Antibody Response in Patients With Gaucher Disease After Repeated Infusion With Macrophage-Targeted Glucocerebrosidase

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Recent clinical data have shown that enzyme replacement therapy with macrophage-targeted glucocerebrosidase (GCR) can be effective in treating type 1 Gaucher disease. Sera from 262 patients, repeatedly infused with GCR, were assessed for the presence of antibodies to this therapeutic protein. Patient serum samples obtained at 3-month intervals were assessed by enzyme-linked immunoabsorbent assay and those with values greater than two standard deviations above the mean value obtained with a pool of normal human sera were further characterized by radioimmunoprecipitation. At the time of these analyses, the duration of patient treatment varied from 3 months to approximately 3 years. Of the 262 patients analyzed, 34 (12.9%) showed IgG antibodies, as confirmed by radioimmunoprecipitation. All patients who seroconverted did so within 1 year of treatment. The predominant antibody developed was the IgG1 subclass. Fourteen patients in the study experienced periodic symptoms suggestive of immediate hypersensitivity. Nine of these 14 patients had antibody to GCR as determined by radioimmunoprecipitation, whereas 5 patients were antibody negative. There was no evidence of the development of IgE antibodies in these 14 patients. The presence of GCR antibodies did not appear to effect efficacy of therapy in any of the patients treated to date.

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Materials and Methods

Patients

Patients with type 1 Gaucher disease received periodic infusions (usually every 2 weeks) of macrophage-targeted placental GCR (approximately 15 to 60 U/kg of body weight). Sera were obtained from patients before the first infusion and at several times after the initiation of therapy, generally at 3-month intervals. Serum samples were stored at −80°C until analyzed. Approval was obtained from the Institutional Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki.

GCR

Clinical-grade GCR was used in developing all the immunosays and in the subsequent monitoring of patient sera. Purified recombinant GCR (rGCR), produced in Chinese hamster ovary cells (Genzyme), was used in experiments to show the protein specificity of patient responses. As noted above, GCR has its carbohydrate structure enzymatically modified to yield a mannosetermminated molecule, whereas rGCR was not modified and consequently had a complex branched oligosaccharide structure.

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Immunologic Methods

Enzyme-linked immunosorbent assay (ELISA). An ELISA was developed as a screening assay for the detection of antibodies to GCR in patient sera. Briefly, microtiter wells of a 96-well microtiter plate (Corning, Corning, NY) were coated with GCR, followed by blocking any unreacted sites on the polystyrene wells with human serum albumin (HSA; Baxter Healthcare Corp, Glendale, CA). Patient sera were diluted 1/100 in dilution buffer (phosphate-buffered saline [PBS], 0.05% Tween 20, 0.01% HSA) and added to each well and allowed to incubate for 2 hours at 37°C. The plates were washed with PBS-Tween followed by subsequent incubation with horseradish-peroxidase (HRP)-conjugated goat antihuman IgG Fc specific antibody (Jackson Immunoresearch Laboratories, West Grove, PA). The presence of GCR antibodies in patient sera were detected using o-phenylene diamine (OPD; Sigma, St Louis, MO) as the conjugate substrate and the color reaction was measured by reading absorbances at 490 nm using a microtiter plate reader (Molecular Devices, Menlo Park, CA). As a control measure, all patient sera were analyzed for reactivity to wells coated with only HSA because this material is used as an excipient in the GCR formulation. None of the patients in the study developed antibodies to HSA as judged by this assay.

Radioimmunoprecipitation (RIP). The presence of IgG-specific antibody was confirmed by RIP. Iodinated GCR was prepared using Enzymobead iodination reagent (BioRad Laboratories, Richmond, CA). Patient sera, normal human serum, or affinity-purified rabbit anti-GCR antibodies were incubated with 125I-GCR overnight, with constant mixing, at 4°C. The following day, sepharose-bound protein A (Sigma) was added to the reaction tubes and incubated for 1 additional hour, with mixing, at 4°C. The immune complexes bound to protein A-sepharose were pelleted by centrifugation, washed three times, and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The samples were boiled and subjected to electrophoresis through a 10% SDS-PAGE separating gel. After drying the gel, immunoprecipitated protein was visualized by autoradiography.

