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Neutralizing antibodies to therapeutic enzymes: considerations for testing, prevention and treatment

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Abstract

Lysosomal storage diseases are characterized by deficiencies in lysosomal enzymes, allowing accumulation of target substrate in cells and eventually causing cell death. Enzyme replacement therapy is the principal treatment for most of these diseases. However, these therapies are often complicated by immune responses to the enzymes, blocking efficacy and causing severe adverse outcomes by neutralizing product activity. It is thus crucial to understand the relationships between genetic mutations, endogenous residual enzyme proteins (cross-reactive immunologic material), development of neutralizing antibodies and their impact on clinical outcomes of lysosomal storage diseases. For patients in whom neutralizing antibodies may cause severe adverse clinical outcomes, it is paramount to develop tolerance inducing protocols to preclude, where predictable, or treat such life-threatening responses.

Lysosomal storage diseases (LSDs) are characterized by mutations in the genetic loci encoding enzymes that cleave carbohydrates or muco-polysaccharides from lysosomal substrate material, leading to their accumulation in the lysosome and ultimately causing lysosomal rupture and cell death. Enzyme replacement therapies (ERTs) are currently the treatment of choice for most LSDs, including Pompe's disease, Gaucher's disease and Fabry's disease. However, as in the case of coagulation factor replacement therapy for patients with hemophilia A and B, ERTs are often complicated by immune responses to the therapeutic enzymes (Tables 1 and 2) that may cause severe adverse clinical effects by neutralizing product activity, altering biodistribution or inducing hypersensitivity responses. The capacity for neutralizing antibodies to abrogate therapeutic effects is clear from multiple examples, none more poignant than that of patients with Pompe's disease, whose motor milestone achievements are reversed upon development of neutralizing antibodies, eventually leading to patient death[1,2]. However, in many other enzyme deficiency disorders with clinical endpoints that take years to manifest, the effects of neutralizing antibodies are not yet clear. For all factor-deficient patients undergoing replacement therapies, it is crucial

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to be able to predict susceptibility to the development of neutralizing antibodies that block efficacy and to develop tolerance inducing protocols to preclude such responses.

For LSDs in which neutralizing antibodies abrogate therapeutic efficacy and lead to adverse patient outcomes, tolerance inducing therapies have been explored in experimental animal models and are being implemented for patients who have developed or have a high risk of developing life-threatening neutralizing antibody responses to replacement enzymes. In addition to ensuring maximal efficacy of ERT by controlling immune responses, there are four major challenges: enhancing delivery of ERTs to the central nervous system and other tissues (such as heart valves and joints) that are only poorly penetrated by enzyme; improving the efficiency of ERT by engineering therapeutic enzymes to enhance cellular and lysosomal entry; developing curative therapies by cellular or gene transfer technologies; and developing effective therapies for patients in advanced stages of LSDs, for which ERT has not been efficacious at reversing the long-term manifestations. This last challenge will certainly involve regenerative therapeutics as well as ERT.

Here we summarize current knowledge regarding the prevalence of antibody responses to ERTs used to treat LSDs, the potential mechanisms by which antibodies can neutralize efficacy of ERTs and the assays best suited to detect and monitor both binding and neutralizing antibodies to therapeutic enzymes. Finally, we discuss the need for immune tolerance inducing therapies to prevent or reverse the neutralizing antibody response in the context of a risk assessment.

Neutralizing antibody responses to ERT

For many factor deficiency diseases, there is now a clearer understanding of the relationships between genetic mutations, protein levels and immune responses. For example, for factor VIII and IX deficiencies, the greater the extent of the genetic mutation, the lower the detectable levels of factors VIII and IX and the higher the levels of both binding and neutralizing antibodies to factor replacement therapies[3]. In hemophilia A patients with complete or near-complete gene deletions that remove multiple domains of the factor VIII protein, neutralizing antibodies (known as ‘inhibitors’) to factor VIII develop at a very high rate (88%); smaller deletions that remove a single domain result in inhibitor rates ranging from 25% to 41%, and minor deletions result in inhibitor rates ranging from 16% to 21% (ref. 4). In factor VIII missense mutations, the inhibitor rate is much lower, typically on the order of ~5%. This low inhibitor rate is attributable to the fact that most of these patients make some factor VIII protein that, though nonfunctional, is presumably known to the immune system as self-tolerance is established. The recurring intron 22 inversion that is seen in 40–50% of patients with severe hemophilia A is associated with an inhibitor rate of only ~20%, which is puzzling in view of the fact that factor VIII protein was not detected by an immunological assay (that is, it was negative for cross- reactive immunologic material, or CRIM) and thus a high rate of immune responsiveness was expected[4,5]. Notably, factor VIII mRNA is translated in lymphoid tissue (such as spleen and peripheral blood lymphocytes) in humans and animals with the inversion mutation, so it is possible that factor VIII peptides are translated at a level sufficient for presentation within the major histocompatibility complex, thereby mediating tolerance through a cellular mechanism[6]. For deletion mutations in which there is no transcript, this mechanism cannot occur. For hemophilia B, the overall rate of inhibitor antibodies to factor IX is much lower, on the order of 5% or less, but there is a significant rate of anaphylaxis (26%) with continuing exposure to factor IX in patients with complete factor IX gene deletions[7,8] (Table 2). In this regard, the immune response to factor IX is different from that to factor VIII because anaphylaxis is quite rare in inhibitor-positive hemophilia A patients despite continued exposure to factor VIII. Of the patients with inhibitors to factor IX, half have a gene deletion and nearly all of the rest have a major

