

Fabry disease, enzyme replacement therapy and the significance of antibody responses

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Abstract Fabry disease is an X-linked disorder caused by a deficiency of α -galactosidase A. This leads to a progressive accumulation of globotriaosylceramide in tissues throughout the body. Cardiac, renal and neurological manifestations are common and life expectancy is significantly reduced relative to the general population. Management of Fabry disease involves the administration of intravenous enzyme replacement therapy (ERT). Two forms – agalsidase alfa and agalsidase beta – have been licensed in certain jurisdictions and are generally well tolerated; however, some patients develop antibodies to the infused enzyme, which may impair the efficacy and safety of treatment. Agalsidase alfa and agalsidase beta are produced in different systems; this leads to certain differences in post-translational modification that may affect immunogenicity. Immunoglobulin (Ig) G antibodies have frequently been reported in patients with Fabry disease receiving ERT; IgG responses are reported in a greater proportion of patients receiving agalsidase beta than in patients receiving agalsidase alfa. IgE antibodies are less common than IgG antibodies, and have not been observed in patients receiving agalsidase alfa. However, these data are difficult to interpret due to methodological differences in the assessment of seropositivity, and in the doses of enzyme used. The clinical impact of the development of IgG antibodies to ERT in patients with Fabry disease remains unclear, due to lack of data and to the marked heterogeneity of patients both in terms of disease manifestations and response to therapy. Further studies that examine the

development of antibodies in patients with Fabry disease and the potential impact of such antibodies on the outcome of ERT are necessary.

Introduction

First described independently by Anderson (1898) and Fabry (1898), and subsequently characterized by Brady et al. (1967), Fabry disease (MIM: 301500) is an X-linked lysosomal storage disorder caused by a deficiency of the enzyme α -galactosidase A (EC: 3.2.1.22). Lack of function of this enzyme results in accumulation of globotriaosylceramide (Gb₃) in tissues throughout the body, and leads to diverse multisystemic clinical signs and symptoms in both affected males and females (Desnick et al. 1995). Manifestations include acroparaesthesiae, hypohidrosis, gastrointestinal disturbances, skin lesions, hearing problems and cardiovascular and renal dysfunction (Linhart and Elliott 2007; Mehta et al. 2004; MacDermot et al. 2001a, b; Hoffmann et al. 2007; Branton et al. 2002). Quality of life is generally impaired (Gold et al. 2002) and life expectancy is reduced relative to the general population by an estimated 20 years in males and 15 years in females (MacDermot et al. 2001a, b) (Waldek et al. 2009; Vedder et al. 2007a). As Fabry disease is an X-linked disorder, carrier females are mosaic for deficient cells and cells with normal expression of α -galactosidase A. α -Galactosidase A activity in serum or plasma and white cells is often within the normal range in females.

Fabry disease, at least in its classically severe form, has been regarded as the second most common glycosphingolipid storage disorder after Gaucher disease. The incidence in males has been estimated to be 1:50,000 (Spada et al. 2006), and estimates of the incidence in the general

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population vary from approximately 1:117,000 to 1:833,000 (Poorthuis et al. 1999; Meikle et al. 1999; Pinto et al. 2004). However, the true prevalence of Fabry disease may be higher. A screening study by Spada et al. reported an incidence, when including so-called later-onset forms of the disease, of approximately 1:3100 to 1:4600 male births (Spada et al. 2006). In addition, some studies in specific patient groups, such as those with end-stage renal failure (Kleinert et al. 2009; Bekri et al. 2005), otherwise unexplained cardiac hypertrophy (Chimenti et al. 2004), or cryptogenic stroke (Rolfs et al. 2005) suggest that these populations may include patients with previously undiagnosed Fabry disease. Other studies have, however, failed to diagnose cases of Fabry disease among similar groups of patients (Brouns et al. 2007; Linthorst et al. 2003). A recent review and meta-analysis (Linthorst et al. 2010) has summarized the findings of screening programs in high-risk groups.

The current management of Fabry disease involves the administration of intravenous enzyme replacement therapy (ERT) to supplement the deficiency of α -galactosidase A. Two therapeutic forms have been licensed in certain jurisdictions: agalsidase alfa (Replagal®; Shire Human Genetic Therapies, Inc.) and agalsidase beta (Fabrazyme®; Genzyme Corp.), which are given along with concomitant treatments for specific disease-related manifestations (Mehta and Hughes 2008). ERT has been shown to be effective in reversing Gb₃ storage in some cell types and treating several of the clinical manifestations of Fabry disease, and is generally well tolerated (Thurberg et al. 2002; Eng et al. 2001a; Schiffmann et al. 2001; Hughes et al. 2008). Nevertheless, a proportion of patients develop antibodies to the infused enzyme and it is important that we consider their role in the safety and efficacy of therapy.

To date, studies of antibody formation towards agalsidase alfa and agalsidase beta show an apparent difference between the two licensed preparations of ERT in terms of the proportion of patients who develop antibodies (Eng et al. 2001a; Schiffmann et al. 2001; Hughes et al. 2008; Schiffmann et al. 2006; Wilcox et al. 2004; Germain et al. 2007; Banikazemi et al. 2007; Eng et al. 2001b). This paper reviews what we know about antibody formation in patients with Fabry disease receiving ERT and explores possible differences between agalsidase alfa and agalsidase beta in this regard.

Antibody formation during protein therapy

A humoral immune response to infusion of protein-based therapies is common. To date, antibody formation has been reported during the therapeutic use of human proteins in a variety of settings (Schellekens 2003; Porter 2001). Antibodies that bind to the infused protein have the potential to

reduce both the safety and efficacy of treatment (Richards 2002); however, as reported on many occasions, the presence of binding antibodies may have no clinical consequences (Schellekens 2003) (reviewed in (Hunt 2009)).

The generation of an antibody response to infused therapeutic proteins may impair the effectiveness of treatment by modifying tissue distribution, metabolism, receptor binding, subcellular trafficking or catalytic activity of the infused protein (Richards 2002; Wang et al. 2008). Such antibodies are known as neutralizing antibodies. Antibodies may also impact on safety. Antibody formation [in particular, immunoglobulin (Ig) E responses] can induce hypersensitivity reactions, such as flushing and urticaria (Richards 2002). They may also contribute to the development of febrile reactions associated with the infusion, as well as the formation of circulating immune complexes that activate the complement system, resulting in a general inflammatory response and cytokine release (Richards 2002).

