Innate and Adaptive Immune Response in Fabry Disease

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Received: 05 May 2014 / Revised: 18 September 2014 / Accepted: 30 September 2014 / Published online: 18 February 2015
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Abstract Fabry disease is an X-linked lysosomal storage disease in which mutations of the gene (GLA) cause a deficiency of the lysosomal hydrolase α -galactosidase A (α -Gal). This defect results in an accumulation of glycosphingolipids, primarily globotriaosylceramide (Gb3) which causes a multisystemic vasculopathy. Available since 2001 in Europe, enzyme replacement therapy consists in the administration of agalsidase, a recombinant form of α -galactosidase A. Enzyme replacement therapy was shown to improve the global prognosis but allowed partial success in preventing critical events such as strokes and cardiac arrests. As in most lysosomal storage diseases, frequent immune reactions have been described in naive Fabry disease patients. Humoral immune responses following enzyme replacement therapy have also been described, with unclear consequences on the progression of the disease. While cost-effectiveness of enzyme replacement therapy in Fabry disease begins to be questioned and new therapeutic strategies arise such as chaperone or gene therapy, it appears necessary to better understand the

immune responses observed in the treatment of naive patients and during enzyme replacement therapy with agalsidase. We propose a comprehensive review of the available literature concerning both innate and adaptive responses observed in Fabry disease. We particularly highlight the probable role of the toll-like receptor 4 (TLR4) and CD1d pathways triggered by Gb3 accumulation in the development of local and systemic inflammation that could lead to irreversible organ damages. We propose an immunological point of view of Fabry disease pathogenesis involving immune cells notably the invariant natural killer T cells. We finally review anti-agalsidase antibodies, their development and impact on outcomes.

Introduction

First described in 1898, Fabry disease (FD) is the second most common lysosomal storage disease (LSD) after Gaucher disease, with a worldwide incidence estimated between 1/40,000 and 1/117,000 live births (Meikle et al. 1999; Fuller et al. 2006). This figure is probably underestimated since late-onset variants have been described, with an expected incidence of late-onset disease as high as 1/3,100 births (Spada et al. 2006). FD is a lysosomal storage disease caused by the reduction or absence of hydrolase α -galactosidase A (α -GalA) activity in lysosomes, causing a systemic intracellular accumulation of neutral glycosphingolipids (GSL), most notably of globotriaosylceramide (Gb3). More than 600 mutations have been identified in the galactosidase alpha (GLA) gene (Xq21.3-q22) (<http://www.ncbi.nlm.nih.gov/omim>; Sakuraba database <http://fabry-database.org/mutants>). A wide

Communicated by: Verena Peters

Competing interests: Declared

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spectrum of clinical symptoms usually begins in the early childhood of FD patients and progressively enriches all along the evolution of the disease. These symptoms include acroparaesthesia, gastrointestinal disorders, angiokeratomas, heat intolerance, hearing impairment, ophthalmologic abnormalities but also proteinuria and glomerulosclerosis leading to end-stage renal disease (ESRD), cardiac hypertrophy and arrhythmia and cerebrovascular disease including transient ischemic attacks and strokes (Mehta et al. 2010). Although X-linked, FD also affects females due to the lyonization process that inactivates one X-chromosome. Affected women present mild to severe phenotype which usually appears around 5 years later than in males (Beck 2006). Before enzyme replacement therapy (ERT), life expectancy in FD patients was 58.2 years in males and 75.4 years in females (Waldek et al. 2009). Death was mostly caused by cardiovascular disease (MacDermot et al. 2001a, b; Waldek et al. 2009). In males, FD is diagnosed by demonstrating a deficiency of α -GalA in plasma or leukocytes (Winchester and Young 2006). In heterozygous females, the gold standard is the genetic analysis since enzymatic assays fail to detect one third of the patients because of the residual enzyme activity (Linthorst et al. 2010; Wang et al. 2007; Wilcox et al. 2012). Whereas “classical FD” consists in a multi-organ pathology, single organ variants are more and more evoked, following systematic investigations in specific medical conditions such as hypertrophic cardiomyopathies, cryptogenic strokes or isolated renal disease (Nakao et al. 2003; Chimenti et al. 2004; Rolfs et al. 2005). Such screenings with molecular testing are thus limited by the existence of variants of unknown significance in terms of pathogenicity and penetrance (Lukas et al. 2013; Thomas and Mehta 2013).