Ig isotyping. An indirect ELISA was used to detect the presence of IgM, IgA, and IgE antibodies. Microtiter plates coated with GCR and blocked with HSA were incubated with patient sera using minimum dilutions previously established in our laboratory. Sera were evaluated at a dilution of 1:2 for IgE response, 1:4 for IgM, and 1:8 for IgA. The plates were incubated for 1 hour at 37°C, followed by washing with PBS-Tween and a subsequent incubation with isotype-specific HRP-conjugated secondary antibody. IgE antibodies were detected with HRP-rabbit antihuman IgE, e-chain specific (Axell, Westbury, NY); IgM antibodies with HRP-goat antihuman IgM, Fc 5p-specific (Axell); and IgA antibodies with HRP-goat antihuman serum IgA, a-chain specific (Cappel, Durham, NC). Immune complexes formed in the wells were detected as in the screening ELISA.

IgG subclass. Patient sera were assayed for the presence of the four IgG subclasses. These assays were performed using the ELISA format described previously. For this analysis, all patient sera were diluted 1:10 in dilution buffer and resultant immune complexes were detected using human IgG subclass-specific HRP-conjugated secondary antibodies (The Binding Site, San Diego, CA).

Serum tryptase RIA. Mast cells have granules that contain pharmacologic mediators and proteolytic enzymes such as tryptase. In addition, these cells have receptors for IgE, which, when bound to its allergen, can directly cause degranulation of these cells. Increases in serum tryptase levels are a consequence of mast cell degranulation and suggest an IgE-mediated immune response. Patients who experienced symptoms of immediate hypersensitivity were evaluated, when possible, for serum tryptase levels. Analyses were performed using the Tryptase RIACT assay (Pharmacia Diagnostics AB, Uppsala, Sweden), following the manufacturer’s instructions.

RESULTS

Analysis of a commercially available pool of normal human sera (NHS; Gibco, Grand Island, NY) was performed to establish the background absorbance value in the ELISA used to screen patient sera for the presence of circulating antibodies to GCR. A mean absorbance value of 0.138 ± 0.041 was obtained from 20 replicate determinations. The upper limit of the normal range of reactivity by human sera in this assay was defined as two standard deviations above the mean value or an absorbance of 0.22. To validate this value, sera obtained from 90 normal human volunteers and pretreatment sera obtained from the first 70 Gaucher patients receiving treatment were analyzed by ELISA. All of the normal human serum samples were determined to be within the established normal range (Fig 1). The Gaucher patient samples gave values within the established normal range; 4 gave absorbance values greater than 0.22. These four samples were further analyzed by RIP. None of the 4 pretreatment patient sera that were above the normal range in the ELISA precipitated radiolabeled GCR and consequently were judged not to contain GCR-specific antibody (data not shown). In contrast, it was shown that in this assay both positive control sera (a rabbit polyclonal antibody to GCR) as well as a patient serum sample that had a high ELISA value after the initiation of therapy precipitated the major component of the radiolabeled GCR; a normal human serum sample having an ELISA value within normal range did not precipitate GCR and served as a negative control for the experiment (Fig 2).

The GCR specificity of the posttreatment human serum sample that provided a positive result in the RIP shown in Fig 2 was confirmed by an inhibition assay in which the serum sample was preincubated with either GCR or rGCR before testing in the ELISA (Fig 3). Preincubation of this RIP-positive serum sample with either source of GCR similarly inhibited antibody binding to GCR coated on the microtiter plate, suggesting the antibody was specific for GCR. In addition, the similarity in inhibition provided by both molecules suggested that the antibodies developed to GCR...
were most likely directed against epitopes on the protein rather than directed against the mannose-terminated oligosaccharides generated by the modification procedure.