The formation of neutralizing antibodies has been reported during treatment with several recombinant human proteins, including insulin treatment in diabetes (Fineberg et al. 1983; Meager 1994), factor VIII treatment in haemophilia (Lusher 2000), erythropoietin treatment in anaemia (Prabhakar and Muhlfelder 1997; Casadevall et al. 2002) and interferon (IFN)- β treatment in multiple sclerosis (Rossman 2004). The development of neutralizing antibodies can result in decreased effectiveness of the infused therapy. For example, neutralizing antibody development during IFN- α treatment of hepatitis or cancer has been associated with non-responsiveness to treatment, shortened duration of response, and disease reactivation (Quesada et al. 1985; Giannelli et al. 1994; Antonelli et al. 1996). Similarly, in patients with multiple sclerosis the efficacy of IFN- β is reduced in individuals who test positive for antibodies (Rossman 2004). However, in many cases, patients who develop neutralizing antibodies during treatment may subsequently become seronegative (Sorensen et al. 2005); that is, neutralizing antibodies are no longer detectable.

The formation of antibodies is affected by several factors. Immune responses may be influenced by a range of variables such as immune competence of the patient, concomitant medication, route of exposure, dose, duration of treatment, the presence/absence of structurally similar endogenous proteins and the characteristics of the infused protein (Huby et al. 2000; Schellekens and Casadevall 2004; Sharma 2007; Schellekens 2002). For example, intravenous administration of a protein is more likely to elicit an immune response than intramuscular administration (Schellekens 2005). The precise structure of the infused protein (e.g. amino acid sequence, glycosylation

profile) may also have implications for antigenicity (Schellekens and Casadevall 2004). Characteristics of the infused protein resulting from the manufacturing process can have an impact: protein production, purification, formulation, and storage and handling, for example, through influencing glycosylation, oxidation and aggregation can all influence immunogenicity (Sharma 2007).

ERT for Fabry disease

The two preparations available for the treatment of Fabry disease are produced in different systems. Agalsidase alfa is produced in a continuous human cell line by *in situ* activation of transcription of the *GLA* gene (Beck 2002). Agalsidase beta is produced by recombinant DNA technology using Chinese hamster ovary (CHO) cells (Keating and Simpson 2007). Both products possess the primary amino acid sequence of the native enzyme (Blom et al. 2003), but there are certain differences in post-translational modification, including glycosylation (Lee et al. 2003; Sakuraba et al. 2006), that may affect immunogenicity.

Post-translational modifications, such as glycosylation, sulphation and phosphorylation can have profound effects on the conformation and physicochemical properties of proteins (for review see (Bekri 2006)). Glycosylation processes play a major role in determining the three-dimensional configuration, stability, distribution and biological activity of proteins (Nalivaeva and Turner 2001). For example, replacement of an asparagine residue by serine at the N215 glycosylation site of α -galactosidase A, with consequent loss of the oligosaccharide chain, results in impaired trafficking of the enzyme to lysosomes (Ioannou et al. 1998; Garman and Garboczi 2004). Furthermore, glycosylation is known to have an impact on the antigenicity of recombinant proteins (Brooks 2004).

Post-translational modifications are species- and cell type-specific (Jenkins et al. 1996), and hence glycosylation patterns in proteins derived from human cells are likely to differ from those derived from cells from other mammalian species. Two lines of evidence indicate that this may be the case with α -galactosidase A preparations. Some glycoproteins produced in CHO cell lines, including α -galactosidase A, have been found to contain the sialic acid *N*-glycolylneuraminic acid as a minor component (<5%) (Matsuura et al. 1998). This sialic acid is not present in human tissues (Chou et al. 2002). Antibodies to *N*-glycolylneuraminic acid are present in at least 85% of healthy humans (Ghaderi et al. 2010) and are implicated in the hyperacute rejection of xenotransplants (Chou et al. 2002; Zhu and Hurst 2002). It has also been suggested that a content of 7% *N*-glycolylneuraminic acid in a protein may enhance its antigenicity in an animal model (Noguchi et al. 1995). Pre-existing high titre antibodies to *N*-

Glycolylneuraminic acid might be expected to give rise to a positive ELISA signal to the enzyme therapy at baseline, before the protein was infused. Such a background reactivity might be reported differently according to the different criteria for determining seropositivity in the assays developed by Shire HGT and Genzyme Corp., as described below. More importantly, such background antibody response to non-human glycans can give rise to immune complex formation, complement activation, antigen presentation (Ghaderi et al. 2010) and result in a more specific later secondary immune response to the protein backbone of the infused therapeutic agent (Galili et al. 1996) as described in a study of vaccine responses.

Incomplete sialylation of glycoproteins has been shown to be associated with the generation of antibodies (Gribben et al. 1990) and also increased clearance of the protein from the circulation (Ashwell and Harford 1982), through uptake by asialoglycoprotein receptors in the liver. There is evidence of differences in sialylation profiles between agalsidase alfa and agalsidase beta (Lee et al. 2003) (Table 1), but as agalsidase alfa exhibits less complete sialylation it might be expected to be more, rather than less, immunogenic.

It has been suggested by Barbey et al. and Vedder et al. that differences in glycosylation (unspecified) between the two agents could give rise to differences in immunogenicity, although whether this is the case still needs to be established (Barbey et al. 2004; Vedder et al. 2008).