Another difficulty in the management of FD comes from the lack of reliable biomarkers. Historically, Gb3 levels in plasma, urine and organs, especially increased in Fabry males, have been used as potential biomarkers of the disease (Schiffmann et al. 2010). But Gb3 plasma levels are normal in the majority of Fabry females; therefore, clinical correlation appears difficult (Vedder et al. 2007). Most of the therapeutic trials have been monitored with Gb3 levels. Lyso(Gb3), the deacylated form of Gb3, is taking over this role of biomarker as it was shown to be highly increased in the plasma of both male and female patients with a relative elevation exceeding markedly that of Gb3 (Aerts et al. 2008). Moreover plasma lyso(Gb3) was shown to be an independent risk factor for white-matter lesions in males and left ventricular hypertrophy in females (Rombach et al. 2010a). Another technique was attempted using flow cytometry: as the membrane antigen CD77 corresponds to Gb3, the quantification of CD77 expression on peripheral blood mononuclear cells (PBMCs) was shown to be correlated with Gb3 accumulation and α -GalA downregula-

tion in vitro (Thomaidis et al. 2009). Evidence-based management of FD is therefore limited by the lack of standardized and reliable biomarkers.

Concerning the treatment, agalsidase alfa (Replagal[®], Shire HGT Inc.) and agalsidase beta (Fabrazyme[®], Genzyme Corp.), two recombinant α -GalA, bimonthly infused, have been shown to reduce Gb3 plasma levels (Schiffmann et al. 2001; Eng et al. 2001). Results on urinary Gb3 levels were less prominent (Schiffmann et al. 2001; Eng et al. 2001). Overall under these enzyme replacement therapies (ERT) and in the short term (from 20 to 25 weeks of treatment), sustained clinical benefits were observed such as reduction of left ventricle mass, stabilization of renal function and reduction of pain with in parallel a reduction of Gb3 contents in kidney, heart and skin (Schiffmann et al. 2001; Eng et al. 2001). Nevertheless, long-term effects (median follow-up of 6.0 years) appear more unobvious with the description of a persistent progression of cardiac fibrosis and renal failure and no significant benefit on cardiac death, renal death and stroke (Weidemann et al. 2013). Two recent reviews even reported no evidence for the use of ERT (El Dib et al. 2013) and only benefits in reducing left ventricular mass but limited renal effects (Rombach et al. 2014). Both reviews only considered six studies. One mechanism suspected in the incomplete long-term response to ERT is the development of immune responses against agalsidase. Actually, immune reactions are described in both treatment-naïve and agalsidase-treated FD patients (Rozenfeld et al. 2009; De Francesco et al. 2013). Antibodies against agalsidase are notably well described, but their role remains unclear (Linthorst et al. 2004; Bénichou et al. 2009; Wilcox et al. 2012). In this review we present the spectrum of the immunological reactions, both innate and adaptive, observed in the natural history of FD and developed against agalsidase therapy, and their impact on outcome in FD.

Proinflammatory Pattern in Naïve-Treatment FD Patients

1. Innate immunity response: the role of invariant natural killer T cells, CD1d/TLR4 pathway and Gb3 deposit. Fabry disease is characterized by a lack of α -GalA causing an accumulation of GSL in the late endocytic and lysosomal compartments. Self-GSL is recognized as an antigen by the invariant natural killer T cells (iNKTs). Indeed, iNKTs characterized by their V α 24-J α T-cell receptor (TCR)-alpha chain rearrangement represent a subset of natural killer T cells. The iNKTs recognize as an antigen self-GSL presented by the major histocompatibility complex (MHC) class I-like molecule CD1d of antigen-presenting cells (APCs) (Spada et al. 1998). Human iNKTs are divided into three subsets according to the CD4 and CD8 expression