gene rearrangement[7]. Patients with factor IX missense mutations who are CRIM-positive have little risk for inhibitor formation.

The correlation between genetic mutations, CRIM status (assessed most sensitively with polyclonal antibodies to either the native protein or a mixture of the native protein and its fragments) and development of neutralizing antibodies observed for coagulation-factor deficiencies has also been observed in ERT for LSDs. In Pompe's disease, for example, deletion and nonsense mutations are associated with an absence of detectable enzyme mRNA under standard reverse transcriptase–PCR conditions, undetectable levels of enzyme protein, generation of high-titer neutralizing antibodies to therapeutic enzyme and adverse clinical outcomes[1,9,10]. In Fabry's disease, 10 of 12 patients with α-galactosidase A activity below 0.5 nmol per mg protein per h generated antibody, whereas only 1 of 6 patients with enzyme activity above 1.1 nmol per mg protein per h did so[11]. In patients with low enzyme activity, nonsense or deletion mutations predominated, whereas in those with higher enzyme activity levels, missense mutations were more common or the patients were heterozygous females. Antibodies from all of these patients inhibited enzyme activity *in vitro*, suggesting that at least a component of the antibody response was directed to determinants in or near the active site. Whether the crucial clinical outcomes of renal failure, myocardial infarction, stroke, neuropathic pain and early mortality are affected by such antibodies is not known and requires additional study, particularly in patients who do not show a significant degree of end-organ damage at the inception of the study. Even so, recent studies have shown that after 12 and 24 months of treatment, urinary globotriaosylceramide levels (the substrate for the enzyme) do not decline in antibody-positive patients but decline in antibody-negative patients. In addition, treatment failure, as indicated by increasing left-ventricular mass or decreasing renal function (glomerular filtration rate), occurred more frequently in antibody-positive patients (six of ten) than in seronegative patients (two of six; ref. 12). The relationship between genotype, protein level and immune response is also clear in the case of haptoglobin deficiency, in which six of ten patients homozygous for a deleted allele of the haptoglobin gene had haptoglobin levels below the detection limits of the assay (3 mg dl^{-1}) and generated IgG and IgE antibodies that produced severe anaphylactic or anaphylactoid responses during blood-product transfusions[13]. This scenario is similar for IgA deficiency[14].

Given the association of severe genetic mutation with absence of protein, development of neutralizing antibodies to the replacement factor, and adverse clinical consequences, it is essential that knowledge of these relationships be obtained early in clinical trials so that the effects of immune responses on disease outcome can be prospectively evaluated. The discovery that CRIM-negative patients generate antibody responses that are unremitting and abrogate the efficacy of a life-saving therapeutic requires the development of clinical protocols to induce tolerance, preferably prophylactically or simultaneously with the onset of treatment, but also in the setting of an ongoing immune response.

In contrast to severe genetic mutations that produce a CRIM-negative status, missense mutations result in production of coagulation factors or enzyme proteins that are functionally defective because of defects in the active site or other crucial parts of the molecule that affect intracellular processing, secretion or stability of the protein[3,15–17]. Missense mutations may cause severe infantile-onset disease but are rarely associated with production of neutralizing antibodies during ERT. This underscores the principle that immunologic tolerance to an endogenous enzyme is associated more strongly with the level of protein, however mutated or nonfunctional, than with an enzyme's bioactivity *per se*. In Gaucher's disease, for example, only 13% of patients developed antibody responses to glucocerebrosidase, and only a small percentage of these developed significant neutralizing activity[18]. This low rate of responsiveness reflects the fact that at least one of the three CRIM forms of the normal enzyme found in healthy individuals was easily detected in most

type 1 Gaucher's patients, though at decreased levels and despite the presence of mutations that markedly reduced enzyme activity[19]. These findings indicate that in analyzing the relationships between genotype, phenotype and antibody response, it is crucial to assess not only the residual enzymatic activity, which may be very low, but also the level of the protein itself using an immune-based assay to detect CRIM.

