Hematopoietic stem cell transplantation improves the high incidence of neutralizing allo-antibodies observed in Hurler's syndrome after pharmacological enzyme replacement therapy

Muhammad Ameer Saif,[1,2]* Brian W. Bigger,[1,2]* Karen E Brookes,[1] Jean Mercer,[3] Karen L. Tylee,[3] Heather J. Church,[3] Denise K. Bonney,[2] Simon Jones,[3] J. Ed Wraith,[3] and Robert F. Wynn[2]

[1]Stem Cell & Neurotherapies, Faculty of Medical and Human Sciences, University of Manchester, Manchester; [2]Department of Blood and Marrow Transplant, Royal Manchester Children's Hospital, Manchester; and [3]Genetic Medicine, St. Mary's Hospital, Manchester, UK

ABSTRACT

Background
Mucopolysaccharidosis type I is caused by deficiency of α-L-iduronidase. Currently available treatment options include an allogeneic hematopoietic stem cell transplant and enzyme replacement therapy. Exogenous enzyme therapy appears promising but the benefits may be attenuated, at least in some patients, by the development of an immune response to the delivered enzyme. The incidence and impact of allo-immune responses in these patients remain unknown.

Design and Methods
We developed an immunoglobulin G enzyme-linked immunosorbent assay as well as *in vitro* catalytic enzyme inhibition and cellular uptake inhibition assays and quantified enzyme inhibition by allo-antibodies. We determined the impact of these antibodies in eight patients who received enzyme therapy before and during hematopoietic stem cell transplantation. In addition, 20 patients who had previously received an allogeneic stem cell transplant were tested to evaluate this treatment as an immune tolerance induction mechanism.

Results
High titer immune responses were seen in 87.5% (7/8) patients following exposure to α-L-iduronidase. These patients exhibited catalytic enzyme inhibition (5/8), uptake inhibition of catalytically active enzyme (6/8) or both (4/8). High antibody titers generally preceded elevation of previously described biomarkers of disease progression. The median time to development of immune tolerance was 101 days (range, 26-137) after transplantation. All 20 patients, including those with mixed chimerism (22%), tested 1 year after transplantation were tolerized despite normal enzyme levels.

Conclusions
We found a high incidence of neutralizing antibodies in patients with mucopolysaccharidosis type I treated with enzyme replacement therapy. We also found that allogeneic hematopoietic stem cell transplantation was an effective and rapid immune tolerance induction strategy.

Key words: immune tolerance induction, hematopoietic stem cell transplantation, Hurler's syndrome, mucopolysaccharidosis, enzyme replacement therapy, allo-antibodies.

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**These authors contributed equally*

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Correspondence:
Robert F. Wynn, Department of Blood and Marrow Transplant, Royal Manchester Children's Hospital, Manchester, M13 9WL, UK.
Phone: international +44.161.7018448.
Fax: international +44.1617018410.
E-mail: Robert.wynn@cmft.nhs.uk

Introduction

Mucopolysaccharidosis type I (MPSI) is a lysosomal storage disorder (LSD) caused by deficiency of α-L-iduronidase (IDUA). The deficiency of IDUA results in intracellular accumulation of dermatan sulphate (DS) and heparan sulphate and a progressive, multisystem clinical disorder. Recombinant human enzyme replacement therapy (ERT) for LSD first became available in the 1990s[1] and is currently used clinically for MPSI, MPSII, MPSVI, Pompe, Gaucher and Fabry disease at a considerable financial cost (estimated at $150,000-300,000 per patient per annum in MPSI).[2] It is essential that the ERT is functionally active (catalytic activity) and able to target and penetrate enzyme-deficient cells. In MPSI, enzyme is taken up by cells via a mannose-6-phosphate receptor-mediated mechanism.

The formation of allo-antibodies has been reported to occur in all LSD treated with ERT.[3] The clinical importance of antibodies in ERT-treated patients is uncertain but the existence of an allo-immune response to infused exogenous enzyme and recombinant human proteins is well described.[4-5] In early clinical trials the reported incidence of IDUA-specific antibodies was low (40%) and there was no evidence of immunoprecipitation of enzyme or inhibition of catalytic activity.[6] Subsequently, a prospective, open-label, multinational study revealed a suboptimal biomarker response in the presence of high antibody titers (> 1:10,000) when compared to that in patients with no allo-immune response.[7] More recent studies in canine models of MPSI demonstrated up to 90% inhibition of enzyme uptake by MPSI fibroblasts by serum taken from dogs that had high titer anti-IDUA antibodies.[8] There is increasing evidence, mostly derived from animal models of LSD, of an inverse correlation between an observed antibody response and metabolic and clinical outcome. Even though the immune response currently reported in patients with MPSI is 91%,[9] the true incidence of functionally active (neutralizing) antibodies is unknown.

The consequences of a refractory immune response can be serious, ranging from treatment failure to catastrophic rapid progression of disease and high mortality in some LSD.[7,10-12] Several clinical protocols for the induction of immune tolerance to exogenous enzyme have been reported for these diseases and others, including hemophiliac disorders[13] in which there is a similar host immune response against a foreign protein. Allogeneic hematopoietic stem cell transplantation (HSCT) can replace the recipient's immune system with that of the donor, thereby tolerizing the individual to the replaced enzyme (pharmacological or cellular). The donor's immune system is naturally tolerized to this protein as the donor possesses normal levels of the enzyme. However, data on allogeneic HSCT as a means of immune tolerance induction are scarce.