**Evaluation of Gaucher Patient Serum Samples**

Sera from 262 patients, including the 70 patients used in the assay validation study, were analyzed by the ELISA after the initiation of GCR therapy. Serum samples were obtained from patients at approximately 3-month intervals. Serum samples having absorbance values higher than the established upper limit for NHS were further evaluated by RIP. Patients were judged to have developed an antibody response to GCR when their serum sample produced a discernable band in the RIP assay by 24 hours. The results of this study are summarized in Table I.

Pretreatment serum samples were available from 191 of 262 patients; the remaining 71 patients had a serum sample drawn within the first 3 months of therapy and were subsequently shown to be antibody negative as judged by RIP. Of the 191 patients, 25 had ELISA values above the normal range before the initiation of therapy, but were shown to be antibody negative by the RIP assay. Seventeen of these pretreatment samples contained sufficient volume for further analysis by RID for total IgG and IgM concentrations. All of these patients exhibited Ig levels above the normal range (as reported by the kit manufacturer), which may have elevated the background of the ELISA, resulting in generation of a false-positive signal in the assay.

Analysis of the posttreatment serum samples of the 262 patients during the course of therapy showed that 73 exhibited ELISA values above normal range. RIP analysis confirmed the development of IgG antibodies to GCR in 34 of these 73 patients. This provided an anti-GCR seroconversion frequency of 12.9% in these treated patients.

**Immunologic Basis for Clinical Symptoms Suggestive of Immediate Hypersensitivity**

Fourteen of the 262 patients studied were reported to have episodic symptoms suggestive of immediate hypersensitivity reactions. Nine of these patients had detectable IgG antibodies to GCR as determined by RIP. The symptoms experienced by these patients were transient and included pruritus, urticaria, upper airway involvement, flushing, and abdominal or chest discomfort.

There was no evidence for the development of IgE antibodies to GCR by either the IgE ELISA or serum tryptase assay in the patients who experienced apparent hypersensi-
ANTIBODY RESPONSE TO GCR INFUSION

Fig 3. Specificity of antibody response. Inhibition of ELISA signal of antibody-positive Gaucher patient serum by preincubation with varying concentrations of GCR or rGCR. Values provided are the average of duplicate determinations. Analysis of best fit curves showed that antibodies react similarly to both molecules.

Figures 4A and B represent control sera. Figure 4A shows the C3 electrophoresis profile obtained with normal human serum, whereas Fig 4B shows the pattern obtained from a postinfusion serum sample of a Gaucher patient who is positive for antibody to GCR, but did not experience any infusion-related complications. In both of these samples, only a small amount of C3 is converted to degradation products, as shown by the small second peak. These results are consistent with the small amount of conversion usually associated with sample handling. This pattern was seen both with Gaucher patients who did not develop antibodies to GCR as well as with those that had developed antibodies but whose infusions proceeded without incident. However, greater than 50% conversion of C3 to its degradation products was seen in sera from the 3 patients who experienced the apparent hypersensitivity reactions during infusion (Fig 4C). These data suggest that immune complexes are formed, presumably GCR antibody/antigen complexes, which in turn initiate activation of the complement cascade and thereby contribute to the immediate hypersensitivity symptoms experienced by these patients.

Patient Serologic Profiles

A comparison of ELISA and RIP results as a function of time for 3 individual patients is shown in Fig 5. These data are representative of the types of serologic profiles observed in patients who seroconverted. The patient shown in Fig 5A lacked antibody to GCR at initiation of therapy. Within 3 months, this patient had developed antibody to GCR, as shown by the positive response in the ELISA and RIP. This patient continued to be treated and at approximately 8 months after the initiation of therapy experienced an adverse reaction during infusion suggestive of an immediate hypersensitivity reaction. The infusion was terminated and hydrocortisone was administered. The patient recovered within 3 to 4 minutes and all symptoms subsided within 10 minutes. Treatment of this patient was temporarily suspended. Subsequent serum samples showed a reduced absorbance value in the ELISA. There was no evidence for the development of IgM, IgA, or IgE antibodies to GCR by ELISA. To eliminate the possibility that the sensitivity of this in vitro determination of GCR-specific IgE may not be adequate to detect clinically relevant IgE antibodies, the patient was skin tested. Epicutaneous and intradermal skin testing with GCR, HSA, and diluent was performed and proved negative in this patient, suggesting that the adverse reaction was not IgE mediated. Therapy was reinitiated and has proceeded without incident.