An additional notable finding in treatment of coagulation-factor disorders and enzyme deficiency states, particularly in CRIM-positive patients, is the evolution of tolerance over time with continued ERT in patients who initially developed antibodies. Tolerance supervened within a 1- to 2-year period in almost all Gaucher's patients maintained on standard therapy[18], as well as in subpopulations of patients with Fabry's disease and mucopolysaccharidosis I[20,21]. In hemophilia A patients, successful immune tolerance induction may occur with protocols where factor VIII is administered alone at high doses (the Bonn protocol)[22] or with cyclophosphamide immunosuppression, intravenous immunoglobulin immunomodulation and possibly extracorporeal immunoabsorption procedures added to continued factor infusions (the Malmo protocol)[23].

The development of tolerance over time with continued factor therapy raises the intriguing possibility that tolerance may spread from conserved B- and T-cell epitopes, present in the mutated protein, to new immunogenic epitopes in the catalytic or receptor-binding regions of the normal protein. This type of tolerogenic epitope spreading could be mediated by regulatory T cells or other mechanisms involving an 'infectious' type of tolerance[24,25]. Tolerance induction with continued therapy is not unique to protein deficiency states, as it has also been observed in patients undergoing chronic treatment with other therapeutic proteins in which there is no known endogenous deficiency (e.g., in chronic treatment with type I interferons)[26].

Notably, tolerance-inducing protocols that are largely successful in patients with CRIM-positive hemophilia A[27] or Gaucher's[18] are not successful in inducing tolerance in CRIM-negative patients with severe mutations; in severe factor IX deficiency and severe Pompe's disease, such protocols have been shown to induce nephrotic syndrome as a result of renal deposition of antigen–antibody complexes[10,28]. These cases make it clear that the bar for tolerance induction is considerably higher in patients who are CRIM negative.

Tolerance to self proteins depends on many factors, chief among which are protein abundance[29–31], the maturational status of the cell on antigen encounter, the affinity of the protein ligand for the receptor, the degree and type of receptor engagement by the self antigen (avidity) and, finally, the presence of concurrent signals that facilitate or diminish the induction of immunity[32–35]. Thus, tolerance to self proteins present at low levels is not robust[32,33,36,37], with potentially self-reactive antibody-secreting B cells deleted only at high levels of protein exposure; at lower levels, B cells may be reversibly nonresponsive, and at the extreme low end of abundance, they may be fully reactive[29,30,34,38]. In contrast, T cells are tolerized at much lower levels of protein[32,39,40], which may be related in part to promiscuous expression of what were once thought to be tissue-restricted antigens in the thymus[41] or to the unique ability of the thymus to present soluble antigens in a highly tolerogenic fashion[42]. For CRIM-negative patients, therefore, the immune response is as one to a foreign protein and does not require breaking of tolerance, whereas in CRIM-positive patients, in whom there is a varying degree of immune tolerance, tolerance must be broken to generate antibody. The mechanism of tolerance is thus crucial in determining the ease with which it can be broken. Specifically, for a robust state of tolerance based on deletion of antigen-reactive T and B cells, induction of an antibody response may not be possible, whereas tolerance based on non-responsiveness of antigen-specific T or B cells that are not deleted may be overcome by various means of immune stimulation[43,44].

The neutralizing immune response that follows administration of the thrombopoietin (TPO) congener pegylated human megakaryocyte growth and development factor (PEG-MGDF) to healthy volunteers underscores the principle that low protein expression can impact B-cell tolerance[30,31,33]. In clinical trials, as few as two doses of PEG-MGDF were sufficient to generate potent neutralizing immune responses that induced severe thrombocytopenia because antibodies to the therapeutic protein cross-reacted to neutralize endogenous TPO, the sole factor responsible for stimulation of platelet production[45]. In some cases, the thrombocytopenia was of longstanding duration and required active tolerance-inducing regimens for remission[46]. In retrospect, the fact that TPO is present only at picomolar levels in blood should have alerted investigators to the possibility that self-tolerance could be broken. Although truncation of MGDF may also have contributed to the cross-reactive immune response that neutralized endogenous TPO, this was probably not a crucial factor, as full-length, unaltered TPO itself was found to be immunogenic in humans and animals, even using species-specific TPO[47,48].

The immunogenicity of human growth hormone (hGH) provides a further clear example of these concepts. The earliest therapeutic source of HGH was cadaver pituitaries; as a result of the extraction methods, the product was heavily contaminated with hGH aggregates to which a large percentage of patients generated antibody. However, such antibody responses did not neutralize hGH activity and were thought to be directed to epitopes associated only within the aggregates[49] and not to the native hormone. In contrast, in a small number of patients, antibody to HGH was neutralizing and abrogated efficacy[50]. The difference in responses of these patient populations was attributed to the etiology of the growth-hormone deficiency. In patients rendered hGH deficient by removal of the pituitary because of trauma or malignancy, there was robust tolerance to unaggregated endogenous hGH, as a result of extensive previous exposure, but not to epitopes unique to aggregated species. In contrast, patients with mutations in the hGH-encoding gene that cause an isolated deficiency of hGH (that is, CRIM-negative patients) were fundamentally nontolerant to naturally conformed hGH and thus raised neutralizing antibodies that targeted the native protein.