with a different inflammatory profile: positive only for CD4 (CD4 iNKTs), positive only for CD8 (CD8 iNKTs) and double negative for CD4 and CD8 (DN iNKTs) (Gumperz et al. 2002). Numerous quantitative and qualitative defects have been implicated in antimicrobial response defects, antitumor immunity and autoimmunity (Sugita et al. 2004; Chuang et al. 2012; Hunn and Hermans 2013). The recognition of GSL induces the release of various proinflammatory cytokines such as interferon-gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α), interleukin 4 (IL-4), IL-5, IL-9, IL-10, IL-13 and IL-17 (Gumperz et al. 2002). It also upregulates CD40l inducing IL-10 and IL-12 secretion by dendritic cells (O'Reilly et al. 2011). A quantitative reduction of iNKTs has been reported in murine models of different LSDs including FD (Gadola et al. 2006; Macedo et al. 2012). In Fabry knockout (KO) mice, an age-associated increase in Gb3 storage was reported (Macedo et al. 2012). Concomitantly, a progressive decrease in iNKT rates was observed, which differs depending on the organs with a more significant reduction in the spleen (around 75%) and affecting primarily the CD4 iNKTs subset. ERT was shown to prevent the progression of this splenic iNKT deficiency in mouse (Macedo et al. 2012). In FD patients, under ERT for most of them, the quantitative reduction in the iNKT pool was not found in PBMCs (Balreira et al. 2008). This absence of disorder in iNKTs has been thought to result from the differences in intracellular trafficking of mouse and human CD1d molecules or the role of ERT. More recently, Pereira et al. reported a decrease in CD4 iNKTs, an increase of DN iNKTs and a reduction in the IL-4 production, without significant difference between ERT-treated and untreated patients (Pereira et al. 2013). Of note, only 4 patients were treated among the 15 included, limiting the impact of ERT. Interestingly, Rozenfeld and Balreira showed a low level of CD1d that could correspond to an increase of internalization and an increase in the expression of MHC class II in monocytes from Fabry patients (Balreira et al. 2008; Rozenfeld et al. 2009). Therefore it seems that the GSL accumulation in FD induces disturbances in iNKT distribution and plays a proinflammatory role via CD1d pathway.

The proinflammatory pattern in FD is well described with an increase of inflammatory cytokines (De Francesco et al. 2013). Urinary Gb3 levels were found to be highly correlated with increased IL-6 plasma levels ($r = 0.971$, $p < 0.01$) in 14 FD patients (Biancini et al. 2012). The supernatant of PBMC samples from 29 Fabry patients showed a significant increase in secreted IL-6 and IL-1 β compared to 15 healthy controls, and dendritic cells (DCs) from the same Fabry

patients revealed a trend towards higher basal levels of IL-1 β ($p = 0.095$) and TNF- α ($p = 0.049$) secretion than controls. This production was abolished in vitro when using a toll-like receptor 4 (TLR4) blocking antibody. TLRs are innate immune receptors that recognize pathogen-associated molecular patterns (PAMPs) of microorganisms. Via TLRs, DCs link innate and adaptive immunity (Iwasaki and Medzhitov 2004). TLR4 enhances DC maturation in a proinflammatory way inducing the secretion of IL-12 and IFN- γ but the inhibition of IL-4 (Caielli et al. 2010). It also triggers iNKT activation by inducing presentation of endogenous GSL by CD1d molecules. (Caielli et al. 2010). Pathological involvements of TLR4 pathway are reported through an endogenous ligand-mediated signal in an antibody-induced arthritis (Kim et al. 2012). TLRs also recognize endogenous ligands. Accumulated gangliosides in GM1 and GM2 gangliosidosis were reported to act as an endogenous ligand for TLR4 (Jou et al. 2006). Hence, Gb3 is questioned to act as an endogenous ligand to TLR4 (De Francesco et al. 2013). In fact, the Gb3 accumulation appears to be the starting point of an innate immunity reaction, involving CD1d and TLR4 pathways which would induce a dysregulation of one of the iNKT subsets and DCs, source of a pathological proinflammatory state with secretion of specific proinflammatory cytokines. We tried to summarize in Fig. 1 the interrelations between Gb3 accumulation, innate immunity cells and cytokines.