Table 1 Differences in sialylated/phosphorylated oligosaccharide distribution between agalsidase alfa and agalsidase beta. Reproduced from (Lee et al. 2003) with permission

Glycosylation site	Oligosaccharide type	Proportion with glycoform (%)	
		Agalsidase beta	Agalsidase alfa
Asn 108	Complex fully sialylated	83	35
	Complex partially sialylated	17	53
Asn 161	Oligomannose/hybrid	75	48
	Containing mannose-6-phosphate	96	69
	Complex	25	52
	Fully sialylated	87	55
Asn 184	Partially sialylated	13	45
	Oligomannose/hybrid	96	66
	Containing mannose-6-phosphate	95	76
	Complex	4	34
	Fully sialylated	50	0

Assessing antibody formation during ERT in Fabry disease

Antibody formation in patients with Fabry disease has been studied in several clinical trials of agalsidase alfa and agalsidase beta. Immunoglobulin (Ig) G antibodies have been seen most frequently, although IgE antibodies have been reported in some patients on agalsidase beta. When interpreting the results of studies using agalsidase alfa and agalsidase beta there are some methodological factors that must be considered. First, the products are licensed for use at different recommended doses: agalsidase alfa is administered at a dose of 0.2 mg/kg every 2 weeks and agalsidase beta at a dose of 1.0 mg/kg every 2 weeks. Secondly, in the majority of studies, different criteria have been used to assess antibody production in response to the two different preparations. It appears that, in the majority of cases, testing in the original trials was carried out by the respective manufacturer (for agalsidase alfa by Shire Human Genetic Therapies, Inc., formerly known as Transkaryotic Therapies, Inc. (TKT), Cambridge, MA, USA, and for agalsidase beta by Genzyme Clinical Specialty Laboratory, Framingham, MA, USA). Although the presence of IgG antibodies has typically been determined by enzyme-linked immunosorbent assay (ELISA) (Eng et al. 2001a; Schiffmann et al. 2001, 2006; Wilcox et al. 2004; Germain et al. 2007; Richards et al. 1993), methodological differences between studies include differences in cut-off points, definitions of seropositivity, sample dilution, and the timing of testing. For instance, in studies of agalsidase beta, patients were determined to be seropositive if IgG concentrations were above the 95% confidence interval, established based on the analysis of sera from a group of healthy human donors, and if results of a radioimmunoprecipitation (RIP) assay were positive (Eng et al. 2001a; Richards et al. 1993; Bénichou et al. 2009). By contrast, IgG seropositivity in patients receiving agalsidase alfa was determined based on the demonstration of a twofold or greater increase in ELISA absorbance relative to the absorbance levels found for the same patient at baseline (Schiffmann et al. 2001, 2006). These contrasting approaches will undoubtedly result in differences in the sensitivity and specificity of each assay for detecting antibody generation in patients receiving ERT. In general, it is not possible to compare the immunogenicity of two recombinant proteins in the same class unless the assay used is fully validated and performed by a third party (Wang et al. 2008). In addition, all patients need to be screened for antibodies against both agalsidase alfa and agalsidase beta.

Antibody formation in single product clinical trials

The formation of IgG antibodies in male patients with Fabry disease is relatively common and has been reported

with both agalsidase alfa and agalsidase beta, although the reported incidence is nearly always higher for agalsidase beta (Eng et al. 2001a; Schiffmann et al. 2001; Hughes et al. 2008; Schiffmann et al. 2006; Wilcox et al. 2004; Germain et al. 2007; Banikazemi et al. 2007; Eng et al. 2001b; Schiffmann et al. 2007) (Table 2). In most patients, antibodies developed during the first 3 months of treatment. In initial clinical trials, 21.4% of adult male patients developed IgG to agalsidase alfa and 53.3–87.9% to agalsidase beta (Eng et al. 2001a; Schiffmann et al. 2001; Eng et al. 2001b). Subsequent studies have reported IgG antibodies in males of 20.0–56.0% for agalsidase alfa and 72.7–91.1% for agalsidase beta (Hughes et al. 2008; Schiffmann et al. 2006; Wilcox et al. 2004; Germain et al. 2007; Banikazemi et al. 2007; Bénichou et al. 2009; Schiffmann et al. 2007; Lubanda et al. 2009). The development of IgG antibodies has also been observed in boys. Two clinical trials conducted in children have reported IgG antibodies in 1 out of 19 boys receiving agalsidase alfa and 11 out of 14 boys receiving agalsidase beta (Wraith et al. 2008; Ries et al. 2007; Ries et al. 2006). The concentration or titre of antibodies decreased over time during continued treatment in many male patients (Schiffmann et al. 2006; Germain et al. 2007; Wraith et al. 2008). In some patients, the antibody response became undetectable (patients are said to become seronegative/tolerized) (64, 66). However, for other male patients there was a persistent IgG antibody response. A small number of male patients (one aged <18 years) with Fabry disease receiving agalsidase beta during clinical trials have also tested positive for IgE antibodies (n=3), to have reactivity to agalsidase beta on skin testing (n=4), or both (n=1) (Wilcox et al. 2004; Banikazemi et al. 2007; Wraith et al. 2008; Bodensteiner et al. 2008). These patients were withdrawn from the clinical trial they were participating in as a precautionary measure, although several subsequently restarted treatment (Bodensteiner et al. 2008). IgE antibodies have not been reported in clinical trials of agalsidase alfa.

By contrast, IgG formation in female patients (in whom α -galactosidase A is expressed by many cells) is relatively rare and has only been reported in adults where the antibody titre is generally low. In all cases reported so far there has been tolerization during continued treatment. One clinical trial reported that 3 out of 8 women receiving agalsidase beta developed IgG antibodies (Banikazemi et al. 2007). In another trial, 1 of 2 women receiving agalsidase beta developed antibodies (Wilcox et al. 2004; Germain et al. 2007). No IgG antibodies have been reported to date in women in trials of agalsidase alfa (Whybra et al. 2009). IgE antibodies have not been reported in female patients receiving either preparation.

Table 2 Summary of findings relating to IgG antibody formation in clinical studies of agalsidase alfa and agalsidase beta

