Mannose 6-Phosphate Conjugation Is Not Sufficient to Allow Induction of Immune Tolerance to Phenylalanine Ammonia-Lyase in Dogs

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Abstract The immune response to exogenous protein has been shown to reduce therapeutic efficacy in animal models of enzyme replacement therapy. A previously published study demonstrated an immunosuppressive regimen which successfully induced immune tolerance to α -L-iduronidase in canines with mucopolysaccharidosis I. The two key requirements for success were high-affinity receptor-mediated enzyme uptake, conferred by mannose 6-phosphate conjugation, and immunosuppression with low-dose antigen exposure. In this study, we attempted to induce immune tolerance to phenylalanine ammonia-lyase by producing a recombinant mannose 6-phosphate conjugated form and administering it to normal dogs according to the previously published tolerance induction regimen. We found that the recombinant conjugated enzyme was stable, could bind to the mannose 6-phosphate receptor with high affinity, and its uptake into fibroblast cells was mediated by this receptor. However, at the end of a tolerance induction period, all dogs demonstrated an antigen-specific immune response when challenged with increasing doses of unconjugated phenylalanine ammonia-lyase. The average time to seroconvert was not significantly different among three separate groups of test

animals ($n = 3$ per group) and was not significantly different from one group of control animals ($n = 3$). None of the nine test group animals developed immune tolerance to the enzyme using this method. This suggests that high-affinity cellular uptake mediated by the mannose 6-phosphate receptor combined with a previously studied tolerizing regimen is not sufficient to induce immune tolerance to an exogenous protein and that other factors affecting antigen distribution, uptake, and presentation are likely to be important.

Introduction

The intravenous administration of recombinant human enzyme replacement therapy has revolutionized the treatment of several lysosomal storage diseases. Recombinant enzyme is currently in clinical use for Gaucher disease; Fabry disease; mucopolysaccharidosis I, II, and VI; and Pompe disease (Kakkis et al. 2001; Wraith et al. 2004; Muenzer et al. 2006; Harmatz et al. 2005; Barton et al. 1991; Eng et al. 2001; Schiffmann et al. 2000). Enzyme replacement therapy improves many aspects of these disorders and in many cases has enabled patients to lead more normal lives.

Administration of recombinant proteins often produces an antibody response, which can have clinical or therapeutic consequences. An immune response to these protein antigens occurs in most patients being treated with replacement enzyme but is usually well tolerated clinically (Wang et al. 2008). There is evidence, however, that antibodies to replacement enzyme can reduce the effectiveness of treatment (Sifuentes et al. 2007; Brooks et al. 1997,

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1998; Turner et al. 2000; Dickson et al. 2008). Modulation of the immune response to the recombinant protein is therefore an important consideration in optimizing enzyme replacement therapy.

Previous studies by our laboratory (E.D.K.) showed that immune tolerance to recombinant human α -L-iduronidase (rhIDU) could be induced in dogs with mucopolysaccharidosis I (MPS I) using a 60-day regimen of the immunosuppressive drugs cyclosporin A and azathioprine in combination with 12 weekly intravenous (IV) rhIDU infusions at a low dose (Kakkis et al. 2004). After completing this regimen, animals did not produce antigen-specific antibodies when challenged with a full treatment dose of rhIDU up to 6 months after the induction period, thereby demonstrating immune tolerance to rhIDU. There were two key requirements for the successful induction of tolerance using this regimen. The first requirement was achieving a sufficient cyclosporin A serum level prior to starting the low-dose tolerizing rhIDU infusions. Study animals that failed to develop cyclosporin A serum trough levels of at least 350 ng/mL or that were not treated with immunosuppression consistently developed strong antibody responses to rhIDU (Kakkis et al. 2004). The second requirement was efficient cellular uptake of rhIDU, which was shown to depend on mannose 6-phosphate conjugation of the enzyme. Mannose 6-phosphate (M6P) modification of N-linked carbohydrates occurs on α -L-iduronidase and other lysosomal enzymes and allows high-affinity receptor-mediated uptake via the mannose 6-phosphate receptor (Dahms et al. 1989). When treated with the above immunosuppressive regimen, dogs failed to develop immune tolerance to dephosphorylated rhIDU or to ovalbumin, a glycoprotein lacking mannose 6-phosphate modification, but could be induced to develop immune tolerance to recombinant human α -glucosidase, an enzyme containing high levels of mannose 6-phosphate on its N-linked carbohydrates (Kakkis et al. 2004). We hypothesized that we could induce immune tolerance to other exogenous proteins by producing an M6P conjugated form and then administering low doses of the modified protein along with the above outlined immunosuppressive regimen.