Assays for detecting neutralizing antibodies

The overall strategy for detecting an antibody response to ERT is similar to that for all protein therapeutics[51,52]. A very sensitive screening assay for binding antibodies is used first, followed by an assay to confirm that all positive responses are antibody mediated and a competition assay using antigen to confirm that all positive responses are specific. Positive samples are then tested for their neutralizing activity in both enzyme activity and cellular-uptake assays and may be further assessed for titer and isotype. In early phases of clinical trials, it is crucial to use a well-qualified assay that should be validated before phase 3 studies[51,52]. It is generally not possible to compare the immunogenicity rates of two products in the same class unless the assay is being done by a third party using an assay that has been validated as equally sensitive and specific for detecting immune responses to both products. Different assays performed by different manufacturers of the same class of product differ with respect to key performance characteristics, such as sensitivity.

Evaluation of immune responses to a protein therapeutic necessitates development and implementation of several immunoassays of proven sensitivity, specificity and robustness. Given the complex biology of ERT, assessment of antibody neutralization in particular requires development of at least two types of neutralizing antibody assay: one that assesses neutralization of enzyme uptake by cells and one that measures the ability to inhibit catalytic activity. These distinct functions are mediated by different structures in the enzyme. Targeting of most lysosomal enzymes to cells, their uptake into cells and their entry into the lysosomes within cells is mediated mainly by binding of *bis*-man-nose-6-phosphate (M6P)

residues on the glycosylated enzyme to specific high-affinity cell surface and lysosomal M6P receptors (M6PR; Fig. 1)[53,54]. Exertion of enzymatic activity depends critically on residues involved in substrate binding and catalysis, and antibodies that neutralize the efficacy of therapeutic enzymes may, accordingly, have specificity for these various functional domains of the enzyme. Furthermore, as some therapeutic enzymes require proteolytic processing within the lysosome to be activated, it is theoretically possible for neutralizing antibodies to bind parts of the enzyme that are targets of lysosomal proteases, thereby blocking the enzyme's conversion to a more highly active form. Antibodies directed to other conserved elements of the enzyme may not strictly neutralize but when present in high titer may inhibit efficacy by diverting the enzyme into a more restricted cell population (for example, into cells bearing Fc receptor, or FcR) or by altering pharmacokinetics[55]. Assays for antibodies that neutralize uptake or catalytic activity should be carried out side by side using the same patient samples, and the results should be analyzed in conjunction with clinical outcomes.

Uptake neutralization assays

Important elements of uptake neutralization assays (Fig. 2a) include the following: selection of model cell lines that express the relevant uptake receptor(s) and do not express FcR; demonstration of assay specificity by competitive inhibition with unlabeled product but not with irrelevant proteins; assessment of uptake inhibition by measurement of the ratio of intracellular enzyme signal (using labeled enzyme) in the presence versus absence of patient serum; and demonstration that uptake loss is mediated specifically by antibody (for example, depletion of immunoglobulin in patient sera restores enzyme uptake). A positive-control antibody for uptake neutralization should be used. Other assay parameters such as cut point (established with a small number of normal sera initially, then with 30–50 pretreatment patient sera) and sensitivity should be rigorously defined per previous recommendations[51].

As has been mentioned, enzyme bound to antibody may be internalized into FcR-bearing cells through the binding of the complex to FcR rather than binding of M6P residues to M6PR[56]. Internalization of enzyme into FcR-expressing cells may interfere with therapeutic efficacy in two ways: by diverting enzyme away from crucial target tissues such as muscle and endothelial cells and, potentially, by diverting enzyme to an endosome dedicated to antigen presentation rather than to the lysosome. Thus, for specific evaluation of uptake inhibition based on antibody to the M6P domain of the enzyme, it is crucial to use a cell line that does not express FcR.

Enzymatic activity–based neutralization assays

Neutralization of catalytic activity (Fig. 2b) should be assessed by mixing serially diluted patient sera into a validated potency assay that measures the activity of the enzyme toward the specific substrate. This assay should be validated as recommended[51,52]; use the natural (as opposed to an artificial) substrate, unless the assay can be fully validated using the artificial substrate; and be conducted at substrate concentrations that fall on the linear part of the activity curve.

Neutralizing antibodies specific for the receptor binding and uptake domain(s) or the catalytic domain will interfere with efficacy of therapeutic enzymes if one of the following conditions apply: the antibody is present in sufficient titer to prevent uptake in relevant cell populations, the antibody diverts enzyme away from the lysosomal compartment in which substrate is degraded and into antigen-presenting cell compartments, the antibody significantly alters pharmacokinetics or the antibody has a catalytic activity that inactivates the enzyme. Indeed, almost half of factor VIII inhibitory antibodies have been shown to proteolyze and so inactivate factor VIII[57]. As the sites of cleavage are heterogeneous,

catalysis is apparently not peculiar to a specific ‘consensus’ site of cleavage in the target protein.