Paper	Duration of study	Method	No. patients developing IgG	Timing of IgG appearance	Maximum titre reported	Final antibody titre/level	Comments
Agalsidase alfa Schiffmann et al. 2001	24 weeks	ELISA ^A	3/14 adult males	–	Low-titre (approximately 1:10)	It was noted that antibody levels decreased over time.	9 patients tested positive by immunoprecipitation assay with titres of approximately 1:2. Low-titre antibodies appeared to have no significant effect on safety or efficacy. Presence of antibodies did not correlate with incidence of IRRs.
	4.0–4.5 years	ELISA [see (Schiffmann et al. 2001)] ^B	14/25 adult males	–	Titres described as relatively low	Seronegative (n=6) Persistent antibody response (n=8)	Persistently positive: serial positive antibody titres including the last assessment made. Transiently positive: development of positive antibody titres that subsequently became negative, including the last assessment. Mean urinary sediment Gb ₃ levels rose in patients with a persistent IgG response, nearly reaching mean baseline levels after 12 months of ERT. Less dramatic effect on plasma Gb ₃ . In those with transient IgG/no antibodies, urine sediment Gb ₃ remained 20% below the mean baseline level. Presence/absence of persistently positive antibodies did not appear to correlate with the magnitude or direction of changes in eGFR in individual patients.
Ries et al. 2007; Ries et al. 2006	26 weeks	ELISA ^C	1/19 boys 0/5 girls	Week 9	1:100	None at subsequent time points week 17 and 26.	Neutralizing <i>in vitro</i> (87%). The patient tested IgG positive again after 1.5 and 2 years
Hughes et al. 2008	2 years	ELISA	3/15 adult males	–	–	–	–
Clarke et al. 2007	10 weeks Various dosing regimens	ELISA ^D	2/18 adult males both were receiving agalsidase alfa 0.1 mg/kg weekly	–	–	–	–
Schiffmann et al. 2007	2–4 years EOW then 2 years weekly	ELISA [see (Schiffmann et al. 2001)] ^B	5/11 adult males	–	–	–	5 patients developed IgG with EOW dosing. No additional patients developed IgG after switching to weekly dosing.

Table 2 (continued)

Paper	Duration of study	Method	No. patients developing IgG	Timing of IgG appearance	Maximum titre reported	Final antibody titre/level	Comments
Whitfield et al. 2005		Semiquantitative time-resolved fluorescence immunoassay. <i>In vitro</i> enzyme inhibition was investigated.	Subgroup of patients from previous trials with declining renal function 3/6 adult males 0/2 adult females	Antibodies were detected pre-treatment in 2 patients and after 2 weeks on ERT in 1 patient	Range, 19–>1000 U/L		No trend for a change in titre in either direction as a result of increasing frequency of infusions. No antibody response in 5 patients. Pre-treatment enzyme inhibition ranges from 0–17% in these patients compared with 0% in the control sample. Post-treatment values ranged from 0–20%. In the 3 patients who developed IgG during treatment, enzyme inhibition <i>in vitro</i> was greater than 85%. In the patient with the most marked antibody response, urine Gb ₃ levels increased and he experienced IRRs. Although the antibody response declined markedly, enzyme inhibition persisted.
Pastores et al. 2007		ELISA	3/19 adult males 0/2 adult females	–	–	–	–
Agalsidase beta							
Eng et al. 2001b	5 infusions Various dosing regimens	ELISA and RIP assay	8/15 males aged ≥16 years	6/9 patients on biweekly dosing seroconverted after 2nd or 3 rd infusion. 2/6 patients on every-48-h regimens seroconverted after 5th infusion (day 9).			The 4 patients who experienced symptoms compatible with hypersensitivity-type reactions had IgG antibodies. The authors suggest that antibodies may not be neutralizing based on the absence of overall changes in pharmacokinetic profile between the first and fifth infusions of agalsidase beta in a small number of patients, some of whom had IgG antibodies; however, this study did not explicitly compare antibody-negative and antibody-positive patients so these results require confirmation.
Eng et al. 2001a	20 weeks double-blind, 6 months open label	ELISA and RIP assay [see (Richards et al. 1993)].	51/58 (56 males and 2 females) aged ≥16 years	–	–	After 12 months, IgG titres had decreased in 15/26 who seroconverted	Serocconversion did not affect the primary (renal capillary endothelial Gb ₃ clearance) or secondary (composite score for microvascular

Table 2 (continued)

Paper	Duration of study	Method	No. patients developing IgG	Timing of IgG appearance	Maximum titre reported	Final antibody titre/level	Comments
Wilcox et al. 2004	30–36 months	ELISA assay ^{E,F}	51/56 males and 1/2 females aged ≥ 16 years at study start Same patient group as in (Eng et al. 2001a)	Approx 6 weeks; median of 63 days to peak titre	Peak titre was 400 in the female who developed antibodies	during double-blind phase: 1 IgG-positive patient with low titre became seronegative during this period. Tolerized (n=6 male, 1 female) Low responders (n=2) Plateaued (n=10) Highest titre at last visit (n=2) Downwards trend (n=30) Withdrew from study (n=1)	endothelial deposits of Gb ₃ in kidney, heart and skin biopsy) efficacy endpoints. Tissue storage scores for renal samples did not differ significantly between those who did and did not seroconvert. Tolerized: no detectable IgG antibodies by ELISA and two consecutive negative RIP assays. Low responders; titre ≤ 800 (1:8 above background). Downward trend: did not tolerate but has at least 1 titre value >800 and a 4-fold decrease in titre from peak to last value. There had been no decline in efficacy in the 2 patients with the highest titre at the 30-month visit as evidenced by skin biopsy histology, plasma Gb ₃ levels and serum creatinine levels. Seroconversion did not affect long-term efficacy as evidenced by persistently normalized plasma Gb ₃ levels, sustained Gb ₃ clearance in skin capillaries and stable renal function.
Germain et al. 2007	up to 54 months	ELISA assay [see (Wilcox et al. 2004)] ^F	51/56 males 1/2 females aged ≥ 16 years at study start Same patient group as in (Eng et al. 2001a)	Within the first 3 months of starting treatment in most cases	–	Tolerized (n=8 male, 1 female) Low responders (titre ≤ 800) (n=1) Plateaued (n=9) Downwards trend (n=32) One patient is missing.	The frequency of IRRs increased in parallel with the seroconversion rate but then decreased over time. Gb ₃ clearance was demonstrated in renal biopsies from patients with antibodies (n=7 patients/samples). Peak antibody titres were similar in patients with and without renal progression. Based on this limited evidence, there does not seem to be a correlation among IgG titres, plasma Gb ₃ levels and patients clinical status.