In children with severe MPSI (MPSI H, Hurler's syndrome) the standard of care is allogeneic HSCT. Engrafted donor leukocytes deliver enzyme (cellular ERT) to the brain. Pharmacological ERT is unable to correct neurological disease because of its inability to cross the blood-brain barrier. It has been our practice recently to give ERT to patients with MPSI H in the interval between diagnosis and transplantation in order to improve the somatic (including cardiac) manifestations of the disease and to prepare the child for transplant conditioning therapy.[14] We have developed quantitative and functional immunological assays and studied the incidence, pattern and impact of the immune response in MPSI patients prior to receiving any treatment and following ERT and HSCT. We have also determined the relationship between these antibody responses and metabolic biomarkers of the disease.

Design and Methods

Patients' samples

Blood samples were collected from 28 MPSI H patients treated and followed up at the Royal Manchester Children's Hospital over a 4-year period. The samples were collected with informed consent and under permission REC 08H101063 of the hospital's ethical commission. The patients' blood samples were processed within 6 h and the serum was separated and stored at -80°C. One group of patients was followed longitudinally and comprised patients who received ERT prior to HSCT (n=8). The second was a cross-sectional group of patients (n=20) studied at least 1 year after HSCT. One patient (patient 5) rejected the donor cells following HSCT and had autologous cells returned to restore hematologic function. This patient subsequently received a second allogeneic HSCT which was successful. Longitudinal data for this patient during both transplants are included in the results. The demographics, enzyme levels, details of HSCT, immune reconstitution, and immunosuppression for all patients in the longitudinal series are presented in Table 1. The transplant details and information on immunosuppression for all patients in the cross-sectional series are shown in Table 2.

Immunoglobulin G enzyme-linked immunosorbent assay

For the immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA), 96-well plates were coated with recombinant human IDUA enzyme (Aldurazyme) (Genzyme, Framingham, USA) in enzyme-carbonate solution (10 μg/mL in 0.1 M sodium bicarbonate, pH 8.5), overnight and blocked with blocking agent containing 1% human serum albumin. The plates were washed with washing buffer [phosphate-buffered saline (PBS), 0.1% Tween at pH 7] three times and patient's serum (50 μL) was added (two-fold serial dilutions) in duplicate and incubated at room temperature for 1 h. The plates were then washed three times with washing buffer, and goat anti-human IgG-horseradish peroxidase antibody (1:5000 dilutions in dilution buffer containing PBS, 0.05% Tween, 0.01% human serum albumin) was added and incubated at room temperature for 1 h. Plates were washed again, o-phenylenediamine dihydrochloride solution was added and the reaction stopped with 2.5 M H_2SO_4. The plates were read at 492 nm immediately. The normal range was determined for each dilution by testing 13 normal sera (from normal volunteers). A positive cut-off value was defined as two standard deviations above the absorbance value for the normal sera at that concentration. To quantify ELISA, the lowest titer with absorbance above the cut-off was reported as a positive serum dilution for that patient's sample. Positive responses were confirmed by western blotting to show the specificity of the IgG antibody to Aldurazyme.

Catalytic inhibition

Functional enzyme activity was measured using 4-methylumbelliferyl α-L-iduronide (4MU) substrate (Glycosynth, Warrington, UK) at 37°C based on previously described methods.[15-16] We modified the protocol to develop and optimize mixing assays in 96-well plates and assessed inhibition of enzyme activity by patients' sera.

The assay was performed in duplicate on the highest titer

patient's sample in the longitudinal series. A series of two-fold dilutions (starting from 30 ng/mL to optimize the catalytic inhibition) of Aldurazyme in dilution buffer (PBS, 0.05% Tween, 0.01% human serum albumin) were mixed with the same volume of patient's sera or normal sera in serial dilutions in 96-well black plates (Sterilin, South Wales, UK). The sera were added at fixed dilutions (1:4) and incubated at room temperature for 2 h in an incubator kept under agitation. The 4MU substrate (20 μL) in substrate buffer (0.4 M formate buffer, pH 3.5, 0.9% NaCl) was added to each well and incubated for 1 h at room temperature in the dark. The enzyme activity in the absence and presence of sera (for patient's serum and normal serum) was measured. Normal serum was used as a standard to compare and quantify the inhibition caused by the antibodies in the patients' sera. The enzyme activity in the presence of a patient's serum was compared with the enzyme activity of the plain enzyme solution for each dilution. We also used IDUA from human embryonic kidney cells (HEK) and mouse liver cells to assess the inhibition of innate enzyme by patients' sera.

The percentage inhibition of the catalytic activity for each enzyme dilution was determined as follows:

$$\% \text{ Inhibition} = 100 - 100 \times \frac{EA_{Ps} - BEA_{Ps}}{EA_{Ns} - BEA_{Ns}}$$

where EA_{Ps} is the enzyme activity in the presence of the patient's serum, EA_{Ns} is the enzyme activity in the presence of normal serum, BEA_{Ps} is the background enzyme activity in the patient's serum and BEA_{Ns} is the background enzyme activity in normal serum.

Cellular uptake inhibition

This assay determines the inhibition of mannose-6-phosphate-mediated enzyme uptake and subsequent activity by binding antibodies in non-FCR expressing, IDUA-deficient fibroblasts.