In theory, enzyme activity may not be influenced if antibody-bound enzyme is directed to the relevant lysosomal compartment, as antibodies have been shown to be degraded within several hours of lysosomal localization[58]. However, the binding of antibody to enzyme may well alter enzyme conformation, rendering it more susceptible to lysosomal proteases and rapid degradation.

Risk assessment of tolerance induction

As with all clinical treatments, tolerance-inducing therapies should be subject to a risk assessment that weighs the risks and benefits. The main risks of tolerance-inducing therapies are infection and malignancy. In the case of CRIM-negative patients with Pompe’s disease who have uniformly poor clinical outcomes despite ERT, the increased risk of infection or malignancy of the tolerance-induction therapy can be justified compared with the fatal outcome accompanying the neutralizing antibody response. Moreover, in experimental models, tolerance induction seems to further improve the activity of ERT in reducing substrate in crucial tissues[59]. For other lysosomal storage diseases, such as Fabry’s disease, in which the endpoints of vascular occlusion and end-organ damage, such as renal insufficiency, may take many years to become fully manifest, the effect of neutralizing antibodies or high-titer binding antibodies are somewhat less understood and require further clinical and laboratory studies. In these diseases, the risk-benefit profile for tolerance inducing therapies is less clear. Nonetheless, prolonged and intensive immune suppression is the treatment of choice for autoimmune disease and prevention of transplant rejection, even in pediatric populations, and prophylactic tolerance induction for soluble protein therapeutics using a variety of treatment regimens, though intensive, is anticipated to be of shorter duration[60,61].

For ongoing neutralizing antibody responses in the CRIM-negative Pompe’s setting, in which nephrotic syndrome may be induced by continued administration of enzyme, tolerance inducing therapies have a favorable risk-benefit ratio; however, in this situation, the immune suppression may be even more intensive and extensive because antibody levels must be markedly reduced to prevent nephrotic syndrome and to facilitate tolerance induction[4,62]. When ERT fails as a result of neutralizing antibodies, and patient outcome is death or severe disability (such as respirator dependency), such a situation clearly warrants vigorous therapeutic efforts at eliminating immunity based on the best available experimental and clinical studies.

Prevention of immunity by tolerance induction

To identify appropriate cellular and molecular targets of tolerance inducing therapies, it is crucial to consider the cellular and molecular mechanisms that generate antibody. Given the preponderance of neutralizing antibodies of the IgG1 and IgG4 isotypes, the consensus in the field is that immunoglobulin responses to protein therapeutics—though rarely investigated—arise from classic T-helper-dependent B-cell mechanisms. This has been verified in limited experimental studies[63–66]. Thus, appropriate cellular targets for tolerance induction include antigen-specific T and B cells and antigen-presenting cells[67]. The antibody response may or may not require the participation of T-cell help after the initiation of a response[65], nor is it clear whether in some settings delayed-type hypersensitivity or cytotoxic T-lymphocyte responses are generated to cells expressing peptides of ERTs in the context of self-HLA. Such cellular responses could hasten the death of the target cells of enzymes.

In addition to tolerance strategies that target the human immune system, it is crucial to eliminate or minimize product quality attributes that contribute to activation of immune responses, such as protein aggregates and the presence of factors that trigger innate immunity through toll-like receptor agonists (such as microbial DNA and endotoxins), as well as factors arising from the container closure system that leach into the product[68]. These factors could contribute to the initiation and maintenance of the immune response in the absence of T-cell help.

Ideally, a tolerance-inducing protocol should produce highly specific and long-lasting tolerance. Targeting T-helper cells by administering a nondepleting CD4 monoclonal antibody induces durable tolerance to aggregated horse immunoglobulin, a potent immunogen, in a relevant nonhuman primate model[69]. Tolerance induced by nondepleting CD4 monoclonal antibody seems to be mediated by $CD4^+$ T-regulatory cells, at least in animal models. In α-glucosidase knockout mice, a short course of methotrexate, an immunosuppressive used widely in the setting of autoimmunity, induces tolerance[59,70]. Methotrexate is also efficacious in inducing tolerance in the setting of a mouse model of Fabry's disease (α-galactosidase knockout)[60]. Furthermore, treatment with cyclosporine A and azathioprine plus α-L-iduronidase induces tolerance in a canine model of mucopolysaccharidosis I[71], despite the known propensity of cyclosporine A to block tolerance induction in T cells[72,73]. However, it is possible that the long-term suppression of T-cell activation by cyclosporine A facilitates tolerance induction specifically in the B-cell compartment by continued high-dose antigen exposure in the absence of T-cell help[74,75]. Similarly, although cytotoxic T-lymphocyte antigen (CTLA)-4 immunoglobulin is known to block the CTLA-4 signaling crucial for tolerance[76–78], prolonged blockade of T-cell help to enzyme-specific B cells by CTLA-4 immunoglobulin may allow the emergence of tolerance in the B-cell compartment.