Figure 5B depicts a patient who did not develop antibody to GCR until after prolonged treatment. At initiation of therapy, this patient had an ELISA value slightly above normal range; however, this patient did not have specific IgG antibodies to GCR because the pretreatment sample was

| Table 1. Summary of Antibody Response in Patients With Gaucher Disease After Repeated Infusion With GCR |
|-------------------------------------------------|----------------|----------------|----------------|
| Patients                         | ELISA          | RIP            | Clinical Event |
| Pretreatment (n = 191)            |                |                |                |
| 25 ANR                           | 0 pos          | 25 neg         |                |
| 166 WNR                          | ND             |                |                |
| Posttreatment (n = 262)           |                |                |                |
| 73 ANR                           | 34 pos         | 9              |                |
| 189 WNR                          | 39 neg         |                | 5*             |

Abbreviations: ANR, above normal range; WNR, within normal range; ND, not done; pos, positive; neg, negative.

* Patients are RIP negative.
maintained a constant value in their response or there were not enough data points to firmly establish a downward trend. There was no evidence that the presence or changing levels of GCR antibodies in the seropositive patients were correlated with product efficacy (data not shown).

Sera from 20 randomly chosen seropositive patients were analyzed for IgG subclass. The sera from these patients were chosen at the time of peak antibody response as judged by ELISA. A summary of this analysis is shown in Table 2. All 20 patients had IgG1 antibodies directed against GCR. The next predominant subclass was IgG3, in which 11 of 20 patients developed these antibodies. Only 3 of 20 and 4 of 20, respectively, developed IgG2 and IgG4 antibodies.

The length of time patients received therapy before developing antibodies to GCR is shown in Fig 6. Of the 34 serum samples from patients that developed specific antibody to GCR, 31 patients had provided a negative serum sample at some time during the previous 3-month interval and were therefore evaluable. Twenty-eight of the 31 patients (90.3%) developed specific antibody within the first 6 months of therapy. None of the patients seroconverted at time points beyond 12 months of therapy.

DISCUSSION

Previous investigators have shown that intravenous infusion of macrophage-targeted GCR can be an effective therapy for treatment of patients with type I Gaucher disease.**'** GCR, the commercially available formulation of placental-derived, macrophage-targeted GCR, has joined a number of other therapeutic proteins that are currently being used to treat a variety of genetic and somatic cell defects in humans. These proteins include human factor VIII to treat hemophilia, α1 antitrypsin to treat hereditary emphysema, growth hormone to treat short stature, and insulin to treat type I diabetes.

Based on the clinical experience with these and other therapeutic proteins, it was anticipated that some fraction of the patients on GCR therapy would develop antibodies to GCR. For example, it has been reported that approximately 15% of the hemophiliac patients treated with plasma-derived, α1 antitrypsin to treat hereditary emphysema.”

We were particularly interested in comparing the presence of circulating antibody in patients treated with GCR with that observed with hemophiliacs treated with human plasma-derived factor VIII. The two therapies are similar in that both involve treatment of genetic defects with repeated intravenous infusions of human tissue-derived proteins.

