Monocyte Dysfunction in Patients With Gaucher Disease: Evidence for Interference of Glucocerebrosidase With Superoxide Generation

By Yair Liel, Assaf Rudich, Ofra Nagaucher-Shriker, Tikva Yermiyahu, and Rachel Levy

Gaucher disease patients are occasionally affected by chronic or fulminant infections. Since Gaucher cells originate from tissue phagocytes, we studied the functional implications of glucocerebrosidase accumulation on phagocytes in Gaucher disease patients. Circulating monocytes and granulocytes from nine type I Gaucher disease patients, and matched controls, were studied. Evaluation of phagocytic activity included (1) maximal superoxide generation rates following stimulation by phorbol 12-myristate 13-acetate (PMA), opsonized zymosan (OZ), or formyl-methionyl-leucyl-phenylalanine (FMLP); (2) nitroblue tetrazolium reduction test (NBT); (3) chemotaxis toward FMLP; (4) phagocytosis of OZ particles; and (5) killing activity against Staphylococcus aureus. Superoxide generation in monocytes following PMA, OZ, and FMLP stimulation was significantly suppressed at 52% ± 15%, 39% ± 9%, and 51% ± 11% of control, respectively. The rate of chemotaxis toward FMLP decreased in both monocytes and granulocytes; however, decreased chemotactic rates were observed in some patients. The abnormality of superoxide generation could be reproduced in a dose- and time-dependent manner in normal circulating monocytes incubated with glucocerebrosidase. Superoxide generation in glucocerebrosidase-conditioned normal monocytes in a cell-free system showed normal superoxide generation, reflecting the integrity of the NADPH oxidase complex itself. These results demonstrate markedly compromised phagocytic functions in circulating monocytes in Gaucher disease patients. These abnormalities can be attributed to accumulation of glucocerebrosidase, since it could be reproduced in normal monocytes incubated with glucocerebrosidase. Similar abnormalities in Gaucher cells throughout the reticuloendothelial system could impair host defense, and may be of particular importance in the pathogenesis of osteomyelitis in Gaucher disease patients.

Therefore, the present study was designed to evaluate the phagocytic function of circulating monocytes and granulocytes in patients with Gaucher disease.

MATERIALS AND METHODS

Patients. Nine patients were studied. The clinical characteristics of the patients are listed in Table 1; all were of type I Gaucher disease and the diagnoses were verified by assessment of leucocyte glucocerebrosidase activity. Calculated mean severity score was 5.1, and it ranged between 1 and 12. The study was approved by the institutional Human Research Committee of the Soroka Medical Center.

Materials. Glucocerebrosidase, ferricytochrome c, phorbol 12-myristate 13-acetate (PMA), formyl-methionyl-leucyl-phenylalanine (FMLP), Ficoll-Hypaque, and zymosan were purchased from Sigma (St Louis, MO). Percoll was obtained from Pharmacia, Uppsala, Sweden. Sodium dodecyl sulfate (SDS) was purchased from Bio-Rad Laboratories (Hercules, CA). Cell-culture media and sera were purchased from Biological Industries (Beit Ha’emek, Israel).

Glucocerebrosidase was freshly dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO added to the cells did not exceed 0.1%. This concentration of DMSO did not affect cell viability and function.

Monocyte separation and culture. Separation of monocytes was performed as previously described by Tarsi-Tsuk and Levy. Hepatinized venous blood was centrifuged at 850 g for 5 minutes. The buffy coat was separated and diluted 1:10 vol/vol with RPMI-1640 culture medium, layered over 25% Ficoll-Hypaque, and centrifuged for 30 minutes at 850 × g for 5 minutes. The separated mononuclear cells were washed three times with Mg2+- and Ca2+-free phosphate-buffered saline (PBS; pH 7.2) containing EDTA (1 mmol/L). The pellet was then resuspended in RPMI-1640 medium to 20 × 10^6 cells/mL. The monocytes were separated from the lymphocytes using a Percoll gradient, which was prepared by vigorous mixing of 15.75 mL Percoll, 1.75 mL PBS × 10, and 11.7 mL PBS × 1. The mixture was centrifuged at 800 × g for 30 minutes at 3°C. The monocyte fraction was separated, washed three times with Mg2+- and Ca2+-free PBS (pH 7.2). This procedure yielded monocytes at 95% purity. Cells were counted and their viability was determined by trypan blue exclusion.
Monocytes were suspended at a concentration of 10⁶ cells/mL in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) 1-glutamine (2 mmol/L) and 1% antibiotic solution (nystatin 12.5 U/mL, penicillin 100 U/mL, and streptomycin 0.1 mg/mL). Cultures were performed at 37°C in a 5% CO₂ atmosphere.