Less well developed at this time are therapeutics for induction of tolerance specifically in the B-cell compartment. Therapies to trigger inhibitory FcR expressed on B cells and antigen-presenting cells[79] and to target B-cell survival and activation factors (such as B-cell activating factor and B-lymphocyte stimulator) are under development. Depleting approaches using rituximab, a chimeric monoclonal antibody with human IgG1 constant domains that depletes mature B cells expressing the CD20 molecule, may be of particular use in the prophylaxis setting, potentially allowing introduction of enzyme at a stage when immature enzyme specific pre- and pro-B cells may be deleted or rendered nonresponsive. Combinations of rituximab and antibodies to B-cell activating factor continue to be of interest[67].

Tolerance induction in the setting of ongoing immunity

More difficult than inducing tolerance in a naive setting is the task of reversing an ongoing robust immune response. For example, in the α-galactosidase knockout mouse model of Fabry's disease, administration of methotrexate leads to sustained reduced levels of antibodies to ERT when administered at the onset of treatment but does not reduce the antibody levels once the immune response is firmly established[60]. The use of rituximab in the setting of an ongoing immune response is supported by studies showing therapeutic effectiveness in some patients with antibody-mediated life-threatening autoimmune diseases, such as antiphospholipid syndrome and thrombotic thrombocytopenic purpura[80–83], as well as other studies showing diminished responses to a neoantigen in patients with chronic renal failure[84]. These studies suggest that rituximab interrupts the pathways driving development of plasma cells and that not all plasma cells have an equally long life span[85]; however, it is not clear whether true tolerance is induced in these settings. Notably lacking from the therapeutic armamentarium are antibodies to target long-lived plasma cells, whose

elimination may be vital in reversing entrenched immune responses. Alemtuzumab, the humanized IgG1-type monoclonal antibody to CD52, depletes $CD38^+$ plasma cells, suggesting that it is a candidate for further evaluation in attempting tolerance induction in the setting of an ongoing immune response. Treatments to eliminate plasma cells, however, may pose a more severe risk of infection due to diminished serum immunoglobulin levels, loss of vaccine immunity and inability to respond to microbial challenges. Use of intravenous immunoglobulin, prophylactic antibiotics and revaccination after successful tolerance induction may all be indicated. A consortium of experts in clinical trials of immune tolerance, the Immune Tolerance Network (National Institute of Allergy and Infectious Diseases) may be of particular value in guiding the development and execution of clinical trials in this setting.

Tolerance inducing protocols elicit particular concerns for children, as exposure to infectious agents (such as Epstein-Barr virus and cytomegalovirus) in the setting of tolerance induction may abrogate long-term protective responses against the pathogen. Infections also have the capacity to sabotage tolerance induction regimens, which rely on antigen exposure in a noninflammatory environment. Thus, the efficiency of the tolerance inducing regimen is of great importance, and particular precautions should be taken to minimize exposure of patients to infectious diseases while on therapy. An additional specific precaution is to avoid delaying the initiation of ERT, as for some of these diseases (such as infantile-onset Pompe's disease), a delay in treatment could lead to dire consequences, including ventilator dependence and death. Thus, rapid identification of CRIM status and entry into an immune tolerance induction regimen is paramount in high-risk patient populations. Moreover, given the rarity of many of these LSDs, treatment of such patients may be optimized by care at centers with extensive clinical expertise.

Conclusions

When using ERT in the setting of LSDs, it is crucial to have sensitive and specific binding antibody assays as well as neutralization assays for both cellular enzyme uptake and catalytic activity. Serial sampling for product-specific antibodies from patients with defined mutations and CRIM status will provide a vital tool in individual patient management and in expanding our understanding of the relationships between genetic mutation, CRIM status, immune response and clinical outcome for each enzyme or factor deficiency disorder.

Moreover, in the setting of ERT for Pompe's disease, it is crucial to define the CRIM status of the patient before onset of ERT, as CRIM-negative patients are expected to develop neutralizing antibody responses that abrogate the efficacy of ERT and are thus candidates for tolerance-inducing therapies. In other LSDs in which antibody responses are generated, it is crucial to conduct clinical and experimental studies in relevant animal models to elucidate the effects of the antibody response on therapeutic efficacy. It is necessary to balance the potential detrimental effects of the antibody response on treatment outcome and the potential adverse effects of the tolerance inducing therapy.