Classic phenylketonuria is an autosomal recessive disease caused by deficiency of phenylalanine hydroxylase in humans that result in elevated phenylalanine levels and a spectrum of subsequent clinical findings including severe cognitive impairment (Mitchell et al. 2011). Phenylalanine ammonia-lyase (PAL), a key enzyme in plant and fungus phenylpropanoid metabolism, catalyzes the degradation of phenylalanine to *trans*-cinnamic acid and ammonia (Koukol and Conn 1961; MacDonald and D'Cunha 2007) and has been studied as a potential alternative therapy to reduce phenylalanine levels in phenylketonuria

(Sarkissian et al. 1999). When PAL was administered in an animal model of phenylketonuria, it was shown to induce a vigorous immune response that degraded the protein (Sarkissian et al. 1999, 2008). The ability to induce immune tolerance to PAL could improve its therapeutic use in humans as well as provide a method for improving the safety and efficacy of recombinant protein therapy with a wide variety of nonmammalian immunogenic protein antigens.

In this report, we tested the hypothesis that mannose 6-phosphate conjugation is necessary to allow induction of immune tolerance to an exogenous protein by producing recombinant phenylalanine ammonia-lyase conjugated with an M6P-containing group and administering it to dogs in low doses along with the previously published immunosuppressive tolerizing regimen.

Materials and Methods

Production of Purified Recombinant PAL

We expressed recombinant phenylalanine ammonia-lyase (RtPAL) in *Escherichia coli* transformed with the yeast *Rhodospiridium toruloides* PAL gene modified to introduce a lysine residue via an R91K amino acid substitution. The protein was secreted in a rich culture medium upon IPTG (isopropyl- β -D-thio-galactoside) induction and purified by hydrophobic interaction followed by anion-exchange chromatography. Purified RtPAL (76.9 kDa) was buffer-exchanged by ultrafiltration/diafiltration using a 30 kDa molecular weight cutoff polyethersulfone membrane (Sartorius Stedim Biotech GmbH, Goettingen, Germany) into Dulbecco's Phosphate-Buffered Saline (D-PBS) (VWR International, Radnor, PA) adjusted to pH 6.9 with hydrochloric acid (HCl).

Conjugation of RtPAL With a Mannose 6-Phosphate Carrier

N-succinimidyl-S-acetylthioacetate (SATA) (Thermo Fisher Scientific Inc., Rockford, IL), a sulfhydryl-containing linker that conjugates to primary amines, was added to 4 mg/mL of D-PBS-buffered RtPAL in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) in a 15:1 mole ratio (SATA:RtPAL) and incubated for 2 h at room temperature to form the RtPAL-SATA conjugate. DMSO and excess SATA were removed by gel filtration using a Zeba desalt column (Thermo Fisher Scientific Inc.) pre-equilibrated with D-PBS adjusted to pH 7.2 with HCl. The purified product was then deacetylated to produce a deprotected sulfhydryl group on SATA by addition of hydroxylamine hydrochloride ($\text{Cl}^-\text{H}_3\text{N}^+\text{OH}$) (Thermo Fisher Scientific Inc.) to 2.3 mg/mL

of RtPAL-SATA in a 100:1 mole ratio ($\text{ClH}_3\text{N}^+\text{OH}:\text{RtPAL-SATA}$) and incubated at room temperature for 75 min.