MPSI fibroblasts (MPSI-A171) from a patient with Hurler's syndrome (genotype W402X/W402X) were established and maintained in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 1% glutamine at 37°C in 5% CO_2. The assays were performed in duplicate. Six-well culture plates were seeded with 1.5×10^5 fibroblasts/well and grown to 95% confluence. The enzyme was diluted in culture medium (Dulbecco's modified Eagle's medium with 1% glutamine) to below half the Km for Aldurazyme (100 ng/mL). Patient's serum or normal serum was added at a volume of 10 μL (1 in 100 dilutions) to the diluted enzyme solution (1000 μL volume) and incubated for 2 h at room temperature. The culture medium was replaced with the enzyme mix medium and incubated for 1 h in 5% CO_2 at 37°C. The cells were washed with PBS and harvested by trypsinization. The cells were re-suspended and washed twice with PBS and a final suspension was made in homogenizing buffer (0.5 M NaCl/0.02 M Tris pH 7-7.5). Following a cycle of freezing and thawing, the cells were sonicated and centrifuged at 2045 g for 10 min. The supernatant was tested for enzyme activity using the 4MU assay described earlier. The protein concentration was determined

Table 1. Patients' demographics (longitudinal series of patients).

N.	Gender	Genotype	Age at HSCT (months)	Age at ERT (months)	Duration of ERT (days)	Type of HSCT	Source of stem cells	Conditioning	GVHD prophylaxis	Duration of immuno-suppression (days)	CD3 count at assessment x10⁶/L	CD19 count at assessment x10⁶/L	Enzyme level at diagnosis	Latest enzyme level post HSCT	Donor engraftment (VNTR)	Complications
1	Male	W402X/ W402X	6.9	4.2	83	MUD	CB	Bu, Flu, ATG	CSA, Pred	184	330	733	0.22	55.5	100%	Autoimmune hemolysis, rhinovirus pneumonitis
2	Male	W402X/ W402X	11.5	9.2	64	MUD	CB	Bu, Cy, ATG	CSA, Pred	250	1400	1470	0.22	27.6	100%	VOD (late), skin GVHD, renal tubular acidosis secondary to CSA
3	Male	W402X/ W402X	12.2	8.5	96	MUD	PBSC	Bu, Cy, Campath	CSA, MTX	172	880	68	0.14	49.9	100%	RSV pneumonitis, pneumothoraces
4	Female	Q70X/ W402X	8.3	4.5	114	MUD	BM	Bu, Cy, Campath	CSA	166	3994	666	0.31	15.7	98%	Cy-induced acute cardiomyopathy
5	Male	Q70X/ W402X	7.5	5.3	87	MUD	CB	Bu, Cy, ATG	CSA, Pred	110	370	160	0.2	38.3	99%	Adenovirus, gastrointestinal & skin GVHD, pneumonia,
6	Male	R628X/ R628X	11.5	8.1	112	Sibling	BM	Bu, Cy, Campath	CSA	165	1748	238	0.05	33.8	100%	VOD, CMV reactivation
7	Female	W402X/ Un-known	13.2	10.2	113	MUD	CB	Bu, Cy, ATG	CSA, Pred	184	854	794	0.09	17.1	100%	Norovirus gastroenteritis
8	Male	R628X/ R628X	5.0	0.9	124	Sibling	BM	Bu, Flu, Campath	CSA, MTX	155	1982	159	0.06	20.1	70%	VOD, adenovirus infection

*MUD: matched unrelated donor; CB: cord blood; BM: bone marrow; PBSC: peripheral blood stem cells; CSA: cyclosporine A; Pred: prednisolone; VNTR: variable number tandem repeats; VOD: veno occlusive disease; GVHD: graft-*versus*-host disease; CMV: cytomegalovirus; Bu: busulphan; Cy: cyclophosphamide; ATG: antithymocyte globulin; RSV: respiratory syncytial virus; Flu: fludarabine.*

using a bicinchoninic acid assay, as described elsewhere.[17] No uptake inhibition was observed with normal sera and hence the percentage inhibition was calculated against plain enzyme solution as follows:

$$\% \text{ Inhibition} = 100 - 100 \times \frac{\text{Enzyme activity of lysate incubated in the presence of patient serum}}{\text{Enzyme activity of lysate incubated with enzyme}}$$

Biomarkers and chimerism analysis

The ratio of DS to chondroitin sulphate (CS), a glycosaminoglycan (GAG) which is not stored in cells in MPSI, is a better marker of disease progression than total urinary GAG.[18-21] We, therefore, studied DS/CS in our longitudinal series of patients. Total urinary GAG and DS/CS ratio were determined using two dimensional electrophoresis (originally described by Whiteman[22]) and LabWorks software (Anachem, Luton, UK).[19] Donor engraftment was evaluated in our clinical laboratory through analysis of variable number tandem repeats using a UVP gel photography system and LabWorks software.

Results

Pattern of immune response

In the longitudinal series of patients, high titers of IDUA reactive antibodies were seen in 87.5% (n=7) of patients during IgG ELISA. Figure 1A shows the immune response after first exposure to ERT up to the time of HSCT in these patients. One patient (patient 8) had detectable IgG antibodies only at a low titer (1:256). These patients were followed up for a median period of 216 days (range, 155-685). The median time between first exposure to ERT and the first positive IgG antibody result was 38.5 days and while that to the peak response was 55.5 days (range, 17-129). Antibody titers in seven patients (immediate post-transplant data were not available for patient 6 in this group) who received HSCT had dropped to less than 1:10,000 by a median time of 101 days (range, 26-137) despite these patients having a normal level of innate

Table 2. Patients' demographics (cross-sectional series of patients).