Preparation of granulocytes. Granulocytes, at 95% purity, were obtained by Ficoll-Hypaque centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes as previously described. Cells were counted and their viability was determined by trypan blue exclusion.

Superoxide generation. The production of the superoxide anion (O₂⁻) by intact monocytes or granulocytes was measured as the superoxide dismutase inhibitable reduction of acetyl ferricytochrome c by the microtiter plate technique as previously described, with modifications. Cells (2.5 x 10⁵/well) were suspended in 100 μL Hank's Balanced Salts Solution (HBSS) containing acetyl ferricytochrome c (150 μmol/L). Superoxide production by the cells was stimulated by the addition of PMA (50 ng/mL), opsonized zymosan (OZ; 1 mg/mL), or FMLP (0.1 μmol/L). The reduction of acetyl ferricytochrome c was followed by the change of absorbance at 550 nm at 2- to 5-minute intervals on a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA). The maximal rates of superoxide generation were determined and expressed as nmol O₂⁻/10⁵ cells/10 minutes using the extinction coefficient E₅₅₀ = 21 mmol/L⁻¹·cm⁻¹.

Nitroblue tetrazolium test. PMA-stimulated nitroblue tetrazolium test (NBT) studies were performed as described previously.11 Cells were incubated for 20 minutes at 37°C with PMA (100 ng/mL) as the stimulant. Cells were then fixed and stained with safranin and the percent positive cells determined.

Chemotaxis. Chemotaxis was assessed according to Nelson et al.1 Agarose was dissolved in sterile, distilled boiling water for 10 minutes. After cooling to 48°C in a water bath, the agarose was mixed with an equal volume of prewarmed 2× minimal essential medium (MEM) with 10% heat-inactivated FCS and 7.5% (wt/vol) fetal calf serum. The dishes were subsequently incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air. Dishes hardened. Six series of three wells, 2.4 mm in diameter and spaced 2.4 mm apart, were plucked out. Cells were suspended in MEM. The dishes were subsequently incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air. Dishes containing granulocytes were incubated for 2 hours; monocytes were incubated for 16 hours. The plates were fixed by addition of 3 mL methanol at 4°C overnight or for 30 minutes at room temperature. After the methanol was poured off, the plates were placed in glacial dehydrate (2.5%) for 30 minutes at room temperature. The agarose gel was removed intact after fixation and the plates stained by Giemsa stain and air-dried. Chemotaxis was defined as the ratio between the linear migration toward the chemoattractant (FMLP) (A) and the control medium (MEM) (B). Phagocytosis. Five x 10⁶ cells were suspended in RPMI-1640 containing 10% heat-inactivated FCS and incubated at 37°C for 1 hour with 5 μL zymosan (1 mg/mL), opsonized by pooled human serum. Subsequently, the cells were smeared and stained with differential Wright-Giemsa. Phagocytosis was determined under the microscope at least 100 cells, and as defined as the percent of cells containing more than two phagocytized particles of OZ.

Bacterial activity. Bactericidal activity of phagocytes (monocytes or granulocytes, respectively, from Gaucher disease patients and matched controls) for Staphylococcus aureus was examined according to a previously described method.14 Briefly, bacterial suspension (10⁹ microorganisms in 100 μL broth) were preopsonized with 10% pooled normal serum in HBSS for 60 minutes at 37°C. The suspensions were centrifuged and the bacterial pellets were resuspended in 1 mL HBSS. One hundred microliters of preopsonized bacteria was mixed with an equal volume of phagocytes suspension (10⁶ cells/mL HBSS), in polypropylene counting tubes (bacteria to cells ratio, 10:1). Serial 10-fold dilutions of the mixtures were prepared immediately after the mixing (time 0), and following incubation, in a shaking bath, for 30, 60, and 90 minutes at 37°C. Triplicate 20-μL samples from the appropriate dilutions were plated on nutrient agar, and bacterial colonies counted after 24-hour incubation at 37°C. Control tests were performed by incubating bacteria without macrophages. The results were expressed as percent killed bacteria (percent decrease in bacterial colonies v control) after 90 minutes incubation (at plateau phase).