BPM6, a proprietarily synthesized molecule consisting of two carbohydrate chains terminated by mannose 6-phosphate groups and connected to a primary amine backbone, was prepared for conjugation to RtPAL-SATA by treatment with succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) (Thermo Fisher Scientific Inc.) in DMSO in a 15:1 mole ratio (SMCC:BPM6) and incubated at room temperature for 45 min to form BPM6-SMCC. Freshly prepared BPM6-SMCC was immediately added to the above prepared RtPAL-SATA in a 15:1 mole ratio (BPM6-SMCC:RtPAL-SATA) to form conjugated RtPAL-BPM6 using standard cross-linking chemistry, incubated first for 2 h at room temperature and then overnight at 4°C. The final product RtPAL-BPM6 was purified by gel filtration and formulated for injection at 1.78 mg/mL in 10 mM Tris (tris(hydroxymethyl)amino-methane) + 140 mM NaCl pH 7.5.

Characterization of RtPAL-BPM6

Protein concentrations were determined by A280 absorbance assay using $0.51 \text{ Lg}^{-1} \text{ cm}^{-1}$ as extinction coefficient. Protein endotoxin levels of the formulated material were undetectable ($<1 \text{ EU/mL}$) as measured using a Limulus amoebocyte lysate spectrophotometric assay (Charles River, Wilmington, MA). Enzymatic activity was measured according to a previously published method using optical absorption at 290 nm to monitor the production of *trans*-cinnamic acid when RtPAL-BPM6 was added to a buffered solution containing L-phenylalanine (Wang et al. 2005).

Molar substitution ratios (MSR) were determined by separate colorimetric assays for primary amines, using Ellman's reagent, and for free sulfhydryl groups, using 2,4,6-trinitrobenzenesulfonic acid (TNBSA) (Thermo Fisher Scientific Inc.), according to the manufacturer's protocols. The MSR homogeneity was investigated with isoelectric focusing (IEF) on a Novex pH 3–7 IEF gel (Invitrogen, Carlsbad, CA), and conjugate phosphorylation was identified after digestion with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Protein aggregation and molecular weight shift upon conjugation were monitored by SDS PAGE gel electrophoresis.

Mannose 6-Phosphate Receptor Binding

To characterize RtPAL-BPM6 binding to the mannose 6-phosphate receptor, a Nunc MaxiSorp ELISA plate (Thermo Fisher Scientific Inc.) was first coated with 4 $\mu\text{g/mL}$ of soluble MPR (Center for Advanced Biotechnology and Medicine, UMDNJ, Piscataway, NJ) in pH 9.5 carbonate buffer and incubated overnight at 4°C, then washed with D-PBS with 0.1% polysorbate-20, and finally

blocked at 37°C for 1 h in D-PBS with 2% BSA and 0.05% polysorbate-20. The blocking solution was discarded and test samples consisting of unconjugated RtPAL, the intermediate RtPAL-SATA, or the conjugated product RtPAL-BPM6 were loaded into plate wells in duplicate and incubated at 37°C for 1 h. Samples of each species were initially prepared at 2 nM concentrations in D-PBS with 2% BSA and 0.05% polysorbate-20 and then subjected to serial twofold dilutions to 0.03125 nM. After washing the plate, we loaded HRP-conjugated rabbit anti-RtPAL IgG (BioMarin Pharmaceutical, Inc., Novato, CA), diluted to 1:5000 in the same buffer, into plate wells and incubated at 37°C for 30 min. Following a final plate wash, wells were incubated at 37°C for 15 min with TMB peroxidase substrate (Bio-Rad Laboratories, Hercules, CA), then 2 N H_2SO_4 was added to stop the HRP-TMB reaction. Finally, absorbance at 450 nm was measured using a SpectraMax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The mean A_{450} values of duplicate wells were plotted versus sample protein concentration to yield binding curves, and single-reciprocal linear transformation (Hanes-Woolf method) and linear regression fit were performed to calculate the K_d (dissociation constant) value.

Cellular Uptake via the Mannose 6-Phosphate Receptor

Human fibroblast cells were treated with 2nM FITC-labeled RtPAL or RtPAL-BPM6 and incubated for 4 h in a tissue culture incubator (37°C, 8% CO_2) and then visualized using confocal fluorescence microscopy to determine intracellular uptake. Additional fibroblast cell incubations were performed with RtPAL-BPM6 in the presence of either free mannose 6-phosphate or free glucose 6-phosphate to test the specificity of MPR-mediated uptake. Fibroblast cell nuclei were counterstained with DAPI (diamidino-2-phenylindole).