N.	Gender	Type of HSCT	Age at transplant (months)	Source of stem cells	Conditioning	Immunosuppression	Duration of immune suppression (days)	Duration of ERT before HSCT	IgG antibody	Donor engraftment (VNTR)	Complications
1	Male	MUD	14.4	PBSC	Bu, Cy, Campath	CSA, Pred	154	120	Negative	100%	GVHD (grade I), idiopathic pneumonitis, CMV reactivation
2	Female	MUD	11.0	CB	Bu, Cy, ATG	CSA, Pred	126	90	Negative	100%	GVHD(grade I)
3	Female	MUD	10.7	PBSC	Bu, Cy, Campath	CSA, MMF	122	72	Negative	100%	GVHD(grade I)
4	Male	MUD	15.4	CB	Bu, Cy, ATG	CSA, Pred	270	110	Negative	100%	Chronic skin GVHD (grade I)
5	Female	Sibling	11.0	BM	Treo, Flu	CSA, MTX	90	150	Negative	100%	EBV reactivation
6	Male	Sibling	17.7	BM	Bu, Cy	CSA, MTX	105	90	Positive (1:256)	100%	No significant problems
7	Male	MUD	21.5	CB	Bu, Flu, ATG	CSA	122	95	Negative	NA	No significant problems
8	Male	MUD	25.4	CB	Bu, Cy, ATG	CSA	229	240	Negative	100%	EBV reactivation, neutropenic sepsis, GVHD, VOD, cardiomyopathy
9	Female	MUD	15.3	BM	Cy, Bu, Campath	CSA, Pred	150	No prior ERT	Negative	100%	GVHD, Sepsis, cardiac arrest
10	Male	Sibling	7.5	BM	C,/Bu	CSA	210	No prior ERT	Negative	100%	No significant problems
11	Male	Sibling	31.3	BM	Bu, Cy	CSA, MTX	115	No prior ERT	Weak positive (1:128)	100%	No significant problems
12	Male	Sibling	10.8	BM	Bu, Cy, ATG	CSA, Pred	140	No prior ERT	Weak positive (1:64)	90%	Neutropenic sepsis
13	Male	Sibling	15.2	BM	Bu, Cy, ATG	CSA	188	No prior ERT	Negative	90%	GVHD skin (grade I), neutropenic sepsis
14	Male	Unrelated	16.6	CB	Cy, Melphalan, ATG	CSA	94	No prior ERT	Negative	45%	PTLPD, neutropenic sepsis
15	Male	MUD	18.8	BM	Cy, Melphalan, ATG	CSA	85	No prior ERT	Negative	100%	CMV reactivation, neutropenic sepsis, EBV reactivation , PTLPD
16	Male	Sibling	11.8	BM	Bu, Cy, ATG	CSA	154	No prior ERT	Negative	70%	Neutropenic sepsis, Gram-negative sepsis
17	Female	MUD	22.7	BM	Cy, Bu, ATG, Flu	CSA	186	No prior ERT	Negative	100%	No significant problems
18	Female	Sibling	13.4	BM	Cy, Bu, ATG, Flu	CSA, Pred	330	No prior ERT	Negative	100%	EBV reactivation, neutropenic sepsis, adenovirus infection, chronic limited skin GVHD
19	Male	MUD	15.0	BM	Bu, Cy, Campath	CSA	NA	No prior ERT	Negative	NA	No significant problems
20	Male	MUD	14.9	PBSC	Bu, Cy, Flu, Campath	CSA	243	No prior ERT	Negative	100%	GVHD (grade I), Gram-negative sepsis, EBV reactivation

*MUD: matched unrelated donor; CB: cord blood; BM: bone marrow; PBSC: peripheral blood stem cells; CSA: cyclosporine A; Pred: prednisolone; VNTR: variable number tandem repeats; VOD: veno occlusive disease; GVHD: graft-*versus*-host disease; EBV: Ebstein-Varr virus; CMV: cytomegalovirus; Bu: busulphan; PTLPD: post-transplant lymphoproliferative disorder; Cy: cyclophosphamide; ATG: antithymocyte globulin; Flu: fludarabine; MTX: methotrexate.*

IDUA. A summary of the immune responses in all these patients is shown in Table 3. The specificity of the ELISA was demonstrated using western blotting, as shown in Figure 1B. This Figure shows that in the absence of antibodies in the pre-ERT sample, there is no antibody binding to Aldurazyme (recombinant IDUA) or Myozyme (recombinant human alglucosidase-α, Genzyme, Framingham, USA). After exposure to ERT, the IgG antibodies in patients' sera only bound to Aldurazyme and not to Myozyme. ELISA results from all patients over the entire follow-up period are shown in Figure 1C base-lined to the first HSCT. None of the patients had detectable antibodies on the most recent ELISA performed a year or more after the HSCT. It is interesting to note that patient 5 in this series had primary graft rejection with autologous hematologic recovery after infusion of stored autologous cells. This resulted in an escalation of the immune response following subsequent exposure to IDUA which was later eradicated by a second unrelated donor transplant. In this cohort, the median CD3 and CD19 counts were 1140×10^6/L (range, 330×10^6/L - 3994×10^6/L) and 452×10^6/L (range, 68×10^6/L – 1470×10^6/L), respectively, at the time of the last assessment.

Analysis of the sera from patients in the cross-sectional group (n=20) showed that 85% (n=17) of patients tested 1 year or more after HSCT had no detectable antibodies despite normal IDUA levels. Eight of these patients had received ERT prior to HSCT. Three patients in this group had weakly positive IgG antibody responses to IDUA (<1:250). Chimerism data were not available for two patients in this group. The majority of patients (n=14, 78%) had achieved full donor engraftment while 22% (n=4) were chimeric with donor leukocyte engraftment of 45%, 70%, 90% and 90%. The median duration of immune suppression for graft-*versus*-host disease prophylaxis after HSCT in the cross-sectional and longitudinal series of patients was 150 days (range, 90-330) and 169 days (range, 110-250), respectively (see Tables 1 and 2). The median time to assessment in this cohort was 66 months (range, 12-181).

