10 was produced by cloning the variable regions of the heavy and light chains (VL, VH) from the original hybridoma into expression plasmids (pFUSE, Invivogen, San Diego CA) containing the mouse antibody heavy or light constant regions, respectively. RNA was isolated with the RNeasy Mini Kit, (Qiagen, CA), and VH and VL genes were amplified by using the SMART Race cDNA amplification kit and Advantage 2 Polymerase Mix according to manufacturer’s instructions (Clontech, Inc.), with CH and CL specific primers (mIgG1R ATCTACAAACCAGCTGAACTGGACCT, mKappaR GTGAGTGGCCTCACAGGTATAGCTTGT). The product was then ligated into a PCR21-Topo cloning vector (Invitrogen, Carlsbad, California, U.S.) and sequenced by ABI 373 Stretch automated sequencing (Applied Biosystems, Foster City, CA). Restriction sites on flanking sides of each variable gene were introduced by PCR and the product was inserted into PCR2.1-T/A TOPO (Invitrogen). VH-gene fragment obtained by digestion with Eco47III, and EcoRI were then ligated into pFUSE-CHl-g-mG1 (Invivogen, San Diego, California). The VL fragment was ligated into pFUSE-CL-Kl-g-mG1 (Invivogen) using Agel/Xhol. The final constructs were verified by sequencing, and co-produced in the FreeStyle 293 expression system (Invitrogen) according to the manufacturer’s instructions. Antibodies were purified
on a protein G HiTrap HP column using ActaPrime Plus (GE Life Sciences) and dialyzed against PBS overnight.

1.5A10 was conjugated to HRP by using the HRP LYNX rapid conjugation kit (Abdserotec, Kidlington, UK) according to manufacturer’s protocol.

Three H435-containing IgG3 variants found in different ethnic groups (accession numbers AJ390272, AJ390276, AJ390279, for variants 1-3, respectively) were produced by ordering a codon optimized heavy constant regions chain from Mr. Gene (Geneart, LifeTechnologies), and cloning into pcDNA3.1. They were then produced in the FreeStyle expression system along a corresponding light chain (GDOB1 kappa, plasmid as was described above).6,16 IVIg was acquired from Sanquin (Amsterdam, the Netherlands, containing 56.6 mg/ml IgG, with 34,20,1.3,1.3 of IgG1-4, respectively).

**Serum IgG quantification**

IgG subclass quantification was performed by nephelometry (Siemens Dimension Vista Nephelometer). IgG3 G3m16 allotype quantification was determined in a sandwich ELISA on Nunc Immunu-maxorp plates using the allotype specific recombinant mouse antibody 1.5A10 (0.625ug/ml in PBS, overnight at 4°C) for coating and 1.5A10-HRP (0.156 µg/ml in PBS 0.05% Tween, one hour room temperature) for detection, and a recombinant IgG3 G3m16 variant 2 as a standard. Samples were diluted in PBS 0.05% Tween and incubated for one hour at room temperature. Wells were washed five times between steps with PBS 0.05% Tween.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5.01 for Windows (GraphPad, Inc., La Jolla, CA). Significance was set at P<0.05. Level of significance is indicated on figures as *: P<0.05, **: P<0.01 and ***=P<0.001.