Animal Background

The study subjects were twelve normal beagles, approximately 6 months old and weighing between 8–12 kg, housed and maintained under the care of a veterinarian at the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The study was reviewed and approved by the institutional animal care and use committee.

Tolerance Induction Regimen

The tolerance induction regimen consisted of treatment with the immunosuppressive drugs cyclosporin A (CsA) (Novartis)

and azathioprine (Aza) (DSM Pharmaceuticals) and tolerizing weekly enzyme infusions as previously described (Kakkis et al. 2004). The CsA dose started at 37.5 mg/kg/day divided twice per day and was adjusted to reach a target serum trough level of ≥ 400 ng/mL. Starting on the first morning of CsA treatment, 5 mg/kg Aza was given once every other day. Weekly intravenous infusions of RtPAL-BPM6 at low dose (0.056 mg/kg) began after 18 days of immunosuppression and continued for 12 weeks. After the third weekly RtPAL-BPM6 infusion, the doses of CsA and Aza were halved every 2 weeks and then discontinued at week 7. At week 13, we began a weekly antigenic challenge dose of unconjugated RtPAL and monitored for the development of an anti-RtPAL IgG antibody response. The RtPAL dose was doubled every week to a goal of 0.5 mg/kg/week at week 16 and was continued at this level for an additional 5 weeks. One week after the final infusion at week 21, serum was collected and infusions discontinued. In the original tolerance induction study, dogs that were successfully tolerized to rhIDU did not produce an antigen-specific IgG response when challenged in this manner (Kakkis et al. 2004).

Determination of Specific Antibody Titers in Canine Serum

Serum samples for antibody analysis were collected weekly prior to infusions and frozen at -20°C for subsequent analysis. Anti-RtPAL IgG antibody titers were measured by an enzyme-linked immunosorbent assay (ELISA) method. In brief, serum samples were first diluted 1:50 then subsequent 1:3 serial dilutions were incubated on assay plates containing wells with adsorbed RtPAL. Specific binding of anti-RtPAL IgG antibodies to the coated wells was detected using a monkey anti-canine IgG secondary antibody conjugated to HRP and measuring absorbance after incubation with a substrate. An absorbance cutpoint was generated for each assay plate based on comparison with pooled serum from untreated animals. The optical density (OD) values of the serial dilutions were compared with this plate cutpoint, and the highest dilution factor that generated OD value above the plate cutpoint was reported as the titer for this sample. The time to seroconversion was defined as the number of elapsed weeks at which an anti-RtPAL IgG antibody titer greater than 1:50 was observed.

Statistical Analysis

Analyses were performed with SYSTAT statistical analysis software (Systat Software, Inc., Chicago, IL). Means and standard deviations were calculated according to standard formulas. We performed analysis of variance (ANOVA) with a post-hoc Tukey-Kramer test, and $p < 0.05$ was considered significant.

Monitoring

The safety of the tolerance regimen and recombinant enzyme infusions was assessed with serum chemistries to evaluate liver and renal function, urinalysis, cyclosporin levels during tolerance induction, physical examination, daily weight, and recording of adverse events. Blood samples were collected every 4 weeks for a complete blood count and chemistry profile. Urinalysis with reagent strips was also performed every other week on a fresh urine sample to monitor parameters such as proteinuria and hematuria. During infusions with the recombinant enzyme, the dogs were monitored for anaphylactic reactions. There were no adverse events in dogs in any experimental group, and all animals survived the study.

Results

Successful stepwise conjugation of RtPAL to the mannose 6-phosphate carrier BPM6 (Fig. 1) was demonstrated by TNBSA assay and Ellman's assay. RtPAL is a homotrimer containing 30 lysine residues per monomer, some of which are buried in the protein core, which serve as the primary amine sites for conjugation to SATA. The free primary amine molar ratios as determined by the TNBSA assay were 14.8 (RtPAL), 11.3 (RtPAL-SATA), and 11.1 (RtPAL-BPM6), indicating that an average of 3.5 lysine molecules per RtPAL monomer were no longer free after the SATA conjugation step. The free sulfhydryl molar ratios, indirect measures of SATA conjugation through the presence of deprotected SATA sulfhydryl groups, were 0.2 (RtPAL), 3.4 (deacetylated RtPAL-SATA), and 0.6 (RtPAL-BPM6), indicating that RtPAL had been conjugated to an average of 3.2 SATA molecules per monomer. Additionally, this result showed that an average of 2.8 SATA ligands per RtPAL monomer had been successfully deacetylated and subsequently bound after the addition of BPM6-SMCC, resulting in loss of previously detected free sulfhydryl groups.