As previously mentioned, ERT could have a beneficial impact on innate immune response in FD in terms of iNKT cell counts (Macedo et al. 2012; Pereira et al. 2013). But no dedicated prospective and controlled study has been done to evaluate the impact of ERT on innate immune response as an endpoint in human. Besides, other therapeutic keys could arise from specific research on innate immunity. For example, lysosomal phospholipase A2 was reported to play a role in the generation of CD1d complexes and could therefore become a possible target like in coronary diseases (Paduraru et al. 2013; The STABILITY Investigators 2014).

But TLRs are not only expressed by immune cells. Endothelial cells, podocytes and kidney tubular epithelial cells also expressed TLR4 (Anders et al. 2004; Banas et al. 2008). Interestingly, Ma et al. recently reported the role of TLR4 activation in diabetic nephropathy (Ma et al. 2014). TLR4^{-/-} diabetic mice had significantly attenuated albuminuria, reduced kidney hypertrophy and glomerular injury. They were also protected from fibrosis and tubular injury. First observed in hepatic fibrosis, TLR4 seems to drive a fibrogenic response through the TGF- β signalling pathway in diabetic nephropathy (Seki et al. 2007; Qian

Fig. 1 Innate response observed in Fabry disease. *APCs* antigen-presenting cells, *Gb3* globotriaosylceramide, *iNKTs* invariant natural killer T cells (CD4+ or DN, double negative subsets), *PBMCs* peripheral blood mononuclear cells, *TCR* T-cell receptor, *TLR4* toll-like receptor 4

et al. 2008). Fabry nephropathy has similar aspects with diabetic nephropathy: glomerular and vascular changes with proteinuria then glomerulosclerosis, interstitial fibrosis and tubulopathy (Barbey et al. 2008). In a human podocyte model, lyso(*Gb3*) increased TGF- β expression, and anti-active TGF- β 1 antibodies decreased fibrosis component such as fibronectin and type IV collagen (Sanchez-Niño et al. 2011). In all, *Gb3* seems to activate the TLR4 pathway in podocytes and immune cells, leading to local inflammation, cellular injuries and interstitial remodelling.

2. *Gb3* deposits induce a pro-oxidative pattern and alterations of the apoptotic pathway.

Accumulation of *Gb3* induces a pathological pro-oxidative state in endothelial cells by decreasing eNOS, enhancing iNOS and COX2 expression and upregulating the expression of cellular adhesion molecules such as ICAM-1, VCAM-1 and E-selectin (Shen et al. 2008; Namdar et al. 2012). Recently, 3-nitrotyrosine (3-NT), a specific marker for reactive nitrogen species and established cardiovascular disease in humans, was even suggested to be a biomarker for the vasculopathy in *GLA* knockout mice and classical FD patients. Indeed, in 13 FD patients, a more than sixfold elevation in 3-NT concentration was observed in comparison with 11 matched controls (Shu et al. 2014). The pathogenesis of vasculopathy in FD remains controversial. Rombach et al. discussed the role of *Gb3* deposits in media layer leading to smooth muscle cell proliferation with arterial

remodelling and shear stress (Rombach et al. 2010b). Increases of ROS and NF- κ B and decrease of NO would then be the results of the angiotensin system activation. Another hypothesis proposed by Shu et al. is based on the sufficient role of *Gb3* deposits to dysregulate eNOS pathway (Shu et al. 2014). Anyway, *Gb3* accumulation triggers a pro-oxidative and pro-apoptotic pattern in endothelial cells and an upregulation of adhesion molecules with pathological effects that lead to diffuse vasculopathy.