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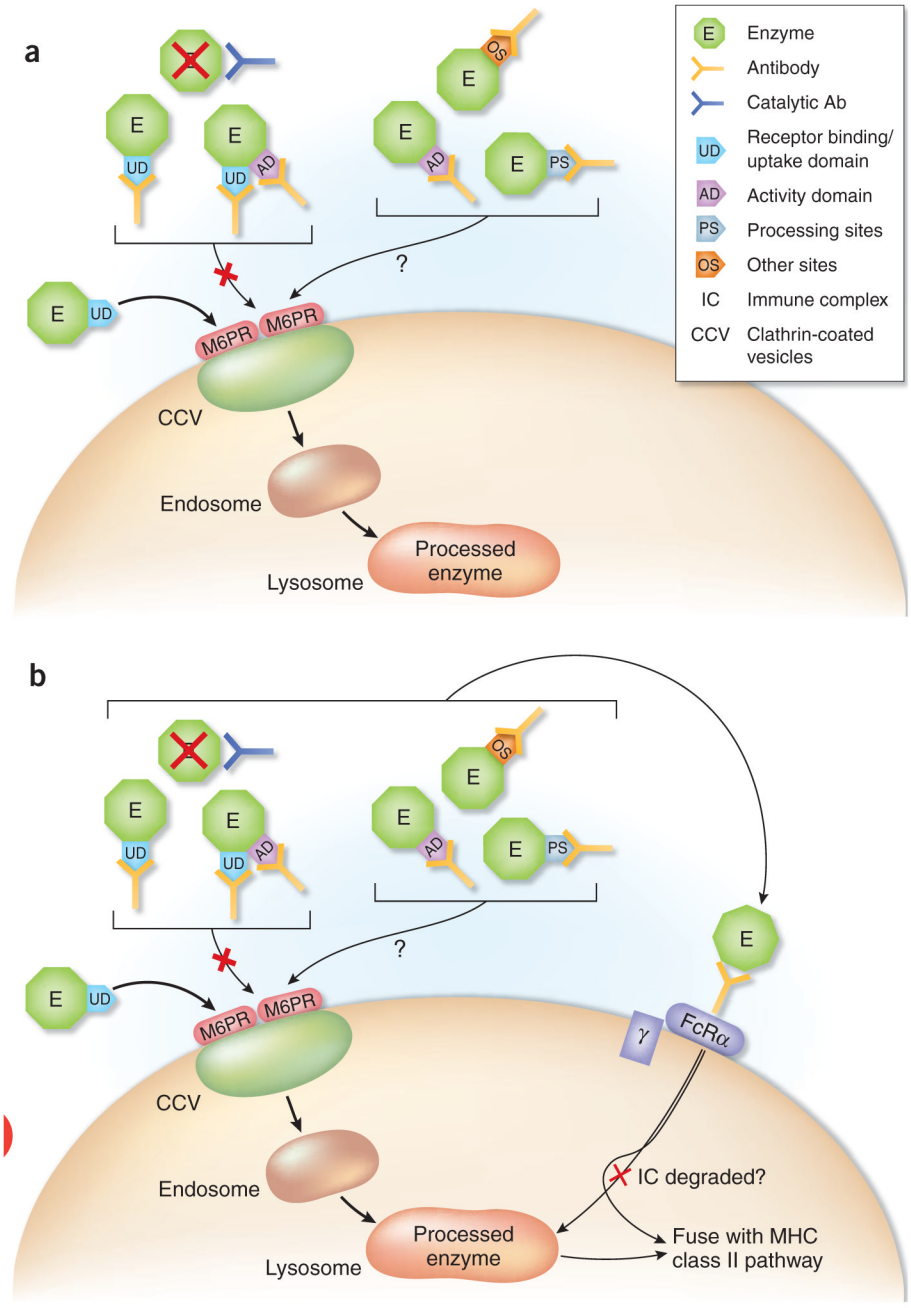

Figure 1.

Impact of neutralizing antibody on enzyme entry and activity in ERT. (**a**) There are several crucial steps by which therapeutic enzymes for LSDs mediate effectiveness. First, lysosomal enzyme (E) is taken up by target cells in an M6PR-dependent fashion, endocytosed by clathrin-coated vesicles (CCV) and fused with endosomes and then with lysosomes, where enzymatic activity is exerted on accumulated substrate. During this migration, the full-length enzyme may be processed into an intermediate form in the late endosomes or lysosomes, and then into the fully activated species in a proteinase-dependent manner[86]. There are several possible outcomes of the binding of lysosomal enzyme–specific antibodies (yellow 'Y'): blockade of enzyme uptake through M6PR by binding to the receptor binding or

uptake domain(s) (UD; domains containing exposed M6P); blockade of enzyme uptake by M6PR and suppression of enzymatic activity by binding to epitopes near the receptor binding domain and the enzymatic activity domain (AD; theoretical); blockade of both uptake and activity domains by separate antibodies specific for each site; degradation of the enzyme by catalytic antibody (red 'Y'); reduction of enzymatic activity by targeting the enzymatic domain; prevention of enzyme maturation by targeting the enzyme protease processing sites (PS); and targeting of other sites (OS) of the enzyme, resulting in conformational or trafficking changes. (**b**) Binding of antibodies to enzyme may redirect the enzyme to FcR-expressing cells, such as macrophages and B cells. Enzyme–antibody complexes internalized through FcRs may prevent proper translocation of functional enzymes to the lysosome. Binding of antibody to other domains of the enzyme may change pharmacokinetics or redirect the enzyme to FcR-expressing cells. IC, immune complex.