Conjugate homogeneity at each step of the RtPAL-BPM6 production process was inferred from IEF electrophoresis (Fig. 2, panel a), which showed an expected distribution of isoelectric point (pI) values for each product corresponding to the distribution of MSR values about the average for each product. The absence of overlap in the pI distribution of RtPAL (Fig. 2, lane 1, panel a) and RtPAL-SATA (Fig. 2, lane 2, panel a) indicated that all RtPAL molecules had reacted with at least one SATA molecule. The decreasing pI values seen as stepwise conjugation proceeds from RtPAL, to RtPAL-SATA, to RtPAL-BPM6 (Fig. 2, lane 3, panel a) are consistent with successive loss of positively charged primary amines (e.g., lysine residues

Fig. 1 RtPAL-BPM6 stepwise conjugation scheme

Fig. 2 Isoelectric focusing (IEF) gel electrophoresis (panel **a**) of intermediate products in RtPAL-BPM6 stepwise conjugation: RtPAL (lane 1), RtPAL-SATA (lane 2), RtPAL-BPM6 (lane 3), RtPAL-BPM6 treated with alkaline phosphatase (lane 4), and IEF Marker (lane 5).

NativePAGE Novex® 3-12 % Bis-Tris Gel (panel **b**): molecular weight marker (lane 1), RtPAL (lane 2), RtPAL-SATA (lane 3), RtPAL-BPM6 (lane 4), and RtPAL-BPM6 treated with alkaline phosphatase (lane 5)

on RtPAL) and addition of negatively charged phosphate groups (e.g., mannose 6-phosphate groups on BPM6). Treatment of RtPAL-BPM6 with alkaline phosphatase is expected to dephosphorylate BPM6 at the terminal mannose 6-phosphate groups and produce a shift to a higher pI (Fig. 2, lane 4, panel a). Taken together, these data are consistent with successful stepwise conjugation of RtPAL to form RtPAL-BPM6. Under native conditions, gel electrophoresis (Fig. 2, panel b) showed monomeric products with the expected molecular weight ranges.

We tested the RtPAL-BPM6 conjugate for stability *in vitro* using IEF and gel electrophoresis following storage at 4°C for up to 2 months and multiple freeze-thaw cycles and found the conjugation to be preserved (data not shown). However, all enzymatic activity of RtPAL was lost during the first conjugation step to form RtPAL-SATA, and the decrease in activity appeared to be a direct function of the SATA:RtPAL molar ratio used in the reaction (Fig. 3). While modification of RtPAL with other bulkier groups has been shown to induce a limited loss of activity, the small

Fig. 3 RtPAL enzymatic specific activity (percentage of unconjugated RtPAL activity) measured as a function of the amount of SATA added (SATA:RtPAL molar ratio) in the first step of the RtPAL

conjugation scheme. The SATA:RtPAL molar ratio used in the conjugation reaction described in text was 15:1 (arrow)

Fig. 4 Mannose 6-phosphate receptor (MPR)-coated ELISA plate wells incubated with RtPAL-BPM6 (solid circles), RtPAL (open circles), or RtPAL-SATA (open triangles), showing saturable binding of RtPAL-BPM6 with a calculated K_d of 0.4 nM

size of the SATA molecule may facilitate its penetration and conjugation at sites within the core of the tetramer where it can induce changes in enzyme folding, substrate binding, or substrate affinity (Wang et al. 2005).

Conjugated RtPAL-BPM6 exhibited saturable binding to the mannose 6-phosphate receptor. The K_d value, defined as the sample protein concentration resulting in 50% of maximal binding, was calculated to be 0.4 nM (Fig. 4). Neither unconjugated RtPAL nor RtPAL-SATA bound to MPR-coated wells, demonstrating that the terminal mannose 6-phosphate residues on BPM6 confer MPR binding specificity.