Another inflammatory mechanism can affect the apoptotic pathway in Fabry cells (DeGraba et al. 2000; Shen et al. 2008). Misfolded proteins can trigger the endoplasmic reticulum (ER) stress apoptotic pathway. This pathway is common to numerous inborn metabolic diseases. For example, missense mutations in *GBA* gene induce production of misfolded enzyme that accumulates in lysosomes of type 1 Gaucher disease patients (Wei et al. 2008). This pathological mechanism would be accessible to chaperone therapy, in which “chaperone” molecules bind to the inactive or unstable misfolded enzymes switching them into a functional shape (Boyd et al. 2013). Surprisingly, a study comparing 32 Fabry patients with 30 healthy controls showed that Fabry PBMCs displayed rather a mitochondrial than ER apoptotic pathway (De Francesco et al. 2011). This phenomenon could be explained by the different types of mutations (nonsense and missense) in patients, which can lead to very different

tertiary structure of proteins and then involve radically different ER stress behaviours. Of note, the type of mutation was not mentioned in the study of De Francesco et al.

Finally, another mechanism affects apoptosis regulation as Gb3 accumulation was associated with an increase of autophagosomes and a decrease of mammalian target of rapamycin (mTOR) and AKT signalling cascades in a human FD podocyte model. The latter are two well-known apoptosis inhibitors (Li et al. 2002; Liebau et al. 2013). Whether only Gb3 accumulation or both Gb3 and misfolded α -GalA are implicated is not fully clarified, but a proapoptotic state mediated by at least the intrinsic mitochondrial apoptotic pathway is described, with a pro-oxidative-associated pattern that could explain in part the systemic vasculopathy observed in FD. The benefits of ERT on these innate immune mechanisms have not been evaluated yet specifically. Although further studies are needed, we can hypothesize that the decrease of Gb3 could reduce inflammation and organ injury.

Immune Adaptive Responses Against Agalsidase in Fabry Disease

1. ERT in Fabry disease

Two different recombinant α -GalA are available since 2001. Agalsidase alfa (Replagal[®], Shire Human Genetic Therapies, Inc., Cambridge, MA, USA) is produced in a human cell line by gene activation, with recommended infusion dosage of 0.2 mg/kg per 14 days (Schiffmann et al. 2000). Agalsidase beta (Fabrazyme[®], Genzyme Corporation, Cambridge, MA, USA) is produced by classical cDNA transduction in Chinese hamster ovary (CHO) cells (Eng et al. 2001), with recommended infusion dosage of 1 mg/kg per 14 days. In the United States only agalsidase beta has been approved, whereas both molecules are available in Europe and many other countries. The Canadian Fabry disease initiative attempted a large head-to-head randomized trial (Sirrs et al. 2014). In this study, the primary endpoint was a composite clinical endpoint consisting of renal events, cardiovascular events, cerebrovascular events or death. No statistical difference in this endpoint was observed, but power was limited by the worldwide shortage of agalsidase beta. In the same way, no clear difference in clinical response and tolerance has appeared from the different long-term surveillance reports (Lidove et al. 2010). Of note, a significant correlation was reported between Gb3 clearance of the podocytes and cumulative agalsidase