Enzyme neutralization by antibodies

Figure 2A illustrates the catalytic inhibition in all patients in the longitudinal series. In this series 62.5% of patients demonstrated catalytic inhibition. The inhibition was more pronounced at lower enzyme concentrations. Three patients (patients 4, 5 and 8) did not show enzyme inhibition at any concentration whereas another two patients (patients 7 and 3) showed over 75% inhibition of enzyme activity at concentrations of less than 1 ng/mL. Enzyme inhibition at higher concentrations (7.5-30 ng/mL) was up to 20% in comparison to that of a standard (normal serum). A similar pattern of inhibition was seen by antibodies when human and mouse enzymes were used (*data not shown*).

To confirm that the inhibition in a patient's serum was

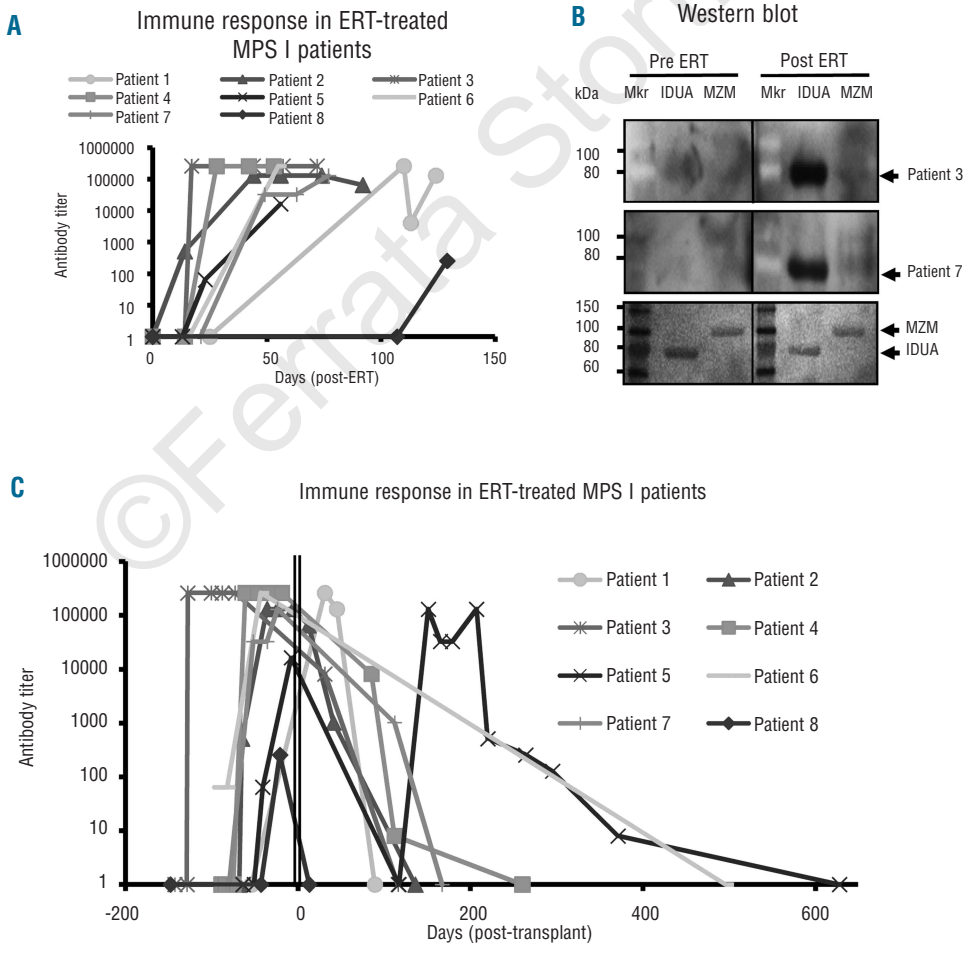

Figure 1. (A) Longitudinal data demonstrating the immune response in eight patients (1-8) in the longitudinal series from start of ERT to just before HSCT are shown baselined to the time of first starting ERT. All patients raised antibody responses to the enzyme. (B) A western blot for two patients depicting the specificity of the ELISA for IDUA (Aldurazyme). In the absence of antibodies in the pre-ERT sample, there was no antibody binding to IDUA or Myozyme (MZM). After exposure to ERT, the IgG antibodies in the patients' serum only bound to IDUA and not to MZM. Mkr (Marker) region shows the position of various bands based on their molecular weight (kDa) according to the ladder. (C) Longitudinal data demonstrating the immune response in eight patients (1-8) in the longitudinal series are shown baselined to the time of first HSCT. Antibody titer is presented on the primary vertical axis in a logarithmic scale. Note a rapid decline in antibody titer after HSCT (following approximately 3 months of ERT). Patient 5 rejected his first graft which was followed by an escalation of antibody titers. This immune response resolved after a second transplant on day 328.

due to antibodies, we assessed the enzyme inhibition in the same patient before and after HSCT i.e. the specimen with the highest titer of antibodies and no detectable antibodies on ELISA. The data confirmed that there was no catalytic inhibition in the absence of antibodies (Figure 2B).

Inhibition of cellular uptake of catalytically active enzyme

Cellular uptake inhibition was also assessed in the samples with the highest antibody titer for each patient in the longitudinal series. Inhibition of enzyme uptake by MPSI fibroblasts was seen in 75% (n=6) of patients in this series. Figure 3A illustrates the results of the uptake inhibition assay in eight patients in the longitudinal series. Two patients (patients 6 and 8) did not show any inhibition. It is interesting to note that one of these patients (patient 6) had evidence of some enzyme inhibition on catalytic inhibition assays. In contrast the two patients (patients 4 and 5) who did not have any evidence of catalytic inhibition showed 25% and 76% inhibition of enzyme uptake, respectively. Patients 2 and 7 who showed over 75% inhibition of enzyme activity also demonstrated high levels of cellular uptake inhibition (over 70%). There was no correlation between the catalytic inhibition and cellular uptake inhibition in the rest of the patients suggesting that the antibodies are polyclonal. The cellular uptake inhibition assay was repeated in non-tolerized specimens (patient on ERT pre-transplantation and with a high-titer immune response) and tolerized specimens (post-HSCT with no antibodies) from the same patient to confirm that the inhibition was due to the antibodies in the serum (Figure 3B).