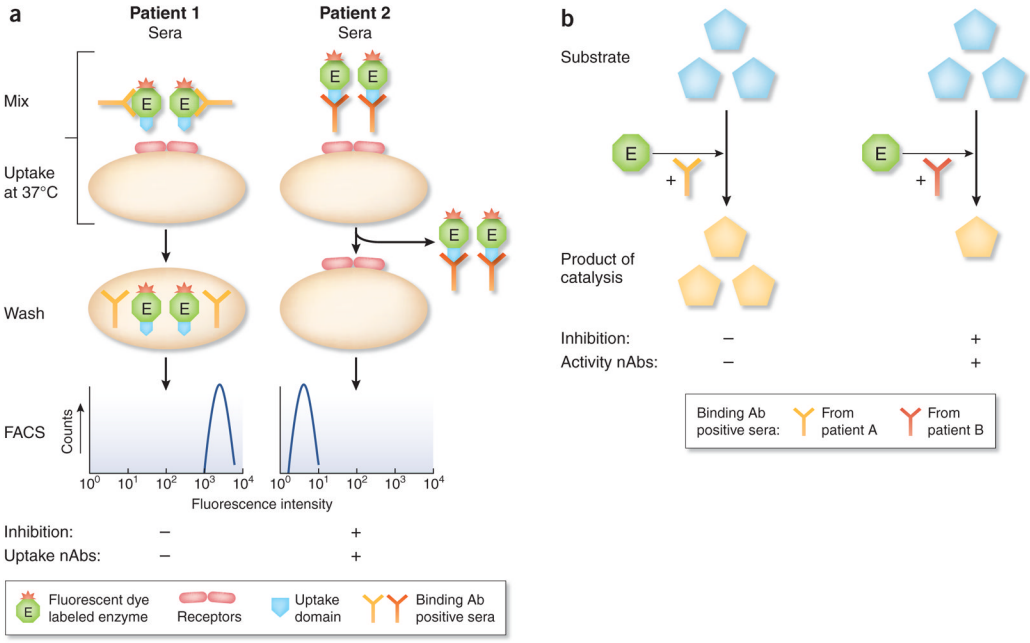

Figure 2.

Neutralization assays. (**a**) Uptake neutralization assay. The assay format presented here is based on flow cytometry and uses enzymes labeled with fluorescent probes and live cells. The specificity of the receptor-mediated internalization of the labeled enzyme should be shown by competition with excess unlabeled enzyme. Approaches using other types of labels could also be developed. Sera (taken before treatment and at various time points after treatment) from all patients who either have detectable binding antibody or clinical decline should be tested with a validated assay. (**b**) Enzyme activity neutralization assay. This can be done without the use of cells. The neutralizing capacity of the patient's serum is measured directly on enzyme activity.

Table 1

Immune responses to replacement lysosomal enzymes

Disease	Product	Enzyme	Product status	Patients with IgG antibody (%)	Reference
Gaucher's	Ceredase	Alglucerase	Licensed	12.8	18
	Cerezyme	Imiglucerase	Licensed	13.8	Product label
Fabry's	Fabrazyme	Agalsidase beta	Licensed	90	20
Hurler's (MPS I)	Aldurazyme	α-L-iduronidase	Licensed	91	55
Pompe's	Myozyme	Acid-α-glucosidase	Licensed	89	Product label
Hunter's (MPS II)	Elaprase	Iduronate-2-sulfatase	Licensed	51	Product label
Maroteaux-Lamy (MPS VI)	Naglazyme arylsulfatase B	*N*-acetylgalactosamine-4- sulfatase	Phase 3 completed	97	87

MPS, mucopolysaccharidosis.

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Table 2

Genetic mutations and development of inhibitors in hemophilia

Disease	Type of mutation	Inhibitor rate	Anaphylaxis rate
Hemophilia A[4,5]	Large deletion	88%	Rare
	Moderate deletion	25–41%	
	Minor deletion	16–21%	
	Intron 22 inversion	20%	
	Missense	5%	
Hemophilia B[8,88]	All mutations	5%	<2%
	Large deletion or minor deletion with stop	50–100% (3/6 or 6/6 patients)	26%