dose ($r = 0,804$; $p = 0,002$) using both preparations (Tondel et al. 2013). Literature supports benefits of an early initiation of ERT, permitting to stabilize kidney function and improve pain, quality of life and the Mainz Severity Score Index (MSSI) developed to quantify the overall severity of FD (Schiffmann et al. 2001, 2003; Eto et al. 2005; Germain et al. 2007; West et al. 2009; Lidove et al. 2010; Engelen et al. 2012). Nevertheless, a prospective follow-up of 66 male patients revealed that despite long-term ERT, glomerular filtration rate (GFR) declined ($p < 0.001$) and cardiac mass estimated by echocardiograms increased ($p < 0.001$), but the risk of developing a first or second major complication (defined by cardiac events such as rhythm trouble, infarction or congestion, cerebral strokes, end-stage renal disease (ESRD) or death) decreased per year of ERT (OR = 0.81 and OR = 0.52 respectively) (Rombach et al. 2013). Progression of the disease under ERT, estimated with GFR and cardiac mass, appears more important in males than in females (Rombach et al. 2013). ERT seems to have less benefit in advanced renal disease such as proteinuria superior than 1 g/day that is associated with continued loss of GFR (West et al. 2009). In advanced FD, the initiation of ERT seems of doubtful clinical benefits (Rombach et al. 2013; Weidemann et al. 2013). Irreversible organ damages such as fibrosis could explain this phenomenon. Furthermore, infusion-associated reactions (IARs) frequently occur with ERT in FD. Fortunately IARs are usually limited to fever and chills (Eng et al. 2001; Linthorst et al. 2004). These were more frequently experienced in patients with anti-agalsidase antibody-positive status (Smid et al. 2013).

2. Humoral responses against ERT: the lack of standardized assays

As mentioned, the main issues in understanding the impact of antibody formation are both the safety and the long-term efficacy of ERT (Smid et al. 2013). Since ERT consists in infused recombinant proteins, the development of a humoral response is expected as previously demonstrated, for example, with recombinant factor VIII in the treatment of haemophilia (Lusher et al. 1993). This problem is also well known in other LSDs, such as Pompe disease. It has been demonstrated that in the infantile-onset form of Pompe disease, a significant proportion of patients (particularly the young cross-reactive immunologic material (CRIM)-negative status patients) develop a robust humoral immune response against the recombinant enzyme, attenuating treatment efficiency (de Vries et al. 2010). To date, in FD no standardized assay exists to detect the presence of anti-agalsidase antibodies (Schellekens 2008;

Lidove et al. 2010). In most of the trials and reports studying the IgG response against ERT, except those of the Dutch team of Aerts, Linthorst et al., antibody analyses were performed by the manufacturer of the infused agalsidase preparation (Linthorst et al. 2004; Ohashi et al. 2008; Vedder et al. 2008; Bénichou et al. 2009; Rombach et al. 2010a, 2012; Lidove et al. 2010; Wilcox et al. 2012). Linthorst et al. then Smid et al. showed that all the antibody-positive patients (assessed by the gold standard ELISA) demonstrated a neutralizing activity (Linthorst et al. 2004; Smid et al. 2013). Smid et al. studied the occurrence of IARs from 1999 to 2011 compared to the antibody status. As all antibody-positive patients first demonstrated neutralizing activity, they admitted the reciprocal proposition defining antibody-positive status with neutralizing tests in the course of the study. To our knowledge this reciprocal proposition has not been proven. Neutralizing technique does not take into account all the possible antibody targets, notably the molecules involved in the intracellular uptake, as evoked in an *in vivo* study in Fabry mice (Ohashi et al. 2008; Rombach et al. 2012). Therefore, comparing the immunogenicity of both treatments appears difficult. Nevertheless, agalsidase alfa seems less immunogenic than agalsidase beta (Lidove et al. 2010). To date, IgE antibodies have only been reported with agalsidase beta, only in males, sporadically and associated with severe IARs (Tanaka et al. 2010). Among the 51 patients receiving agalsidase beta in a double-blind placebo-controlled trial, one experienced anaphylaxis and had positive serum IgE test result and one experienced urticaria with throat congestion and had positive skin test results (Banikazemi et al. 2007). It could be the result of the production process using CHO, with a higher percentage of fully sialylated oligosaccharides and a higher level of phosphorylation (Lee et al. 2003; Bekri 2006; Ohashi et al. 2008; Deegan 2012; Ramaswami et al. 2012). In the latest report of postmarketing surveillance database on agalsidase beta, 73% of the patients developed IgG antibodies towards the exogenous enzyme (Wilcox et al. 2012). Concerning agalsidase alfa, 24% of the patients developed IgG antibodies (Lidove et al. 2010; Keating 2012). The different dosages of the two preparations could explain in part these results as Rombach reported no significant difference in antibody formation between the two preparations when using the same dosage of 0.2 mg/kg for both groups (Rombach et al. 2012). Increasing agalsidase dosage could therefore be associated with the development of antibodies limiting the expected dose-related benefits. All the data mention that females develop fewer antibodies, probably because of the