**RESULTS**

Mouse 1.5A10 can be used to detect and quantify G3m16 in serum and IVIg. The recombinant mouse monoclonal 1.5A10 was found to be highly specific for G3m16 positive sera (typed and designated by the previous as G3m(s,t) nomenclature) (Fig. 1A). To further investigate the specificity of this monoclonal antibody, the three known naturally occurring H435 containing IgG3 allotypes (G3m15, G3m16 variants) (Fig. 1B) were produced as recombinant monoclonal antibodies for quantification of the H435-containing allotypes in human sera. The only difference found in the Fc portion between these variants, was the variation at position 292 (CH2 domain), while the other differences were found in the CH1-domain. The differences at position 192 and 193 in the CH1-domain stem from an isoallotypic variation in variant 1, as the 192N and 193F are a known hallmark of IgG2.
Figure 1. Characterization of 1.5A10, an anti-IgG3 G3m(t)/G3m16 monoclonal. A) 1.5A10 recognized sera typed as G3m(s,t) specifically, but not G3m(s,t)-negative sera (G3m(b) or G3m(g), the full classical Gm allotyping is indicated also for the G1m (z,a,f) and G2m allotypes (n)). B) The sequence difference of three naturally occurring IgG3 allotypes that contain histidine at position 435. Top, the position (EU numbering). From left, the nomenclature used here (variants 1-3), the simplified nomenclature, amino acid sequence, rightmost the accession number of these variants. C) 1.5A10 recognizes G3m16 in IVIg, or G3m16-negative sera spiked with 1.5 µg/ml recombinant G3m16-V2 IgG3. D) 1.5A10 recognizes H435-containing IgG3 variant 2 and 3 equally, but not V1 nor other non-H435-containing IgG3 allotypes (G3m(g)=G3m21 and G3m(b)=G3m5, previous designation versus WHO nomenclature, respectively. Based on the IgG3 allotype specificity, and the non-reactivity with V1 and other isotypes in panel A), we conclude that 1.5A10 recognizes W292, as the alloisotypic variation 192N193F in V1, a hallmark of IgG2, is not recognized by 1.5A10. Therefore 1.5A10 recognizes not G3m15, but G3m16, also known as G3m(t).
1.5A10 recognized G3m16 (variant 2, Fig. 1B) in G3m16-negative sera spiked with recombinant G3m16, and also recognized G3m16 in IVIg at low but easily detectable concentration, with the detection limit in the ng/ml range (Fig 1C). Using the purified recombinant G3m16 variant, we calculated the G3m16 level in IVIg to amount to 1.5 µg/ml, or 0.11% of total IgG3 in the IVIg. 1.5A10 also detected variant 3 to similar degree as variant 2, but did not recognize variant 1 (Fig. 1D). As 1.5A10 did not recognize IgG2 or other isotypes (Fig. 1A-B), we conclude that the epitope recognized is encoded by W292, also known as G3m(t) epitope or G3m16 (WHO nomenclature).10

G3m16 transport in IVIg treated mothers

To estimate the relative placental transport of G3m16 compared to other IgG3, we made use serum samples of women undergoing IVIg (containing G3m16, Fig. 1B) treatment due to the presence of anti-platelet antibodies).17 This method was chosen because the frequency of G3m16 was previously estimated to be 0.9% in the Dutch population.7 We first analyzed what effect the IVIg treatment had on the overall IgG transport across the placenta. We found that the amount of IgG transported over the placenta was directly dependent on maternal levels of IgG (p<0.0001), but in IVIg treated patients, the transport pathway was apparently saturated at approximately 15 mg/ml IgG (Fig. 2A). This is in agreement with the saturating nature of FcRn, first proposed by Brambell.18,19 For the relative IgG transport, we noticed a clear and significant influence of the maternal IgG concentration on the relative transport also under non-saturating conditions; when the IgG levels were higher in the mother, relatively less IgG was transported (p= 0.0034 for non-IVIg treated, p=0.0072 for IVIg treated) (shown for IgG1 in Fig. 2B). With IVIg, these differences became more pronounced (difference between slopes were significantly different, p<0.0001), suggesting this to be due to saturation of both the FcRn-dependent recycling and placental transport.19 As expected, IgG3 transport was significantly lower than IgG1 transport for IVIg treated mothers (Fig. 2C). Transport of G3m16 tended to be higher than for IgG3 total and equal to that of IgG1; however, this tendency was not statistically significant from IgG3 total (Fig. 2D). We detected a high variation in the concentrations of all IgG subclasses measured in the six IVIg-treated pairs, with total IgG values ranging from normal to three times higher than normal, in agreement with a high dose IVIg treatment (Fig. 2C). This was presumably because of difference between the last IVIg treatment and delivery, since IVIg is cleared over time, particularly in the first days when IgG concentration is high enough (~15 mg/ml) to saturate the FcRn-recycling pathway.19

