Impaired Microbicidal Capacity of Mononuclear Phagocytes From Patients With Type I Gaucher Disease: Partial Correction by Enzyme Replacement Therapy

By László Maródi, Rita Káposzta, Judit Tóth, and Aranka László

The higher susceptibility to serious bacterial infections in patients with Gaucher disease (GD) may be due in part to defective function of phagocytic cells. We studied five patients with GD (type I) and examined the ability of granulocytes and mononuclear phagocytes from these patients to phagocytose and kill Staphylococcus aureus and to generate superoxide anion (O$_2^-$) on stimulation with fully opsonized bacteria. Serum-opsonized staphylococci were ingested equally by phagocytic cells from patients and controls. In the presence of normal serum, the extent of killing of S aureus and the release of O$_2^-$ by granulocytes over incubation periods of 90 minutes and 30 minutes, respectively, were also equivalent for patients and controls. However, we found that killing of viable bacteria and release of O$_2^-$ by the patients' monocytes was significantly lower than that in cells from controls (p < .05 for both). The magnitude of differences in killing and O$_2^-$ release between patients' cells and those from controls was even more profound with monocyte-derived macrophages. Enzyme augmentation with macrophage-targeted glucocerebrosidase preparation for 6 months at doses from 7.5 to 10 U/kg/wk resulted in significant increases of functional activities and O$_2^-$ generation of monocytes and macrophages along with hematologic and hepatosplenic improvements. These data suggest that mononuclear phagocytes from GD patients are defective in their ability to kill bacteria and to generate reactive oxygen intermediates. Our data also suggest that enzyme substitution may improve functions of monocytes and macrophages in patients with GD that should make them more resistant to severe bacterial infections.

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Table 1. Pretreatment and Posttreatment Hematologic, Biochemical, and Hepatosplenic Findings in the Study Patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pretreatment</th>
<th>Posttreatment (3 mo)</th>
<th>Posttreatment (6 mo)</th>
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<tbody>
<tr>
<td>Hemoglobin (g/L)</td>
<td>107 ± 8</td>
<td>123 ± 11*</td>
<td>125 ± 12*</td>
</tr>
<tr>
<td>Platelets (x10^9/L)</td>
<td>144 ± 80</td>
<td>186 ± 148</td>
<td>195 ± 137</td>
</tr>
<tr>
<td>Acid phosphatase (U/L)</td>
<td>27 ± 12</td>
<td>22 ± 11*</td>
<td>21 ± 10*</td>
</tr>
<tr>
<td>Hepatic volume (mL/kg)</td>
<td>72 ± 17</td>
<td>ND</td>
<td>60 ± 11</td>
</tr>
<tr>
<td>Splenic volume (mL/kg)</td>
<td>44 ± 6</td>
<td>ND</td>
<td>35 ± 8</td>
</tr>
</tbody>
</table>

Data represent mean ± SEM.

* Values significantly different (P < .05) from those obtained before treatment.

teflon beakers (Savillex, Minnetonka, MN) at 37°C and 5% CO2 for 5 days.16 Viability of cultured cells remained greater than 96% (trypan blue exclusion). Monocytes cultured for 5 days are referred to here as monocyte-derived macrophages or macrophages.

The mean percentage of monocytes in fresh suspensions or suspensions cultured for 5 days, respectively, was 19% ± 6% (mean ± SEM; range, 10% to 28%) and 17% ± 5% (range, 14% to 22%) for the patients’ cells and 22% ± 5% (range, 12% to 34%) and 21% ± 6% (range, 13% to 30%) for control cells, as determined by Giemsa and esterase stainings; less than 0.2% of cells were granulocytes.

In developing the assays described below for phagocytosis and killing of Staphylococcus aureus, results with cells prepared and cultured as described above were equivalent to results with preparations of greater than 85% monocytes; results did not vary with the percentage of mononuclear phagocytes.17

Preparation of human serum. Normal human serum (NHS) was prepared from blood collected from healthy donors under aseptic conditions. The serum was stored at −70°C in small aliquots. Heat-inactivated serum was prepared by heating NHS for 30 minutes at 56°C.

Bacteria. S. aureus (type 42D) was cultured overnight and resuspended to a final concentration of 10⁷ bacteria/mL.14 Gelatin (0.1%) was added to the KRPD to enhance viability and decrease aggregation. Bacterial suspension was thoroughly vortexed before use.