Immune response and disease biomarkers

Longitudinal biomarker data (DS/CS ratio) were available for six patients in this series. Figure 4 shows the relationship between antibody titers and DS/CS ratio over the follow-up period. Patient 2 showed an immediate improvement in DS/CS ratio after starting ERT. This improvement in biomarkers was halted by a high-titer immune response. This pattern continued even after the HSCT was performed and a further improvement in DS/CS ratio resumed only after the immune response was completely abrogated (Figure 4A). The DS/CS ratio in patient 3 stayed high despite HSCT, with a continuing high-titer immune response. The slight improvement in DS/CS ratio seen in this patient was suboptimal and could be considered as a therapeutic failure at this stage. The DS/CS ratio after resolution of the immune response is not yet available for this patient (Figure 4B). The improvement in biomarker levels in patient 4 (Figure 4C) was significant after ERT was commenced; however, this progress was halted and partially reversed after a high-titer immune response was generated. The subsequent improvement in DS/CS ratio followed resolution of the immune response. Patient 5 rejected his first transplant and was given a second transplant which was succcessful. His ERT was interrupted during this period. The initial improvement in biomarker levels plateaued during the high-titer immune response despite restarting ERT. After the second HSCT, the biomarker levels showed some deterioration despite ERT and HSCT and improved only after the immune response was completely resolved (Figure 4D). Patient 6 showed a suboptimal biomarker response to ERT during the high-titer immune response (Figure 4E). The DS/CS ratio in patient 7 dropped significantly after ERT but further follow-up data were not available during the immune response and following the HSCT (Figure 4F).

Discussion

The immune response to replacement recombinant human proteins has been widely reported.[4] However, the clinical impact of antibodies ranges from no significant effect[23] to almost complete failure of therapy.[24] Hemophilia A and B are examples of congenital disorders in which the immune response (inhibitory antibodies) can make the

Table 3. Summary of immune responses in the longitudinal series of patients.

Median time to first positive ELISA test	38.5 days (range 14-129)
Median time to highest immune response	55.5 days (range 17-129)
Median time to immune tolerance	101 days (range 26-137)
Incidence of high titer antibodies	87.5% (7/8)
Incidence of catalytic inhibition	62.5 (5/8)
Incidence of cellular uptake inhibition	75% (6/8)
Immune tolerance 1 year post-HSCT	100% (28/28)

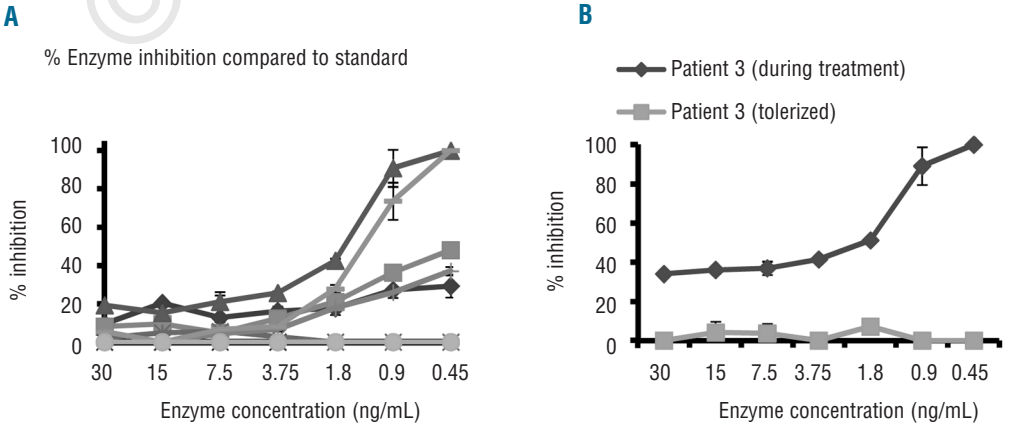

Figure 2. (A) Catalytic enzyme inhibition in eight patients (1-8) compared to a standard (normal serum) across various enzyme concentrations. (B) Inhibition due to tolerized and non-tolerized serum. Non-tolerized serum taken from a patient (patient 3) on ERT before HSCT with high titer antibodies is compared to tolerized serum taken from the same patient a year after HSCT (with no antibodies) which shows no significant inhibition.

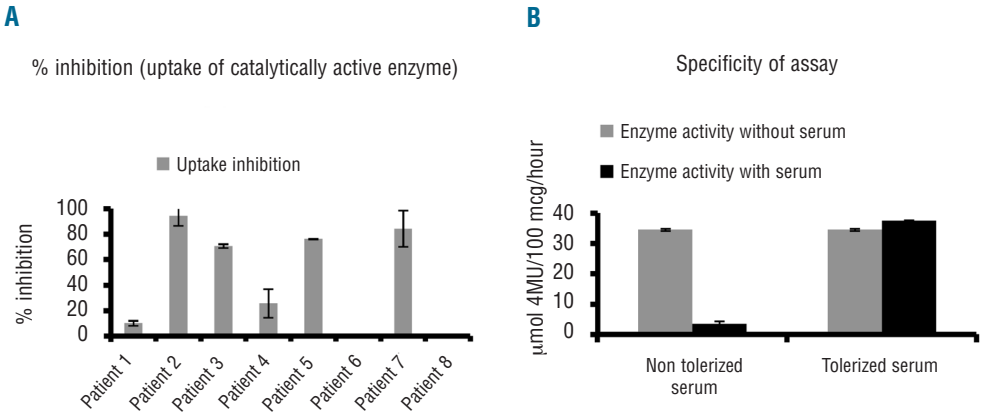

Figure 3. (A) Cellular uptake inhibition in all patients (1-8) in the longitudinal series is shown. Patients 6 and 8 showed no cellular uptake inhibition. (B) Tolerized serum (post-transplant with no antibodies) showed no inhibition compared to significant inhibition by non-tolerized serum (prior to transplant) in the same patient.