We observed MPR-mediated cellular uptake of the recombinant conjugated enzyme when human fibroblast cells were incubated with fluorescently labeled RtPAL-BPM6. There was noticeably increased intracellular fluorescence (Fig. 5, panel a) as compared with negligible fluorescence when the cells were incubated with unconjugated labeled RtPAL (Fig. 5, panel b). Intracellular fluorescence decreased markedly when fibroblast cells were incubated with RtPAL-BPM6 in the presence of free

mannose 6-phosphate (Fig. 5, panel c), consistent with inhibition of RtPAL-BPM6 uptake via the MPR owing to excess M6P. By contrast, there was no apparent inhibition of MPR-mediated uptake when fibroblast cells were incubated with RtPAL-BPM6 in the presence of free glucose 6-phosphate (Fig. 5, panel d).

To test our hypothesis that high-affinity intracellular uptake via the MPR permits immune tolerance induction to RtPAL, we administered low-dose RtPAL or RtPAL-BPM6 to normal beagles concurrently treated with an immunosuppressive tolerance induction regimen. In the first experiment, six dogs were assigned to two groups, a control group (“Group 1,” $n = 3$) and a test group (“Group 2,” $n = 3$). The test group (Group 2) received the previously published tolerance induction regimen consisting of immunosuppression until week 7 and tolerizing infusions of RtPAL-BPM6 weekly for 12 weeks, while the control group (Group 1) received identical RtPAL-BPM6 infusions without immunosuppression (Kakkis et al. 2004). After the 12-week tolerance induction period, both groups of dogs were challenged with increasing doses of the unconjugated

Fig. 5 Fibroblast cells incubated with 2nM FITC-labeled RtPAL-BPM6 (green, panel **a**) or un conjugated FITC-labeled RtPAL (green, panel **b**). Fibroblast cells were also incubated with FITC-labeled RtPAL-BPM6 in the presence of free mannose 6-phosphate (M6P,

panel **c**) or free glucose 6-phosphate (G6P, panel **d**). Fibroblast cell nuclei are counterstained with DAPI (blue), and background intracellular autofluorescence is also visible (red)

Fig. 6 Schematic diagram of tolerance induction (weeks 1–12) and antigen challenge (weeks 13–21) regimen. Cyclosporin A (CsA) and Azathioprine (Aza) dosing are indicated by the full height gray bars marked weekly. After 18 days of immunosuppression, test group dogs began receiving tolerizing low dose weekly infusions of RtPAL-BPM6 (black bars, beginning at week 1). After 2 weeks of combined full dose immunosuppression and low dose tolerizing enzyme

infusion, immunosuppressive dosing was decreased to half the full dose beginning at week 3, then to one fourth the full dose beginning at week 5, and then discontinued at week 7. At week 12, tolerizing low dose weekly RtPAL-BPM6 infusions were discontinued, then at week 13 antigen challenge with un conjugated RtPAL (striped bars) was started and doubled every week to the goal dose at week 16, then continued at that dose until week 21

antigen RtPAL, and anti-RtPAL IgG antibody levels were measured (Fig. 6). Tolerized dogs were predicted to have minimal or no change from anti-RtPAL baseline levels while non-tolerant dogs demonstrated seroconversion with enzyme-specific antibody production. Seroconversion was defined as a specific anti-RtPAL IgG antibody titer of $> 1:50$.

Control dogs all developed a rising anti-RtPAL IgG titer soon after starting the low-dose RtPAL-BPM6 infusions in the absence of immunosuppressive treatment (Fig. 7), with an average time to seroconversion of 4.7 weeks (Table 1).