Table 2 (continued)

Paper	Duration of study	Method	No. patients developing IgG	Timing of IgG appearance	Maximum titre reported	Final antibody titre/level	Comments
Banikazemi et al. 2007	Up to 35 months	ELISA and RIP assay [see (Wilcox et al. 2004)] ^F	40/55 males and 3/8 females aged ≥ 16 years	—	—	Tolerized (3 female, 1 male).	One patient lost to follow-up
Bénichou et al. 2009	Up to 5 years	ELISA and RIP assay	104/122 males 6/12 females Includes patients from (Eng et al. 2001a) and (Banikazemi et al. 2007)	Median interval from treatment start to peak titre was approximately 3 months (range, 1.3–30.0 months).	Peak titre in males ranged from 100–409,600. Peak titre in women ranged from 100–800.	Titres declined over time for most seropositive patients. Based on calculations from data: Tolerized (n=15) Last three available titres lower than peak titre but did not tolerate (n=66) Less than peak (n=8) Same as peak (n=3) Last-available titre for 7 patients ranged from 100 (the minimum dilution) to 800.	This study divides patients into those with low-, intermediate- and high-titre antibodies. Results are discussed in main text. Presence of IgG was not associated with impaired clearance of plasma or dermal Gb₃ Five of the 6 patients who experienced IRRs had antibodies, but there was no correlation between IRR frequency and IgG titre. The only serious adverse event in the study was an IRR in a 16-year old boy. He had an IgG titre of 800. Antibodies developed after the fourth infusion, but the reaction occurred at the fifteenth infusion: the reaction had some features of a type 1 hypersensitivity reaction, but had other characteristics more likely related to autonomic dysfunction associated with the condition. Serocconversion was not associated with elevations in plasma or dermal Gb₃. The decline in IgG antibody titres parallels the decline in IRRs; however, IgG antibodies alone do
Wraith et al. 2008	48 weeks	ELISA and RIP assay [see (Wilcox et al. 2004)] ^F	11/14 boys 0/2 girls	Mean of 56 days after treatment start. Antibodies first detected after 2nd to 6th infusion	≤ 800 (n=5) Range, 1600–6400 (n=6)		

Table 2 (continued)

Paper	Duration of study	Method	No. patients developing IgG	Timing of IgG appearance	Maximum titre reported	Final antibody titre/level	Comments
Lubanda et al. 2009	6 months at 1.0 mg/kg EOW and 18 months at 0.3 mg/kg EOW	ELISA and RIP assay [see (Wilcox et al. 2004)]	18/21 adult males	During the first 12 weeks of treatment. Peak titres ranged from 100–25,600.		Tolerized (n=3) Low responders (titre ≤ 800) (n=2) ≥4-fold reduction in titre from peak value (n=8) Reduction <4-fold (n=5)	not predict susceptibility to IRRs. The highest antibody titres were in patients with coding region deletions (c.674_733 and c.717_718) that predicted truncated versions of the α -galactosidase A protein. The three patients who remained seronegative had either the p.Ile317Thr or the p. Glu341Gly substitution, and the three patients who tolerated had an p.Ile317Thr, p.Glu341Gly, or p. Asn34Ser substitution.
Ohashi et al. 2007	Median 30 months treatment in those with antibodies and 21 months in those without	ELISA and RIP assay [see (Wilcox et al. 2004)] ^F	7/14 males	At least once during the study	Maximum titre during study ranged from 3200–204,800. Antibodies described as low-titre in 3 patients (maximum, 3200, 6400 and 12,800) and high-titre in 4 patients (maximum, 12,800, 12,800, 51,200 and 204,800)		The study compared seropositive (n =7) and seronegative (n=7) patients; patients who were seropositive had significantly more advanced disease (p=0.019) No patient tolerated during the study. At 12 months, urinary Gb ₃ levels were normalized in 3/7 patients with IgG and all seronegative patients. Normalization of Gb ₃ appears to be delayed in seropositive patients. Response was noted to be better in those with low-titre antibodies (see definition in previous column) Note that, although urinary Gb ₃ levels did not differ significantly at baseline, mean levels in those with antibodies were twice those in patients who did not have antibodies (23.3±14.3 versus 11.6±11.4, respectively).
Comparisons of agalsidase alfa and beta							
Linthorst et al. 2004	Up to 12 months	ELISA Neutralizing activity was also assessed.	11/16 adult males 0/2 adult females	–	Up to 1/32,768 based on figure in paper	There was no decline in titre (defined as a 4-fold reduction or more) in 4 patients. In some	All 7 patients who experienced IRRs were IgG positive. There was no correlation between genotype (either nonsense or missense mutation) and

Table 2 (continued)

Paper	Duration of study	Method	No. patients developing IgG	Timing of IgG appearance	Maximum titre reported	Final antibody titre/level	Comments
Vedder et al. 2007b	24 months	ELISA [see (Linthorst et al. 2004)] ^G	10/16 adult males (4/8 on agalsidase alfa; 6/8 on agalsidase beta) 0/13 adult females	–	Titres only reported for 6-month time point (range, 1/64 to 1/32,768 for agalsidase alfa and 1/256 to 1/16,384 for agalsidase beta)	patients there was a tendency for titres to decline (titre reduction between 2 and 4-fold).	the development of IgG antibodies. 3 patients with residual enzyme activity did not develop antibodies. All antibodies showed complete cross reactivity. IgG-positive sera inhibited in vitro enzyme activity by 65–95%. Antibody levels were only assessed in patients from one of the 2 centres. Infusion-related side effects were seen in 3/10 antibody positive male patients (1/4 on agalsidase alfa, 2/6 on agalsidase beta). The induction of antibodies did not correlate with the occurrence of treatment failure (6/10 patients with and 2/6 patients without antibodies showed treatment failure).
Vedder et al. 2008	12 months	ELISA [see (Linthorst et al. 2004)] Neutralizing activity was also assessed.	18/28 males (4/10 agalsidase alfa 0.2 mg/kg EOW, 6/8 agalsidase beta 0.2 mg/kg EOW, 8/10 agalsidase beta 1.0 mg/kg EOW) 0/24 females after 6 months	–	Maximum titres for a subgroup of 5 patients can be calculated from a figure (range, 1/4096 to 1/32,768).	–	Neutralizing activity was present in 17/18 male patients. No neutralizing activity was noted for one patient with very low antibody titres. The serum dilutions that resulted in 50% reduction in enzyme activity (IC50) did not differ between the different three treatment groups. There was a strong correlation between antibody titre and IC50 values (r=0.80, p<0.001). No correlation was found between the presence of antibodies and residual α-galactosidase A in leukocytes. There was no relationship between the presence of a nonsense or missense mutation and the occurrence of antibodies.