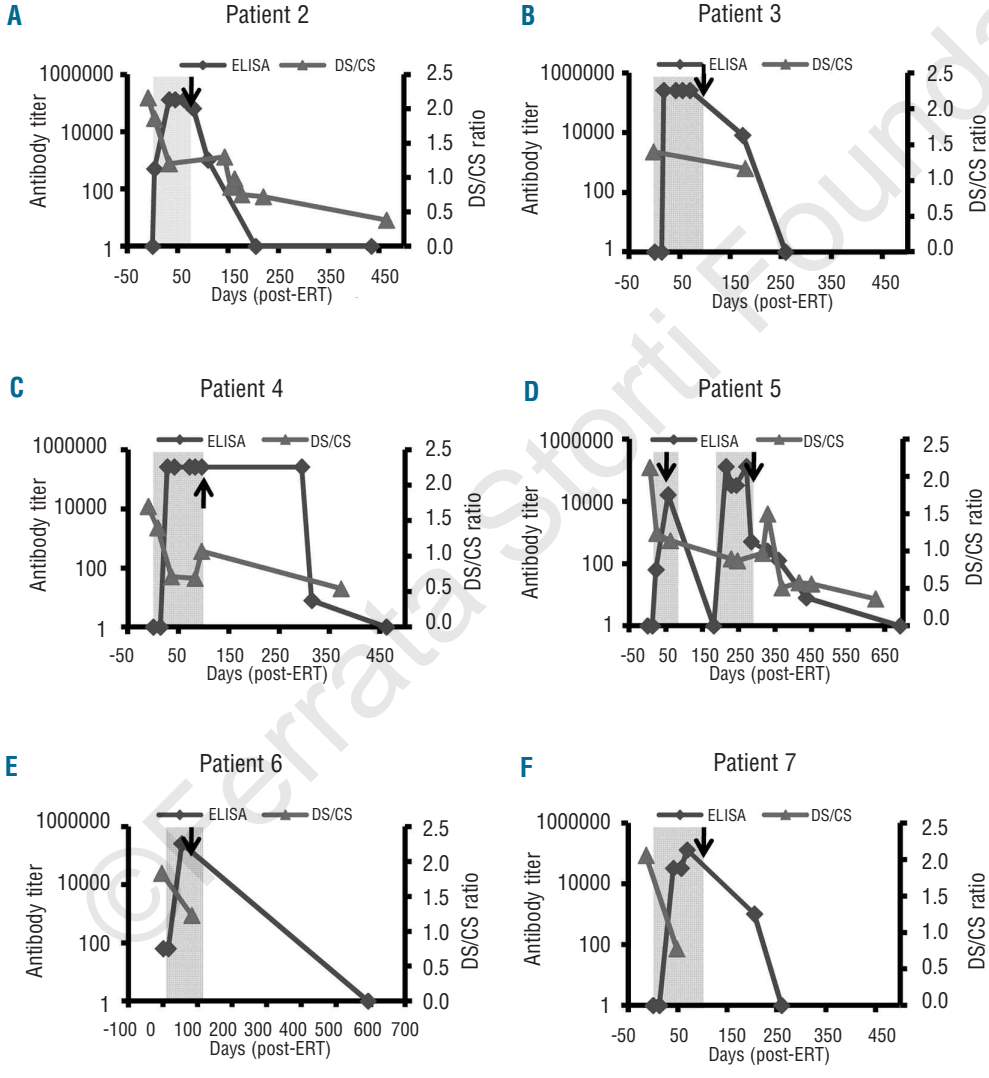

Figure 4. Correlation between biomarkers (DS/CS ratio) and the antibody titers in six patients in the longitudinal series (patients 2-7). The shaded areas represent the period of ERT and black arrows point to the time of HSCT. (A) Patient 2 showed an immediate improvement in the DS/CS ratio after starting ERT which was halted by high titer anti-IDUA antibodies. The biomarker levels improved after the immune response was abrogated. (B) Patient 3 showed minimal improvement in biomarkers despite HSCT and ERT during the high-titer immune response. (C) The improvement in biomarker levels in patient 4 was significant after ERT was commenced; however, this progress was arrested and reversed after the high titer immune response was generated. The biomarkers improved after resolution of the immune response. (D) Patient 5 rejected his first HSCT and had a second successful transplant. The initial improvement in biomarker levels plateaud during the high-titer immune response despite ERT (note a brief period when ERT was interrupted after the first failed HSCT) which improved only after the immune response was completely resolved after the second HSCT. (E) Patient 6 showed a suboptimal response to ERT during the high titer immune response. (F) The DS/CS ratio in patient 7 dropped to a significant level but further follow-up data were not available for the period during the immune response and following the HSCT. Biomarker data are not available for patients 1 and 8.

replacement therapy (recombinant human factor VIII and IX) virtually ineffective. It is noteworthy that the true impact of neutralizing antibodies can only be measured accurately using robust functional assays, such as the Bethesda assays for hemophilia inhibitors. It is considerably more challenging to study the impact of antibodies in LSD because of the absence of standardized immune assays and the marked phenotypic heterogeneity in the natural course of a rare illness. While we studied only two mechanisms of enzyme neutralization, it is hypothesized that antibodies against ERT can inhibit the treatment by several mechanisms.[25] The polyclonal nature of the IgG antibodies makes it imperative to develop multiple functional assays to encompass the full range of effects due to antibodies.[26] Despite the heavy financial burden of ERT, there have been no collaborative efforts to develop and standardize quantitative and functional assays. As a consequence the true incidence and impact of these allo-antibodies remain unknown.