Cell-free superoxide generation assay. Superoxide generation was measured as previously described.15 Membranes were solubilized in a buffer containing sodium deoxycholate (1.16%), glycerol (50%), Na₂SO₄ (1 mmol/L), CaCl₂ (1.2 mmol/L), and Na-glycine (20 mmol/L), pH 7. The final concentration of solubilized membranes was adjusted to 5 x 10⁹ cell-equivalents per mL. Ten microliters of the membrane solution (5 x 10⁹ cell-equivalents) and 10 μL of monocyte cytosol (10⁶ cell-equivalents), at a final volume of 100 μL reaction mixture, were added to wells of a chilled microtiter plate. The reaction solution contained the following: acetyl ferricytochrome c (0.15 μmol/L), MgCl₂ (4 mmol/L), flavin adenine dinucleotide (FAD; 10 μmol/L), EGTA (1 mmol/L), NADPH (200 μmol/L), and arachidonic acid (40 μmol/L), in 75 mmol/L potassium phosphate, pH 7.0. Control wells also contained superoxide dismutase (2.5 mg). The reaction was monitored at 22°C. Light absorption at 550 nm was determined at 2-minute intervals.

Estimation of cell number. Estimation of the number of viable cells was performed by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphe-
nul tetrazolium bromide (MTT) reduction assay. The blue formazan produced, in this assay, by mitochondrial dehydrogenases is proportional to the number of viable cells.

After removal of the culture medium from cells grown in 96-well microplates, 200 μL MTT (5 mg/mL) was added to each well and incubation was performed for 1 hour at 37°C. Subsequently, the MTT solution was removed and replaced with 100 μL DMSO. The plates were vigorously shaken for 20 minutes on a shaking plate (Pathodx rotator; Diagnostic Products), at room temperature, to ensure homogenous dissolution of the formazan granules, and the optical density of each well was measured on a Thermomax Microplate Reader using a test wavelength of 550 nm and a reference wavelength of 630 nm. The results of parallel estimations, under different experimental conditions, by direct counting of trypan blue excluded cells and MTT colormetric method strongly correlated with each other (r = .98).

**Statistics.** Statistical evaluation was performed using unpaired Student’s t tests. Results are expressed as the mean ± SE.

## RESULTS

Figure 1 illustrates the kinetics of superoxide generation in circulating monocytes and granulocytes from a representative patient and matched control, stimulated with PMA, OZ, or FMLP. The results show considerable reduction of superoxide generation in the patient’s monocytes, while normal superoxide generation was maintained in the patient’s granulocytes.

Figure 2 presents the cumulative results of maximal superoxide generation rates in monocytes and granulocytes, stimulated with PMA, OZ, or FMLP, in nine Gaucher disease patients and corresponding controls. Superoxide generation rates in monocytes from the patients were significantly lower than in controls for PMA (3.35 ± 1.04 vs 6.18 ± 1.12 nmol O₂/10⁶ cells/10 minutes; P < .0001), OZ (1.49 ± 0.45 vs 5.0 ± 0.84 nmol O₂/10⁵ cells/10 minutes; P < .0001), and FMLP (0.81 ± 0.21 vs 1.8 ± 0.23 nmol O₂/10⁶ cells/10 minutes) (Fig 2A). Stimulated superoxide generation in granulocytes from Gaucher disease patients did not differ from that of controls (Fig 2B).

The individual results of phagocytic functions in monocytes and in granulocytes are listed in Tables 2 and 3. Maximal superoxide generation rates in monocytes expressed as percent of matched control in each individual experiment, were reduced in eight of nine patients (Table 2). There was no apparent correlation between the severity of the clinical presentation (Table 1) and the extent of the reduction in superoxide generation in peripheral blood monocytes, or between superoxide generation rate in response to either of the inducers used.

NBT, phagocytosis, chemotaxis, and staphylococcal killing were studied in part of the patient group (Table 2). In monocytes, NBT and phagocytosis were significantly reduced. Staphylococcal killing by monocytes was studied in

Fig 1. Kinetic data of PMA-, OZ-, or FMLP-stimulated superoxide generation in circulating monocytes and granulocytes from a representative patient and matched control.
MONOCYTE DYSFUNCTION IN GAUCHER DISEASE

In granulocytes, the mean superoxide generation rates, NBT, phagocytosis, chemotaxis and staphylococcal killing were normal. However, decreased chemotaxis was observed in two of seven patients (Table 3).