Table 2 (continued)

Paper	Duration of study	Method	No. patients developing IgG	Timing of IgG appearance	Maximum titre reported	Final antibody titre/level	Comments
This study compared antibody positive and antibody negative patients. See text for more information.							
^A Plate ELISA technique based on a goat anti-human IgG secondary antibody. For the immunoprecipitation assays, serum was diluted 1:2 and pre-incubated with purified α-galactosidase A. Complexes were precipitated with protein sepharose A beads and analysed by Western blot. ^B A positive response was defined as an absolute absorbance ≥ 0.04 units and a time-point to baseline absorbance ratio of ≥ 2.0. ^C As in B, but >0.04 rather than ≥ 0.04. ^D As in B, but >2.0 rather than ≥ 2.0. ^E Serum was screened for the presence of IgG antibodies using and ELISA and results were confirmed by RIP assay. Quantitation of the antibody was done by titrating antibody reactivity with the ELISA assay, following a 2-fold dilution scheme starting at 1/100. ^F Patients with positive results in both assays were considered to be seropositive for IgG antibodies. IgG titres are reported as the inverse of the highest serum dilution yielding a positive ELISA result. ^G A dilution of 1:8 was determined as the lower detection limit for antibodies. eGFR, estimated glomerular filtration rate; IRR, infusion-related reaction; RIP, radioimmunoprecipitation.							

Established immunological theory would predict that classically affected males who generally express undetectable quantities of α -galactosidase A and who have no cross-reactive immunological material would be more likely to develop antibodies (Hunt 2009). Consistent with this theory, females who express wild-type residual α -galactosidase A levels in cells in which the mutant parental X-chromosome is inactive, are very much less likely to develop antibodies. Although the prevalence of antibodies is higher in men with classic Fabry disease than in women, there has been no consistent relationship between cross-reactive immunological material (CRIM) status and antibody response (Richards 2002; Vedder et al. 2008; Ohashi et al. 2007; Linthorst et al. 2004). A recent study in another lysosomal storage disorder Pompe disease, found that the effects of CRIM status on outcome appear to be mediated by antibody responses (Kishnani et al. 2010). Longer term studies in larger groups of patients are needed in order to investigate the relationship between patient characteristics (CRIM, genotype and residual enzyme activity) and outcomes (antibody titres, tolerance and clinical effects) in Fabry disease.

Although, overall, these studies suggest that there may be a difference in the proportion of patients receiving agalsidase alfa and agalsidase beta who develop antibodies, it is difficult for conclusions to be drawn due to the aforementioned differences in dose and in methodology and criteria for determining antibody response.

Antibody formation in comparative clinical trials/studies

Three published studies that were centred around a randomized controlled open-label clinical trial have investigated IgG antibody formation in response to agalsidase alfa and agalsidase beta using ELISA assays that differed only in relation to the therapeutic protein used to coat the wells (agalsidase alfa or agalsidase beta, as appropriate) (Vedder et al. 2008; Linthorst et al. 2004; Vedder et al. 2007b). The two preparations were directly compared at the same dose (0.2 mg/kg every 2 weeks) in a 24-month two-centre clinical trial that assessed the development of antibodies during the first 12 months of treatment in patients attending one of the two centres (Vedder et al. 2007b). Consistent with previous studies, most male patients (10 out of 16), but no female patients (0 out of 13) who were tested developed IgG antibodies. Among the male patients, antibodies were seen in 4 out of 8 receiving agalsidase alfa and 6 out of 8 receiving agalsidase beta. There was no significant difference between the groups in the proportion of males developing antibodies. After 6 months of treatment, titres ranged from 1:64 to 1:32768 in agalsidase alfa-treated and from 1:256 to 1:16 384 in agalsidase beta-treated patients. After 12 months, antibody

titre had declined in only two male patients, both receiving agalsidase beta.

Two studies from the centre in which antibody formation had been assessed during the clinical trial have compared the two preparations at the 0.2 mg/kg dose every 2 weeks with agalsidase beta at 1.0 mg/kg every 2 weeks (Vedder et al. 2008; Linthorst et al. 2004). In the first study, by Linthorst et al., antibody formation was reported in 4 out of 6 males receiving 0.2 mg/kg agalsidase alfa, 4 out of 5 males receiving 0.2 mg/kg agalsidase beta and 3 out of 5 males receiving 1.0 mg/kg agalsidase beta in biweekly doses (Linthorst et al. 2004). Neither of the two females in the study developed antibodies. Titres were high in all 11 males who developed IgG antibodies in the course of 6 months of treatment (range, approximately 1/2000 to 1/30 000 based on inspection of graphic data presented in the paper) and titres did not differ between patients receiving agalsidase alfa and those receiving agalsidase beta or between patients receiving different doses. Antibodies showed complete cross-reactivity: patients who developed antibodies during treatment with one enzyme developed approximately the same titre of antibodies to the other enzyme. Linthorst et al. have suggested that this demonstration of cross-reactivity, in combination with the absence of differences in the prevalence of antibody formation between the two preparations in this study, supports the supposition that the two enzymes are very similar (Linthorst et al. 2004). However, this conclusion was based on small numbers of patients studied within each of the treatment groups. Furthermore, demonstrating cross-reactivity by equal titres does not necessarily mean that the substances are structurally identical and equally antigenic. To explore the relationship between cross-reactivity and structure, epitope mapping and other methods such as the Auchterlony double immunodiffusion test are needed.

In the second and larger of the two studies, antibodies developed in 18 of 28 male patients: 4 out of 10 receiving 0.2 mg/kg agalsidase alfa biweekly, 6 out of 8 receiving 0.2 mg/kg agalsidase beta biweekly and 8 out of 10 receiving 1.0 mg/kg agalsidase beta biweekly (Vedder et al. 2008).¹ Antibodies were not detected in any of 24 females. The development of antibodies was significantly more frequent in males receiving 1.0 mg/kg agalsidase beta than in those receiving 0.2 mg/kg agalsidase alfa biweekly ($p < 0.005$); however, there were no significant differences in antibody frequency between patients receiving 0.2 and 1.0 mg/kg agalsidase beta biweekly and between patients receiving either preparation at the 0.2 mg/kg biweekly dosage. On the basis of these results, it is not possible to

determine whether the higher frequency of antibodies in patients receiving agalsidase beta than in those receiving agalsidase alfa is due to differences in the antigenicity of the two products.