The IgG3 G3m16 transport advantage was most noticeable in mothers with highest total IgG, i.e. before significant catabolism of this non-endogenously expressed allotype occurred. Non-G3m16 IgG3 levels dropped less fast from the circulation (Fig. 2C, slopes between IgG3 and G3m16+ IgG3 were significantly different, p=0.032), in agreement with its endogenous production.
Figure 2. Transport of IgG across human placenta in IVIg treated mothers: G3m16 versus other IgG3 allotypes. Transport of H435-containing IgG3 across the placenta was estimated in pregnant G3m16-negative women undergoing high-dose IVIg therapy. Normal pregnancies in G3m16-negative women served as controls. A) The total amount found in cord blood was dependent on maternal IgG levels in normal pregnancies. At approximately 15-20 mg/ml, placental transport was saturated in IVIg-treated mothers, presumably because of saturation the FcRn-transport pathway. B-C) The affect maternal IgG concentration on relative IgG1 (B) and IgG3 (C) transport with or without IVIg. Note that paired samples can be identified for IgG3 total and G3m16-IgG3 in (C) as they have the same y-axis value. D) Comparison of the transport of IgG1 versus IgG3 total and G3m16 only in IVIg treated mothers.
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Figure 3. Transplacental transport of IgG subclasses and G3m16 in G3m16- and G3m16+ individuals in pregnancy. Transport of IgG subclasses and G3m16 across the placenta expressed as a % of IgG1 transport within pairs. Both G3m16- and G3m16+ individuals were included for analysis of IgG1, IgG2 and IgG4 as both groups showed similar transport rates for these subclasses (n=31), but only G3m16+ were used for analysis for transport of G3m16. Only G3m16- pairs were used for transport analysis of non-G3m16 IgG3 (IgG3).

G3m16 is transported across the placenta equally well as IgG1

To quantify G3m16 transport directly in normal pregnancies, paired mother and fetal samples were screened for G3m16 positivity in the area of Guangzhou, China, where we found the frequency of the G3m16 allotype to be 7.6% in normal donors (10 out of 132). IgG subclass and G3m16 allotype concentrations were measured in 6 G3m16 positive and 27 G3m16-negative healthy mother/cord blood pairs. As expected, IgG1 transport was significantly higher than that of IgG2 and IgG3 in G3m16- mothers, while IgG4 transport was similar to IgG1 (Fig. 3A). Transport of G3m16 was however significantly increased compared to IgG3 transport (P<0.0001, Fig. 3A) and was similar to that of IgG1 (NS).

DISCUSSION

Of the human immunoglobulin isotypes, only IgG has a prolonged half-life, and is actively transported across the placenta, providing the fetus with humoral immunity before birth. However, in cases when the mothers generate immune response against cellular material from the fetus (foreign paternal antigens), IgG immune responses can have debilitating or even fatal consequences if not treated. The most recognized cases are immune responses against the red blood cell or platelet antigens, e.g. RhD, Kell or human Platelet Antigen 1a.4,5

It is known that IgG3 has as short half-life and limited placental transport. We have previously shown that IgG3 has a normal half-life in G3m16 positive individuals.
and can mediate stronger myeloid- and complement-mediated responses than IgG1. This suggests that fetuses and newborns of G3m16 positive women are more at risk of developing serious clinical symptoms in IgG3 prevalent responses. In the present study we now prove that the G3m16 allotype is more efficiently transported across the placenta.