The titers reached a plateau by week 7 and remained roughly constant for the rest of the observation period. There was no sustained significant change in titer when the control group was challenged with increasing weekly doses of RtPAL beginning at week 13, and these dogs had no evidence of a systemic inflammatory response, hypersensitivity reaction, or anaphylaxis. In comparison, test dogs in Group 2 had a relative delay in seroconversion, developing rising anti-RtPAL IgG titers at an average of 7.3 weeks (Table 1) when the immunosuppressive portion of the tolerance regimen ended (Fig. 7). The difference in average

Fig. 7 Anti-RtPAL IgG antibody titers (OD units/ μ L) measured by ELISA for control (“Group 1,” dotted lines) and test (“Group 2,” solid lines) dogs during the tolerance induction phase (weeks 1–12) and the antigen challenge phase (weeks 13–21). Test group dogs received the last dose of immunosuppression at week 6, and all dogs began

receiving unconjugated RtPAL at week 13. Seroconversion cutoff titer of 1:50 is marked by the thin dashed line. One dog in the test group (*) showed a delay in developing a rising antibody titer after the end of immunosuppression

Table 1 Average time to seroconversion, defined as the time at which an anti-RtPAL IgG antibody titer above 1:50 was detected in the serum of dogs treated with tolerizing infusions of RtPAL-BPM6 or RtPAL while receiving immunosuppressive therapy lasting 7 or 9 weeks (rows 2–4). The first row shows the control group, which was treated with tolerizing enzyme infusions and no immunosuppression

	Study group	Tolerizing infusion (0.056 mg/kg/week)	Weeks of tolerizing infusions	Duration of immunosuppression (weeks)	Weeks of full dose CsA + Aza	Time to seroconvert (average weeks $\pm$ SD)
IMMUNE TOLERANCE REGIMEN– PUBLISHED DURATION	Group 1 ($n = 3$)	RtPAL-BPM6	12	N/A	N/A	4.7 ± 0.6
	Group 2 ($n = 3$)	RtPAL-BPM6	12	7	2	7.3 ± 0.5 ($p = 0.89$)
IMMUNE TOLERANCE REGIMEN– LONGER DURATION	Group 3 ($n = 3$)	RtPAL-BPM6	12	9	4	7.3 ± 5.5 ($p = 1.0$)
	Group 4 ($n = 3$)	RtPAL	12	9	4	8.7 ± 7.1 ($p = 0.98$)

p-values correspond to comparison of average times to seroconvert between test groups (rows 2–4) and control group (row 1); all p-values for comparison of average times to seroconvert between test groups were nonsignificant

time to seroconversion between the test and control groups was not statistically significant (Table 1). One dog in Group 2 (*, Fig. 7) showed a marked delay in developing a rising antibody titer and maintained a low seropositive anti-RtPAL IgG titer even after immunosuppression was discontinued at week 7. When challenged with increasing doses of unconjugated RtPAL, this test dog began producing anti-RtPAL IgG antibodies after week 13, although at a slower rate than other animals.

To improve the success of tolerance induction, we tested the effect of extending the initial immunosuppressive period. A second test group (“Group 3,” $n = 3$) received the same tolerizing infusions with RtPAL-BPM6 and an additional 2 weeks of full dose CsA and Aza (weeks 1–4, immunosuppression discontinued at week 9), and a third test group (“Group 4,” $n = 3$) received tolerizing infusions with RtPAL-SATA as well as the additional 2 weeks of full dose CsA and Aza (Table 1). Group 3 dogs also developed

anti-RtPAL IgG antibodies during the tolerance induction period with an average time to seroconversion of 7.3 weeks that was not significantly different from the control group. Group 4 was added as a necessary control, to determine whether RtPAL-SATA without conjugation to BPM6 could induce immune tolerance. Group 4 dogs developed anti-RtPAL IgG antibodies during the tolerance induction period as expected, with an average time to seroconversion of 8.7 weeks that also did not represent a statistically significant difference from Group 1.

Discussion

Our results show that BPM6-conjugated RtPAL contains approximately 3 BPM6 molecules per RtPAL monomer, is stable for injection after prolonged storage, and is taken up in vitro by fibroblast cells by an MPR-mediated

mechanism, but that animals receiving tolerizing infusions of RtPAL-BPM6 according to a previously published tolerance induction regimen do not develop immune tolerance to RtPAL. In addition, extending the duration of immunosuppressive treatment during the tolerance induction period did not improve the success of tolerance induction or significantly delay the average time to seroconversion. Among all groups of animals studied, the administration of immunosuppressives to the test groups during the tolerance induction period was the only clinically relevant distinguishing factor. However, the longer average time to seroconversion in each test group was not a statistically significant difference in comparison with the control group.