Of note, studies to date do not suggest that the presence/absence of antibodies or their titre is affected dramatically by changes in dose. Data from a subgroup of patients who were switched from a 0.2 mg/kg dose (agalsidase alfa or agalsidase beta) to a 1.0 mg/kg agalsidase beta weekly dose showed that antibody titre remained high or increased further following switch in those with pre-existing antibodies (Vedder et al. 2008); however, interpretation is complicated because data from both products were combined. No antibodies developed in patients who were previously antibody-negative. This is consistent with data from an open-label study of agalsidase alfa in which the presence of antibodies and their titre did not change in patients switched from receiving agalsidase alfa every other week to weekly (Schiffmann et al. 2007).

The significance of IgG antibody formation in patients with Fabry disease

Inhibition of enzyme activity

In general, studies that have tested the effects of IgG antibodies formed in response to either agalsidase alfa or agalsidase beta on α -galactosidase A activity have found them to have neutralizing activity towards the enzyme *in vitro* (Germain et al. 2007; Vedder et al. 2008; Ries et al. 2007; Linthorst et al. 2004; Ohashi et al. 2008; Whitfield et al. 2005). In these studies, neutralising activity was usually determined by the ability of patient serum to inhibit activity of commercial enzyme *in vitro*. In one of the studies comparing the two preparations, neutralizing activity was not detected in a patient with very low-titre antibodies (Vedder et al. 2008). However, the clinical relevance of this *in vitro* neutralizing activity on the therapeutic situation *in vivo* remains unclear. When groups receiving different doses and products were combined, this study also found a positive correlation between ELISA antibody titres and neutralizing activity as measured by the serum dilution that resulted in a 50% reduction of enzyme activity (IC_{50}) ($r = 0.8$, $p < 0.001$). It is of interest to note, however, that in one patient receiving agalsidase alfa, enzyme inhibition was reported to persist at a high level despite a marked decline in the antibody response (Whitfield et al. 2005). In addition, sera from some patients with Fabry disease who have not received ERT has been shown to inhibit enzyme activity to a very small degree (maximum 17%) (Whitfield et al. 2005). This level of inhibition is similar to that reported for normal individuals in a previous study of ERT with

¹ 29 of the 31 patients receiving the 0.2 mg/kg biweekly dose had been part of the previous head-to head clinical trial.

αglucuronidase in patients with Gaucher disease and is believed to represent non-specific inhibition (Rosenberg et al. 1999). These data suggest that it may be difficult to assess the precise relationship between antibody titre and degree of inhibition of enzyme activity using current assays for measuring these two variables. The first study to compare the neutralizing potential of antibodies produced in response to both products was the study by Linthorst et al. (2004). IgG-positive sera from patients with high-titre antibodies induced marked *in vitro* inhibition of enzyme activity, ranging from 65–95%. There was no difference in the inhibitory effect between the groups receiving agalsidase alfa and agalsidase beta. More recently, inhibition of cellular enzyme activity has been demonstrated *in vivo* in a mouse model of ERT with co-administration of agalsidase beta and serum from a patient with high-titre antibodies. This may be caused by either reduced cellular uptake or intra-lysosomal inhibition of enzyme activity after cellular uptake (Ohashi et al. 2008).

Clinical and surrogate outcomes

Caveat

While it is clear that high-titre IgG antibodies produced in response to either agalsidase alfa or agalsidase beta can have an inhibitory effect on α-galactosidase A activity *in vitro*, the relationship between these findings and clinical response in humans is more difficult to assess. This is partly due to the marked heterogeneity between patients both in terms of disease manifestations and response to treatment. There is also very little published data on the relationship between clinical outcome and the presence of antibodies. The majority of studies have instead assessed the effects of antibodies on surrogate measures of efficacy by investigating differences in the clearance of Gb₃ from urine and tissues from patients with and without antibodies.

The most consistent finding has been an association between the presence of IgG antibodies and a decreased treatment effect on urinary Gb₃ excretion (Schiffmann et al. 2006; Vedder et al. 2008; Ohashi et al. 2007; Linthorst et al. 2004). The association between antibodies and plasma Gb₃ levels is less clear (Schiffmann et al. 2006; Bénichou et al. 2009). Slight elevations in plasma Gb₃ levels have been reported in association with high titre antibodies in some studies (Bénichou et al. 2009); other publications report no association between antibodies and plasma Gb₃ (Wilcox et al. 2004; Germain et al. 2007; Wraith et al. 2008). Tissue Gb₃ clearance has also been shown to be impaired in patients with antibodies in only a few studies (Bénichou et al. 2009); however, most publications report successful clearance despite the presence of antibodies (Thurberg et al. 2002; Wilcox et al. 2004; Germain et al. 2007).

Clinical outcomes

Generally, studies including clinical outcomes have shown that antibody levels decreased over time and had no clinically significant effects on safety and efficacy (Schiffmann et al. 2001, 2006; Bénichou et al. 2009). Schiffmann et al. reported that the presence or absence of a persistently positive antibody response did not appear to correlate with the magnitude or direction of changes in eGFR in individual patients receiving agalsidase alfa (Schiffmann et al. 2006). Similarly, in the study by Bénichou et al. there was no correlation between antibody titres and the onset of clinical events, or between antibody titres and abnormal plasma concentrations of Gb₃ in patients receiving agalsidase beta (Bénichou et al. 2009). However, preliminary data from the Canadian Fabry Disease Initiative suggest that the presence of antibodies *per se* (irrespective of titre) may be associated with worse clinical outcomes (M West, personal communication). Data so far show that antibody-positive patients experienced a greater number of clinical events (cardiac, renal, stroke and death) than antibody-negative patients (25% vs 20.2%). In some studies, it has been noted that the majority of patients who experience infusion-related reactions also have IgG antibodies (Eng et al. 2001b; Wraith et al. 2008); however, only a minority of male patients are antibody negative. In one study it was reported that initially the frequency of infusion-related reactions increased in parallel with the seroconversion rate (Germain et al. 2007), but it is hard to draw conclusions from these limited data.