Measurement of phagocytosis. Phagocytosis of bacteria was determined by measuring the decrease in the number of viable extracellular microorganisms following incubation of 3 x 10⁸ phagocytic cells/mL with 5 x 10⁸ bacteria/mL in the presence of 10% NHS. Phagocytosis assays were performed at 37°C and 4 rpm rotation. At 0 and 60 minutes, samples were removed from the phagocytic mixture and extracellular bacteria were separated from phagocytes by differential centrifugation for 6 minutes at 75g. Phagocytosis was expressed as the percentage of decrease from the initial number of viable extracellular microorganisms determined by colony counts in the supernatants.14

Bactericidal assay. Phagocytic cells and bacteria were washed and suspended in KRPD at a bacterium-to-cell ratio of 1:1, with a final cell concentration of 5 x 10⁷/mL. The phagocytic mixture was incubated at 37°C under slow rotation (4 rpm) in the presence of 10% NHS. Samples were removed at 0 and 60 minutes and assayed for total number of viable bacteria. Granulocytes were lysed in distilled water containing 0.01% bovine serum albumin; monocytes and macrophages were lysed by freezing the cells in liquid nitrogen and thawing in a waterbath three times. Bacterial killing was expressed as the percentage of decrease from the initial number of viable intracellular and extracellular bacteria.14

Measurement of superoxide anion (O²⁻) release. The release of O²⁻ from phagocytic cells was quantitated as the superoxide dismu-
tase-inhibitable reduction of ferricytochrome c (type III; Sigma Chemical Co, St Louis, MO).15 S. aureus (2 x 10⁶) was added to an equal number of mononuclear phagocytes in cell suspension in KRPD with 80 μmol/L cytochrome c, with or without 50 μg/mL superoxide dismutase. Incubation was for 30 minutes in the presence of 10% NHS at 37°C with rotation (4 rpm). The reaction was stopped by shaking the tubes in crushed ice followed by centrifugation at 4°C. Supernatants were transferred to microtiter plates (200 μL/well; Nunc, Femron, Denmark) and reduction of ferricytochrome c was measured spectrophotometrically at 550 nm by using a computerized enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Menlo Park, CA). In selected experiments, O²⁻ generation by granulocytes or monocytes was measured by stimulation with formyl-methionyl-leucyl-phenylalanine (FMLP) for 15 minutes.

Expression of data. Results are expressed as the mean ± SEM. Each in vitro experiment was performed in triplicate. Statistical significance was determined by Student’s t-test and ANOVA. Where appropriate, values obtained were also analysed by using Wilcoxon’s matched-pairs signed-ranks test, Friedman’s two-way ANOVA, or Kendall’s coefficient of concordance.18 We set P < .05 as the threshold for significance.

RESULTS

No adverse effects of enzyme infusions were noted during the 6-month course of replacement therapy. All five patients tolerated the treatment without any clinical or subjective complications. No bleeding episodes were observed in the patients during the 6-month treatment with glucocerebrosidase. However, before treatment, one to five episodes of bleeding occurred each year in the three thrombocytopenic patients; one of the splenectomized patients with normal platelet counts also presented with three or four episodes per year of severe epistaxis and gingival bleeding.

None of the patients suffered from severe infection during the treatment period. However, recurrent sinopulmonary infections caused by pyogenic bacteria occurred as a significant problem in four of the five patients before treatment. Deep soft tissue infection in the left arm with general symptoms of toxicity had occurred in one of the splenectomized patients.

Hematologic and hepatosplenic responses. All patients showed an increase in the hemoglobin (Hb) level after 3 months of treatment, and comparison of the initial and 3-month values showed that the difference was significant (Table 1). The platelet counts in the two splenectomized patients was normal at the time of enrollment and it increased further after treatment was started. The three patients with enlarged spleen remained thrombocytopenic throughout the study period. However, the bleeding tendency in these three patients subsided despite the persistence of thrombocytopenia. The serum acid phosphatase level decreased substantially in each patient from the beginning of treatment and the differences between initial and 3-month values were significant (Table 1).

After 6 months of treatment, hepatic volumes decreased by 35% and 19% in the two splenectomized patients, respectively. Small reductions in liver size were achieved in patients with a spleen, but the decrease in splenic volumes in these patients was pronounced (Table 1).