In recent years, the functionally active nature of allo-antibodies and the catastrophic effects of the alloimmune response in some LSD[11-12] were recognized and led to efforts to develop an effective and quick immune tolerance induction regimen. These strategies had variable outcomes and serious side effects were reported in some cases.[13,27-29] There is a risk that intensifying the tolerance induction protocols by combination chemotherapy and immune suppression would result in significantly increased morbidity, as has been reported in hemophilia.[30,31] Development of immune tolerance with allogeneic HSCT has been reported in various autoimmune disorders and to date over a thousand HSCT have been carried out for this indication.[32] The use of low intensity conditioning regimens, the ability to manipulate the graft and the very low rate of transplant-related mortality have led to the use of allogeneic HSCT as a long-term tolerance induction strategy in some solid organ transplant recipients.[33,34] The role of HSCT as an immune tolerance induction mechanism in patients with neutralizing allo-antibodies has not yet been established.

All the patients in our study had the severe phenotype of MPSI H (Hurler's syndrome). Seven patients were homozygous for null mutations, conferring a high risk of allo-immune responses. Consistent with previous reports, we found a high incidence of antibodies in this subgroup. All transplant recipients in the longitudinal series cleared their antibodies to clinically insignificant levels within a median period of 101 days despite normal enzyme levels. None of the 20 patients in the cross-sectional group had a high-titer immune response 1 year or more after HSCT and cessation of immunosuppression. Full donor engraftment was not required for immune tolerance as some patients were tolerized despite achieving only mixed chimerism. These data confirm that allogeneic HSCT is an effective and quick mechanism of inducing immune tolerance. These data also have important implications for CD34 gene therapy. In animal models of LSD and hemophilia, stem cell transplantation after hematopoietic stem cell-based gene therapy tolerized the immune system to the infused protein.[35,36]

The majority of patients (62.5%) demonstrated evidence of some catalytic inhibition by antibodies. Even though the catalytic inhibition appears weak at high enzyme concentrations, this *in vitro* inhibition may reflect partial neutralization of infused enzyme due to the very short half-life of Aldurazyme (3 h in the absence of antibodies) and a mean maximum plasma concentration (C_{max}) of 1.2-1.7 μg/mL (ALID-014-02: A phase II Open-Label Clinical Trial of Aldurazyme). The inhibition of endogenous (human and mouse) enzyme by antibodies suggests cross-reactivity of anti-IDUA antibodies to endogenous IDUA. This signifies a need for HSCT-induced immune tolerance, as the presence of antibodies at high titers after HSCT would potentially neutralize the cellular therapy (enzyme delivered by allogeneic HSCT).

The cellular uptake inhibition can be demonstrated at a much higher enzyme concentration. We used enzyme concentrations of less than half the Km (100 ng/ml) to optimize the uptake inhibition and serum at 1:100 dilution. Corrected for dilution, this is equivalent to just over six times the C_{max} (maximum plasma concentration after infusion) of Aldurazyme, making the treatment virtually ineffective in some patients with high-titer immune responses. The cellular uptake inhibition at a higher enzyme concentration compared to catalytic inhibition suggests stronger inhibition of the mannose-6-phosphate binding sites (and enzyme uptake) than the catalytic site of recombinant IDUA by anti-IDUA antibodies. Our study looked at the antibody neutralization of enzyme in a specific group of MPSI patients with a greater propensity to develop a high-titer immune response. It is possible that the variable effects and polyclonal nature of anti-IDUA antibodies might have resulted in underestimation of the effects of antibodies in previous studies because of the pooling of data from various groups of patients. It is, therefore, important to assess these patients individually.

Analysis of biomarker data and antibody titers show that the recovery is slowed or, in some cases, reversed in the presence of a high-titer immune response. Generally there appears to be a close relationship between the two, although the DS/CS ratio and biomarker responses usually lagged behind antibody responses by several days, confirming the *in vivo* neutralization of ERT by antibodies.

In conclusion, our data show that the high-titer immune responses in MPSI H patients treated with ERT can neutralize replacement therapy in a significant proportion of patients. In the past, the heterogeneous clinical course of the disease compounded by a lack of robust biomarkers and reliable functional immune assays made it very difficult to evaluate the effect of antibodies in LSD patients treated with ERT. There is now a dire need to standardize quantitative and qualitative immune assays in these patients. Given the remarkable improvement in the outcome of HSCT, this therapy is now a viable therapeutic modality as a mechanism for inducing immune tolerance in patients with refractory immune responses to ERT and other replacement therapies by substituting the enzyme-naïve immune system with that of the donor. The generation of high-titer neutralizing antibodies to ERT prior to HSCT makes it unnecessary to continue ERT infusions in the presence of an immune response, particularly if the transplant is carried out early after the diagnosis and in the absence of any serious co-morbidities. However, inevitably, some clinically unstable patients will benefit from ERT to optimize their clinical status prior to HSCT. Close biochemical and clinical monitoring of the immune response in ERT-treated LSD patients can help to determine the optimum therapy for this group of patients.

Authorship and Disclosures

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