Multiple methods have been studied to reduce the immunogenicity of PAL while maintaining its enzymatic activity. These include structural modification with polyethylene glycol (PEG) and engineering resistance to chymotrypsin for oral delivery (Wang et al. 2005; Sarkisian et al. 2008; Kang et al. 2010). These methods rely on evasion or bypassing of the immune response to a foreign and immunogenic therapeutic protein. Inducing immune tolerance to PAL by increasing cellular uptake with mannose 6-phosphorylation has not been previously studied as a method to improve therapeutic efficacy.

The cation-independent MPR is an integral membrane protein that also binds insulin-like growth factor II and functions in the sorting of newly synthesized lysosomal enzymes as well as endocytosis of extracellular lysosomal enzymes and proteins bearing M6P (Kornfeld 1992; Gary-Bobo et al. 2007). This receptor has been found on a variety of cells including macrophages and was shown to mediate uptake of M6P-conjugated bovine serum albumin, suggesting that conjugating immunostimulant molecules with M6P could lead to uptake by and activation of macrophages (Roche et al. 1985). However, it remains unclear to what extent the MPR is expressed on antigen-presenting dendritic cells in the periphery, which are thought to be required for T-cell-dependent B cell activation by taking up, processing, and presenting antigen to T helper cells in the context of major histocompatibility complex II (MHC II) (Sauerborn et al. 2010).

While it is not known whether M6P modification increases uptake of the conjugated protein by antigen-presenting cells, it is likely that this modification alters the distribution of the conjugated protein among the target tissue and immune system compartments. Immunogenicity of biopharmaceuticals depends on structural parameters such as sequence variation and glycosylation as well as pharmacokinetic parameters such as route of administration, dosage, and duration of exposure (Schellekens 2002). Thus the effectively altered exposure kinetics conferred by

M6P conjugation may be important in forming immune tolerance although it is clear that this is not sufficient.

It is interesting to note that in two animals from the first test group (Group 2, Table 1) receiving the 7-week course of immunosuppression, the rise in antigen-specific IgG titers closely followed discontinuation of immunosuppression. This timeline could suggest that antigen presentation and T cell activation may not have been blocked by the immunosuppression, but rather B cell proliferation and antibody production were reversibly suppressed. In the single animal from Group 2 that showed a relative delay in mounting an antigen-specific IgG response until challenged with unconjugated RtPAL, tolerance to RtPAL-BPM6 may have been achieved transiently but was not sustained upon exposure to the more immunogenic RtPAL. Since high-affinity cellular uptake mediated by BPM6 conjugation did not appear to reduce exposure to the immune system in the first two animals, it is possible that this RtPAL modification altered the structure of the conjugated protein in a manner that initially reduced its immunogenicity to the third dog in Group 2. Thus when this dog was exposed to the unmodified RtPAL antigen starting at week 13, it developed a rising anti-RtPAL IgG titer over an immunologically appropriate timescale. This sort of structural alteration is consistent with the observed loss of RtPAL activity upon conjugation with BPM6.

Our research shows that PAL can be successfully expressed in a recombinant form and conjugated to the M6P carrier BPM6, and that this modification enables high affinity binding to the MPR and specific MPR-mediated uptake into human fibroblast cells. Conjugating RtPAL with BPM6 was not sufficient to allow induction of immune tolerance in animals receiving immunosuppression and tolerizing infusions of RtPAL-BPM6 according to the regimen described in Kakkis et al. 2004. PAL is not an endogenous canine enzyme and so there is no naturally occurring central immune tolerance, as would be the case for a self-antigen. Further study is needed to determine a method to induce immune tolerance to RtPAL.

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Synopsis

We produced mannose 6-phosphate-conjugated recombinant phenylalanine ammonia-lyase (PAL), administered it to normal dogs using a previously published tolerizing regimen, and found that, despite demonstrated M6P receptor binding and cellular uptake, all test animals failed

to develop immune tolerance to PAL and produced a marked antibody response.

Competing Interest Statement

The coauthors TL, BZ, PT, SC, JF, and JFL are employees of BioMarin Pharmaceutical. EDK is a former employee of BioMarin and is now Chief Executive Officer of Ultragenyx Corporation.

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