residual enzyme activity due to their heterozygous status (Linthorst et al. 2004; Bénichou et al. 2009; Rombach et al. 2012). During a 1-year follow-up of 59 treated patients, 0/30 females but 17/29 males developed antibodies (Rombach et al. 2012). Antibodies towards agalsidase alfa and beta seem to show a complete cross-reactivity (Linthorst et al. 2004). It seems that men with nonsense mutations develop more antibodies than those with missense mutations, although certain missense mutations such as R342Q are associated with a high rate of seroconversion (Wilcox et al. 2012). Antibodies towards agalsidase seem to develop within 6 months of ERT with a median delay of around 6 weeks (Wilcox et al. 2004; Smid et al. 2013). They can become undetectable after previous positivity, patients then “tolerized” (Rombach et al. 2012; Wilcox et al. 2012). The effectiveness of immunosuppressive therapy in LSDs has already been described. Banugaria et al. reported that immune tolerance induction with rituximab, methotrexate and intravenous immunoglobulins increased the effectiveness of ERT in CRIM-negative infantile Pompe disease patients (Banugaria et al. 2013). Dickson et al. reported another example of such benefits in canine mucopolysaccharidosis I, when combining azathioprine and cyclosporine to ERT (Dickson et al. 2008). Interestingly, while the global impact of antibodies remains unclear, Garman et al. observed in 2004 a reduction of antibody response against agalsidase beta with methotrexate in Fabry mice, at the quite high dose of 10 mg/kg (Garman et al. 2004).

3. Impact of anti-agalsidase antibodies on enzymatic activity *in vitro* and FD biomarkers

As previously mentioned, Linthorst et al. first reported that all IgG-positive sera by ELISA assay showed a marked *in vitro* inhibition of enzyme activity (alfa and beta), ranging from 65 to 95% (Linthorst et al. 2004). Only scarce information is available concerning the effects of antibodies on intracellular enzymatic activity. Anti-agalsidase beta antibodies were found to be associated with an inhibition of enzyme activity in cultured fibroblasts of FD patients (Ohashi et al. 2008). A statistically significant association was also reported between high anti-agalsidase beta IgG titres and Gb3 deposition in dermal capillary endothelial cells in 54 males (Bénichou et al. 2009).

On the other hand, the impact of anti-agalsidase on biomarkers in FD is the source of a growing literature, being associated with an increase of urinary Gb3 levels without clear effect on Gb3 plasma levels (Schiffmann et al. 2006; Banikazemi et al. 2007; Vedder et al. 2008). The interpretation of these results is limited because much of the urinary Gb3 derives from podocytes and

tubular cells heterogeneously accessible to ERT. Indeed, agalsidase delivery to podocyte lysosome depends on the high-efficiency uptake by mannose-6-phosphate (M6P) receptor targeted by the M6P pattern of the recombinant enzyme but also on the megalin and sortilin uptakes, independently from M6P (Prabakaran et al. 2011). These secondary receptors could, however, be limited by specific neutralizing antibodies. As previously mentioned, lyso(Gb3) now appears a better biomarker of FD activity. A recent work conducted with 59 adult “classical” Fabry patients, from the Dutch cohort, treated by either agalsidase alfa or beta, reported that plasma lyso(Gb3) was significantly higher all along the 6-year follow-up in seropositive patients for anti-agalsidase antibodies determined by neutralizing tests (seronegative status was assessed when 5 µL of plasma neutralized less than 50% of the enzyme activity *in vitro*) (Rombach et al. 2012). In the same report, urinary Gb3 decreased only in seronegative patients. Interestingly, in seropositive males switched from an agalsidase beta dose of 0.2 mg/kg/per 2 weeks to 1.0 mg/kg per 2 weeks, plasma lyso(Gb3) and Gb3 additionally declined after 1 year ($p = 0.028$). Nevertheless, urinary Gb3 levels did not change, and biomarker levels remained higher than in seronegative patients ($p < 0.02$) (Rombach et al. 2012). All data are therefore concordant with a negative effect of anti-agalsidase antibodies on biomarkers of FD activity.