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**Fig 4.** Two-dimensional immunoelectrophoresis of sera for C3 degradation products. Complement activation products were identified in 3 patients whose serum samples were appropriately processed for this analysis. C3 degradation products may contribute to the immediate hypersensitivity symptoms experienced by these patients. (A) Normal human serum. (B) Serum from a seropositive patient with Gaucher disease immediately after infusion that proceeded without incident. (C) Serum from a seropositive patient with Gaucher disease immediately after experiencing an adverse event during infusion.
Fig 5. Patient serologic profiles. ELISA and RIP results were compared from 3 individual patients who are representative of the patterns obtained. Serum samples obtained from patients at the designated time points were analyzed in the ELISA. The solid horizontal line above the abscissa corresponds to an absorbance value of 0.22 and identifies the upper limit of the normal range of the assay. The insert represents RIP results for the samples. Patients who develop antibody tend to show a brisk antibody response that generally decreases over time.

However, an interesting difference between the two therapies is that the predominant IgG subclass observed with patients who develop antibodies to factor VIII is IgG4, whereas the predominant IgG subclass for patients who develop antibodies to GCR is IgG1 (Table 2).

This may explain in part the differences in immune-mediated complications observed between seropositive hemophiliac patients and seropositive Gaucher patients. As noted above, development of antibodies to factor VIII often results in immunoneutralization of the therapeutic efficacy of the protein. This is probably because these patients produce adequate titers of anti-factor VIII IgG4 to bind to the major-
ity of factor VIII molecules available in circulation. The fact that IgG4 antibodies are not particularly effective in fixation of complement may explain why these patients rarely suffer other types of immune-mediated complications. Unlike the situation observed with hemophiliacs, we have no evidence that Gaucher patients who have developed circulating antibodies to GCR experience a significant diminution in therapeutic response to treatment. This may be attributable to the significant differences between dosages of the two therapeutic proteins as well as the predominant IgG subclass elicited.

The basis for development of antibodies to GCR or to factor VIII is not clear. It is worth noting that the frequency of such reactions is significantly higher than those frequencies reported for patients treated with other recombinant or human tissue-derived therapeutic proteins. Differences in frequency of reactions may reflect some aspect of dosing, protein size, denaturation, and aggregation. However, it is also possible that the development of immune responsiveness in patients with genetic diseases therapeutically treated with the native human protein is a consequence of not being tolerized to the wild-type form of the molecule during fetal development. Thus, the therapeutic protein is viewed as a foreign entity against which an immune response is elicited. We are attempting to further examine this latter hypothesis by investigating whether there is a correlation between the genotype of seropositive Gaucher patients and the epitope specificity of the anti-GCR antibodies they have developed.

One of the most intriguing observations made as a consequence of this study is the decrease in ELISA and RIP signals seen in many patients that seroconverted during the course of their therapy but continued treatment. We suspect that this may be due to the induction of immune tolerance in these patients. Again, similar observations have been reported in several hemophilia patients treated with factor VIII who developed antibodies to the therapeutic protein but received increased doses of factor VIII to overcome the immunoneutralization. These observations are significant because they provide reason to continue treatment of patients who have developed immune responses to the therapeutic protein of interest. This is obviously based on the assumption that potential immune-mediated complications can be managed during this time. Fortunately, patients who have developed antibodies to GCR have been effectively managed with antihistamines during the time they continued treatment.

Based on the clinical complications observed in certain seropositive patients and the fact that these patients appear to have developed primarily IgG antibodies to the protein, suggests that complement fixation by immune complexes may play a role in the reactions observed. This hypothesis is supported by the limited data we have obtained analyzing the sera of patients obtained immediately after clinical complications for complement degradation products. The results of these analyses indicate that, in certain patients, evidence of C3 degradation products can be shown, whereas these patients lack elevated serum tryptase or a positive skin test.

In addition, an interesting observation was made regarding the development of circulating antibody to GCR as a function of the length of time on therapy. Current evidence indicates that 90% of the patients who develop antibodies to GCR do so within the first 6 months of therapy. None of the patients analyzed thus far have seroconverted beyond 12 months of therapy. Although this represents a relatively small group of individuals within this study (ie, 31 patients), if this trend is confirmed, patients who have not developed antibody within 1 year of treatment may be good candidates for home care therapy with GCR.

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