Surrogate outcomes

Urinary Gb₃ levels generally remain elevated in patients who are persistently antibody-positive relative to patients who are antibody-negative or transiently antibody-positive in studies of both preparations (Schiffmann et al. 2006; Linthorst et al. 2004). However, in a study of patients who tested positive for antibodies at some time during treatment with agalsidase beta, it was suggested that normalization of Gb₃ levels may just be delayed in antibody-positive patients: Gb₃ levels eventually normalized in 3 out of 7 cases described as having low-titre antibodies (maximum IgG titres, 3200, 6400 and 12800) over a 30-month period (Ohashi et al. 2007). Furthermore, in some antibody-positive patients the increase in urinary Gb₃ levels has been transitory (Whitfield et al. 2005) and, in one case, appeared to be independent of the level of serum enzyme inhibition (Whitfield et al. 2005).

It has been suggested that the impairment in urinary Gb₃ clearance that has been reported in patients with IgG antibodies can be overcome by increasing the dose of ERT (Vedder et al. 2008; Lubanda et al. 2009). Vedder et al.

report superior clearance in antibody-positive patients receiving 1.0 mg/kg agalsidase beta every 2 weeks for 12 months compared with antibody-positive patients receiving 0.2 mg/kg of treatment (data on agalsidase alfa and agalsidase beta combined) every 2 weeks (Vedder et al. 2008). On the basis of these results, Vedder et al. concluded that the higher dose is more effective in reducing Gb₃ levels than the lower dose, citing that this finding is analogous to the experience with recombinant products in patients with haemophilia A (Wight et al. 2003) and in animal models of Fabry disease (Ohashi et al. 2008). However, this interpretation has been criticized, in that the pooling of data on agalsidase alfa and agalsidase beta is inappropriate because the two drugs have not been shown to be identical (Mehta et al. 2008). In a subsequent study, urinary Gb₃ levels were significantly decreased by high (1.0 mg/kg) but not low (0.3 mg/kg) doses of agalsidase beta in patients with intermediate antibody titres, suggesting that there may be an interaction between dose and antibody titre (Lubanda et al. 2009). In patients with no or low titre antibody responses both doses were effective, and in patients with high titre, neither dose led to a reduction in levels of this surrogate marker. Further investigation is needed to assess the precise relationship between the two products, antibody formation and urinary Gb₃ clearance.

In general, the clinical relevance of impaired clearance of Gb₃ is unclear because there is little correlation between urinary, plasma or tissue Gb₃ concentrations and the clinical manifestations of Fabry disease (Whitfield et al. 2005; Gupta et al. 2005; Bekri et al. 2006). Furthermore, clinical improvement has been reported in some patients despite continued elevations in Gb₃ levels (Young et al. 2005; Mills et al. 2004), but this finding was in only two boys, based on subjective pain scores and was compounded by the high dose of agalsidase beta used (2 mg/kg). When interpreting the findings relating to urinary Gb₃ it is also important to bear in mind recent data that suggest Gb₃ clearance from renal tubular cells in response to ERT is governed by luminal uptake of filtered enzyme by a megalin receptor-mediated mechanism, rather than a mannose-6-phosphate-mediated, endocytotic mechanism operating on the 'plasma' side of the tubular cells (Christensen 2002; Christensen et al. 2007). This finding is of relevance to data on substrate clearance as urinary Gb₃ is thought to originate from shed renal tubular cells. It can be speculated, therefore, that reduced urinary excretion of Gb₃ may reflect impaired uptake of enzyme by megalin receptors on the luminal aspect of the renal tubular cells, possibly due to diminished filtration at the glomerulus of large enzyme-antibody complexes. Thus, changes in urinary Gb₃ may not be a good indicator of other, more therapeutically important, mannose-6-phosphate-mediated treatment effects.

The plasma concentrations of newly described soluble marker lyso-Gb₃ fall on enzyme treatment in a dose-dependent manner. They also appear to be influenced by antibody response, with greater falls in antibody-negative patients (van Breemen et al. 2011). If future studies support the clinical validity of lyso-Gb₃ as a marker of disease pathogenesis, then these recent findings will be seen to mark a step closer to demonstrating an effect of antibody response on clinical outcomes.

Overcoming immune responses

The loss of detectable specific humoral immune responses to the infusion of agalsidase alfa or agalsidase beta has been reported to occur spontaneously in some patients with Fabry disease (Schiffmann et al. 2006; Germain et al. 2007). Tolerance to foreign proteins can also be induced therapeutically (Clark et al. 2009). Two immunological strategies have been employed in animal models to induce tolerance: low-zone and high-zone (Hunt 2009). Low-zone tolerance may be developed by initially giving very small amounts of the antigen and progressively increasing the quantity and frequency of the administration of antigen in an attempt to 'switch off' the immune system. High-zone tolerance involves the repeated administration of very large doses of the antigen to generate *in vivo* antibody unresponsiveness, also known as 'cumulative immunological paralysis' [for example (Howard and Mitchison 1975)]. A recent review proposed that the concepts of low-zone tolerance and of 'acquired immunological privilege' may be of most relevance for consideration in lysosomal storage disorders (Hunt 2009). Studies of gene therapy in animals also reinforce the potential of inducing immune tolerance in LSDs using low concentrations of the antigen: gene therapy resulting in low-level continuous expression of lysosomal enzymes *in vivo* may in turn suppress immune inactivation in response to the foreign protein expressed by transduced cells (Ziegler et al. 2007; Passini et al. 2007) or to ERT (Sun et al. 2007). Immunomodulatory drugs such as cyclosporin and azathioprine have been used successfully to induce tolerance to ERT in a canine model of MPS I (Kakkis et al. 2004). This area of research may well provide us with information on how best to induce immune tolerance in patients with Fabry disease.

Conclusions

Antibody formation appears to be common during treatment with recombinant α -galactosidase A, as with other human proteins. Although both agalsidase alfa and agalsidase beta have been associated with IgG formation, the

reported incidence of antibodies has generally been higher for agalsidase beta. Nevertheless, interpretation of the effects of antibody formation on the safety and efficacy of the two preparations is complicated by the different methodologies that have been employed in various studies. Comparison of antibody formation to both products at their recommended doses is currently underway as part of the Canadian Fabry Disease Initiative (Sirrs et al. 2009). The Fabry Antibody Standardization Initiative aims to address existing methodological issues by developing standardized antibody assays to allow the scientific community to assess antibody data consistently (Schellekens 2008). These developments should facilitate further studies that will provide important insights into the potential impact of antibody formation on the outcome of ERT in Fabry disease.

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