4. Impact of anti-agalsidase antibodies on clinical outcomes

As previously mentioned, evidence of a pathogenic role of Gb3 accumulation is growing. The differences between the decreases of Gb3 and lyso(Gb3) depending on the presence of antibodies lead to believe that antibodies have a negative impact on clinical outcomes. Nevertheless, anti-agalsidase antibodies (alfa and beta) seem to have no influence on GFR slopes and cardiac mass nor the apparition of new white-matter lesions on cerebral magnetic resonance imaging (Schiffmann et al. 2006; Bénichou et al. 2009; Rombach et al. 2012). Some IARs were associated with high titre of antibodies, required hospitalization and caused significant disabilities or death (Smid et al. 2013). In this study, seropositive status was assessed by a neutralizing test that may be unsuitable for such safety studies. However further studies are needed with larger cohorts and standardized assays to determine the entire impact of these antibodies on clinical outcomes.

5. Cellular immune response against agalsidase

To our knowledge, nothing is known about the possible cellular immune response triggered by agalsidase and its effects.

Conclusion

As Fabry disease is nowadays increasingly diagnosed, the description of the mechanisms involved in its pathogenesis, notably the innate immune reactions, appears essential. Numerous observations support the evidence of a proinflammatory state in Fabry cells, with a probable direct role of Gb3 which would activate TLR4 and CD1d pathways and enhance inflammatory cascades via iNKTs. Notably described in endothelial cells, this may in part explain the observed vasculopathy. TLR4 also enhances TGF- β expression in podocytes leading to the remodelling of the extracellular matrix and secondary irreversible damages such as fibrosis and could explain in part the Fabry nephropathy. Surprisingly, the description of the effects of ERT on these reactions is lacking.

Long-term and early-initiated ERT with both agalsidase alfa and beta appear to improve clinical outcomes when compared to placebo controls. Nevertheless, it prevents neither critical events nor irremediable progression of the disease. Anti-agalsidase antibodies are associated with a negative influence on biomarkers, enzymatic activity and intracellular Gb3 deposits, but the consequences on clinical outcomes are not perfectly clear, partly due to the lack of data and standardized assays.

Understanding the immune response in FD becomes a priority issue in the light of ERT limits. The identification of immunological pathways in FD physiopathology can lead to the development of targeted therapeutics. A better understanding of the immune response developed towards ERT could allow a better management of its effect and the development of new complementary strategies such as antioxidant, chaperones, gene therapies and/or immunosuppressive strategies.

Synopsis

Gb3, TLR4, invariant natural killer T cells and anti-agalsidase antibodies are key actors in immune responses observed in Fabry disease.

Compliance with Ethics Guidelines

Conflict of Interest

Wladimir Mauhin has received travel fees from Shire.

Olivier Lidove has received travel fees and speaker honoraria from Sanofi-Genzyme and Shire HGT and speaker honorarium from GSK.

Elisa Masat declares that she has no conflict of interest.

Federico Mingozzi declares that he has no conflict of interest.

Kuberaka Mariampillai declares that she has no conflict of interest.

Jean-Marc Ziza declares that he has no conflict of interest.

Olivier Benveniste declares that he has no conflict of interest.

Informed Consent

Not concerned.

Animal Rights

Not concerned.

This article does not contain any studies with human or animal subjects performed by any of the authors.

Details of the Contributions of Individual Authors

WM, OL, EM, FM and OB: conception and design, analysis, interpretation of data and drafting the article

WM, OL and OB: revision

KM and JMZ: design, analysis and interpretation of data
Guarantor: OB

All the authors contributed to this manuscript and approved its revision and its submission.

This article has not been previously proposed for submission or publication.

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