To answer this question, an ELISA was set up to quantify H435-containing IgG3, using the monoclonal 1.5A10. To characterize the exact epitope recognized by 1.5A10, the three H435-containing IgG3 allotypes G3m15 (V1), and two G3m16 allotypes (V2-3) were expressed as recombinant IgG3. As only V2 and V3 were recognized we concluded that 1.5A10 is G3m16 specific, recognizing the g3m(t) epitope encoded by W292, missing from the variant 1. This may preclude the usage of this monoclonal to determine the frequencies of H435-containing IgG in populations. However, although the (s) allotype (encoding for the H435-containing IgG3) has been found to occur without (t) (our variant 1, Fig. 1B), this has only been found in the Khoisan populations of African (G3m15), where the G3m16 allotype is also more frequent. Importantly, the G3m(t) epitope occurs exclusively together with the G3m(s) epitope. Thus, the 1.5A10 monoclonal recognizes only H435-containing IgG3, and can be used to quantify and type H435-containing IgG3, except in the G3m15 (G3m(t)-) individuals in the Khoisan population of Afrika, and should therefore not compromise the validity of the study.

The frequency of G3m16 allotype in Europe is extremely low. However, we took advantage of the fact that pregnant women suffering from FNAIT are routinely transfused with IVIg, which is a pooled IgG product from over 10,000 donors, and therefore containing all minor IgG allotype, including G3m16. Given its frequency of 0.9% in the Netherlands, one might expect similar, or even higher, levels of G3m16 to reside in IVIg due to its prolonged half-life. However, Hammarström and colleagues have shown that variants of the germ-line γ3 promotor can affect switching in some IgG3 allotypes, perhaps explaining the relative low serum levels of IgG3 in G3m16+ individuals. We did find that G3m16+ mothers receiving IVIg have detectable amounts of G3m16 in their sera, as well as in the cord blood. However the concentration of the G3m16 allotype decreased faster in time than total IgG1 and IgG3 after infusion. This was because that while IgG1 and total IgG3 were equally subject to catabolism, especially at FcRn-saturating conditions at >15-20 mg/ml, their levels were also replenished by endogenous production. This skews the ratio of different IgG subclass and allotype maternal transport, making the results difficult to analyze. While we detected a tendency towards a more efficient transplacental transport of G3m16, which was not significantly different from IgG1 transport, G3m16 transport was not also not significantly different from transport of IgG3 total. We concluded that the IgG measurements in women treated with IVIG indicated that G3m16 was transported better than non-G3m16 IgG3. However, the results were influenced by IVIg-dosage and time of injection, due to extremely complicated kinetics involving
catabolism in the mother and child, transplacental transport, saturation of all those pathways (saturation value may differ between individuals due to FcRn promoter polymorphisms)²² and endogenous expression of IgG3.

To solve this, we screened paired mother cord samples from the Guangzhou Blood Centre in China, since the frequency of G3m16 is much higher in Asia than in Europe (~8% according to our findings).¹⁰

We analyzed four healthy G3m16 positive paired mother/cord blood samples together with 27 G3m16- pregnancies. In this way we were able to verify that H435-containing G3m16 was transported better than other arginine containing IgG3, and indeed equally well as IgG1. This concurred with our previous observations on G3m16 that it also has an extended half-life in vivo when compared to other IgG3 allotypes; a mechanism which is also mediated by FcRn.⁶

In conclusion, we have verified that the histidine in position 435 has a great influence on the FcRn mediated placental transport of IgG. Since most IgG3 allotypes have an arginine at that position, interaction with FcRn is less pH dependent, causing it to lose competition for FcRn mediated transport and recycling. As a result, IgG3 has a short half-life and is inefficiently transported over the placenta, compared to other IgG subclasses. The half life and, as we show here, the maternal transport of H435-containing IgG3 of the G3m16 allotype is equal to that of IgG1, indicating FcRn to be the primary or sole receptor responsible for materno-fetal transport.⁶ Fetuses and newborns of G3m16 (and the less common G4m15) positive individuals might therefore be more susceptible for adverse HDFN in cases where IgG3-responses are common, as IgG3 tends to have higher FcγR- and complement mediated activities than the other IgG subclasses.⁶,²³ This is particularly true for anti-D responses, but also other antigen mismatches, including AB0, where IgG3 responses are regularly observed.⁴,⁵

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18. Brambell FW. The transmission of immunity from mother to young and