Phagocyte responses. We studied phagocytosis and kill-
IMPAIRED MICROBICIDAL CAPACITY

Table 2. Effect of Glucocerebrosidase Treatment on Functional and Metabolic Activities of Phagocytic Cells From the Study Patients

<table>
<thead>
<tr>
<th>Cells</th>
<th>Phagocytosis of S. aureus* (%)</th>
<th>Bacterial Killing of S. aureus* (%)</th>
<th>Release of Superoxide† (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>90 ± 5</td>
<td>77 ± 6</td>
<td>64 ± 5</td>
</tr>
<tr>
<td>After treatment</td>
<td>85 ± 4</td>
<td>80 ± 7</td>
<td>66 ± 4</td>
</tr>
<tr>
<td>Controls</td>
<td>92 ± 4</td>
<td>86 ± 5</td>
<td>66 ± 7</td>
</tr>
<tr>
<td>Monocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>85 ± 6</td>
<td>45 ± 9</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>After treatment</td>
<td>87 ± 10</td>
<td>66 ± 12†</td>
<td>28 ± 5†</td>
</tr>
<tr>
<td>Controls</td>
<td>90 ± 5</td>
<td>83 ± 6</td>
<td>41 ± 5</td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>76 ± 7</td>
<td>6 ± 4</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>After treatment</td>
<td>68 ± 9</td>
<td>13 ± 4†</td>
<td>8 ± 3†</td>
</tr>
<tr>
<td>Controls</td>
<td>79 ± 4</td>
<td>41 ± 7</td>
<td>14 ± 4</td>
</tr>
</tbody>
</table>

Data represent mean ± SEM.
* Incubation of phagocytes and S. aureus was for 60 minutes in the presence of 10% NHS.
† Incubation of 2 × 10⁶ phagocytes and 2 × 10⁹ opsonized bacteria was for 30 minutes.
‡ P < .05 compared with pretreatment values. The values of phagocytic and bactericidal activities and release of superoxide anion were compared only with the pretreatment values.

As illustrated in Table 2, the extent of ingestion of S. aureus and release of O₂⁻ by granulocytes and mononuclear phagocytes from the patients before and after 6 months of treatment with glucocerebrosidase, and we compared results with those obtained with cells from age-matched controls.

In the absence of opsonizing serum, phagocytosis of bacteria was negligible (<2% of inoculum) and no killing could be detected over 60 minutes of incubation. Phagocytosis by granulocytes or mononuclear phagocytes was negligible at 4°C or in the presence of 0.2 mol/L sodium fluoride (<13% ingestion over periods of 60 minutes; n = 4 for both). Incubation of the cell pellet for 5 minutes at 37°C in the presence of lysostaphin (10 μg/mL) to lyse extracellular S. aureus reduced the total number of cell-associated (i.e., ingested and adhered) bacteria by 9.4% ± 2.6% and 12% ± 3.5% with granulocytes or mononuclear phagocytes, respectively (mean ± SEM, n = 6), as compared with incubation of the cell pellet for 5 minutes in medium only. These control experiments supported the interpretation that the decrease in the number of viable extracellular bacteria mainly represented phagocytosis.¹⁹

As illustrated in Table 2, the extent of ingestion of opsonized S. aureus was normal before treatment for all types of phagocytic cells and did not change significantly during treatment. Killing and O₂⁻ release over incubation periods of 60 minutes and 30 minutes, respectively, by granulocytes were also equivalent in patients and controls (Table 2). However, killing of bacteria by monocytes or macrophages from the patients was less effective and these cells released significantly lower amount of O₂⁻ compared with control cells under the same experimental conditions (Table 2).

FMLP-induced O₂⁻ release by granulocytes from the five study patients was also comparable with that of controls before glucocerebrosidase treatment was introduced (Table 3). However, release of O₂⁻ by the patients' monocytes was significantly lower than that of control monocytes on stimulation with FMLP (Table 3). These data confirm that mononuclear phagocytes from patients with GD are deficient in their ability to release superoxide.

Experiments performed after 6 months of regular weekly infusions of glucocerebrosidase showed a significantly higher degree of killing of S. aureus by monocytes from the five patients when compared with pretreatment values (Table 2). In addition, the data showed enhancement of monocyte O₂⁻ release that coincided with improvements in the bactericidal capacity of these cells. Experiments with macrophages from these patients gave similar results (Table 2).

DISCUSSION

The present results show the efficacy of glucocerebrosidase administered as 10 U/kg/wk for 3 months and as 7.5 U/kg/wk for another 3 months. The patients are currently being treated with 5 U/kg/wk glucocerebrosidase. This dosage was planned for the next 6 months, to be followed by further reductions if clinical improvements are sustained.

It was the intent of this study to gain a better understanding of host defense mechanisms in patients with GD. In addition, we hoped to gain insight into the effect of long-term enzyme substitution with macrophage-targeted glucocerebrosidase on phagocytic cell function. The results presented here elucidate some aspects of functional and biochemical activities of granulocytes and mononuclear phagocytes and enhance our understanding of the immunologic basis of the decreased resistance to bacterial pathogens in patients with this disorder.

We showed that granulocytes and mononuclear phagocytes from GD patients have a normal capacity to ingest S. aureus opsonized through the combined effect of Igs and complement in NHS. Using opsonized zymosan, Lie1 et al.¹¹ recently reported that phagocytosis was decreased in the monocytes from GD patients. Variations in experimental design, including cell concentration, cell-to-particle ratio, or differing requirements for full opsonization, might explain the apparently differing results.¹¹ Based on our previous analysis of the phagocytosis assay system used here, our data suggest normal function of Fc and C3 receptors on both

Table 3. FMLP-Induced Release of Superoxide Anion by Granulocytes and Monocytes From the Study Patients

<table>
<thead>
<tr>
<th>Phagocytic Cells*</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes</td>
<td>49.5 ± 7.3 (5)</td>
<td>53.7 ± 8.6 (7)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>4.2 ± 0.9 (5)†</td>
<td>9.4 ± 2.8 (6)</td>
</tr>
</tbody>
</table>

Experiments were performed before glucocerebrosidase treatment was introduced. Results are expressed as means ± SEM for the indicated number (n) of experiments.
* Incubation of 2 × 10⁶ phagocytes was for 15 minutes in the presence of 10⁻⁹ mol/L FMLP.
† Values different at level of P < .01 compared with controls.
granulocytes and mononuclear phagocytes from GD patients. 14

Opsonic receptors for IgG and complement also function to promote killing of S. aureus as effectively in GD granulocytes as in control cells. In concert with the normal bactericidal function, no suppression of O2" generation in granulocytes was detected in this study. However, random migration and chemotactic response of granulocytes to various stimuli appear to be impaired in GD patients. 9, 11 This selected functional deficiency may represent differential modulation of migration and opsonophagocytic killing by patients’ granulocytes exposed to glucocerebrosides.

We showed here that killing of ingested S. aureus by monocytes from GD patients was markedly decreased compared with normal cells. In an attempt to gain insight into the biochemical basis of the deficient killing of bacteria, we studied the activity of the respiratory burst and found that generation of O2" was significantly decreased in monocytes. These findings are in agreement with those reported by others 9, 11 and suggest that predisposition of GD patients to bacterial infections may be related to decreased functional capacities and metabolic activities of blood monocytes.

The present study provides, for the first time, evidence for significant dysfunction of monocyte-derived macrophages in GD. All experiments with monocytes and macrophages were performed in the presence of lymphocytes, but phagocytosis, killing, and O2" release are expressed only by true phagocytes during the short assay times used. We believe that the presence of nonphagocytic cells in the assay system has not obscured the endpoints studied, and there are data indicating that the presence of lymphocytes to coculture can improve the viability and functional capacity of mononuclear phagocytes. 20

The precise mechanism of the impaired oxidative burst and bactericidal capacity in mononuclear phagocytes in type I GD disease remains to be elucidated. Extracellular glucocerebrosides may inhibit O2" generation by monocytes. 11 Alternatively, the decreased O2" release by mononuclear phagocytes might be a function of quenching by the increased amount of lipids by these cells. Whatever its basis, this deficiency has broad implications if present in vivo, because a decreased capacity of mononuclear phagocytes to kill bacteria could predispose patients to the deep-seated pyogenic infections frequently observed in GD. 6, 12

To our knowledge, there is no previous report on the effect of enzyme augmentation on functional and metabolic activities of monocytes and macrophages from patients with GD. We report here that enzyme substitution in GD patients results in partial correction of the bactericidal defect and the impaired O2" generation by mononuclear phagocytes. The degree to which treatment with glucocerebrosidase improves host defense against pyogenic bacteria in GD patients is not certain. However, the in vitro findings reported here suggest that enzyme augmentation may have a beneficial effect on bactericidal function of monocytes and macrophages along with clinical, hematologic, and hepatosplenic improvements in these patients.

In this country, only the five patients reported here are on glucocerebrosidase replacement therapy. Because GD is rare and the number of treated patients in each single center is few, further evaluation will require a prospective, controlled study of the use of glucocerebrosidase treatment in such patients to determine the real clinical importance of this treatment in terms of incidence and severity of deep-seated bacterial infections.

ACKNOWLEDGMENT

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