To evaluate the specific role of glucocerebroside in the pathogenesis of monocyte dysfunction in Gaucher disease, stimulated superoxide generation was studied in normal monocytes following incubation with different concentrations of glucocerebroside (Fig 3), and for different periods of time (Fig 4). The results indicate dose and time dependency for the inhibitory effect of glucocerebroside on the stimulated superoxide generation rate. Maximal inhibition, with either of the inducers, was observed at a glucocerebroside concentration of 10 µg/mL (Fig 3). Significant inhibition of stimulated superoxide generation in monocytes incubated with glucocerebroside (25 µg/mL) was first observed after 12 hours for PMA and OZ, and after 24 hours for FMLP. Suppression progressed consistently thereafter for 48 hours (Fig 4).

Further studies were performed to determine the mode of glucocerebroside-induced defect in superoxide generation in Gaucher disease patients, and whether it resulted from modification of the NADPH enzyme complex itself, or was related to abnormalities of the signal transduction pathways which lead to its activation. Figure 5 presents results of stimulation of superoxide generation in circulating monocytes with SDS, a stimulator of superoxide generation independent of protein kinase C. PMA-induced superoxide generation rate was significantly lower in the patient, compared with a normal control (2.26 ± 0.1 % vs. 9.69 ± 0.85 nmol O2/106 cells/10 minutes; P < .0001). With SDS, superoxide generation rate in monocytes from the Gaucher disease patient, markedly increased to 6.36 ± 0.54 nmol O2/106 cells/10 minutes, similar to the rate obtained from the control (6.89 ± 0.6 nmol O2/106 cells/10 minutes).

Table 2. Individual Results of Monocyte Superoxide Generation, NBT Reduction, Phagocytosis, Chemotaxis, and Staphylococcal Killing in Nine Gaucher Disease Patients

<table>
<thead>
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<th>Patient No.</th>
<th>PMA (%)</th>
<th>OZ (%)</th>
<th>FMLP (%)</th>
<th>NBT (+)</th>
<th>Phagocytosis (+)</th>
<th>Chemotaxis (A/B)</th>
<th>Killing (%)</th>
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<td>31 ± 7</td>
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<td>1.7 ± 0.3</td>
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<td>Control</td>
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<td>46 ± 5</td>
<td>1.8 ± 0.4</td>
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<td>P (patients v controls)</td>
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* Results are the mean ± SE.
Normal monocytes incubated for 48 hours with glucocerebroside (20 \( \mu \)g/mL) exhibited a significantly reduced superoxide generation rate compared with controls (Fig 6A). However, in a cell-free system, in the presence of arachidonate, the activity of the mixture of cytosol and membranes from the glucocerebroside-treated monocytes was similar to that of untreated monocytes (Fig 6B). Mixing of membranes from untreated monocytes with cytosol from treated monocytes and vice versa produced similar activity (data not shown).

DISCUSSION

Sphingolipids constitute important components of the outer layer of biological plasma membranes. Among their known biological roles, sphingolipids participate in protection of the cells against environmental fluctuations; they constitute blood group antigens, form specific binding sites for viruses, and are involved in cell-to-cell interactions, in the modulation of membrane bound receptors function, and in intracellular signal transduction.

The present study provides, for the first time, unequivocal evidence for significant monocyte dysfunction in Gaucher disease, most consistently expressed by markedly suppressed superoxide generation. This abnormality was observed in a large majority of Gaucher disease patients, regardless of the clinical severity of disease. Notably, in the present study, as well as in a recently published study, superoxide generation in granulocytes was not affected. Consequently, NBT reduction and staphylococcal killing were considerably reduced in monocytes and were normal in granulocytes. Phagocytosis, which is unrelated to superoxide production, was decreased in the monocytes and was normal in the granulocytes. Chemotaxis, both in monocytes and granulocytes, was occasionally reduced, but the mean chemotactic activity was not different from control (Tables 2 and 3). These results indicate that cell functions, other than superoxide production, may also be deficient.

Our studies with normal monocytes, indicating dose-dependent suppression of superoxide generation following incubation with glucocerebroside (Fig 3), emphasize the role of glucocerebroside uptake by the cells in the pathogenesis of the monocyte dysfunction. The concentration of glucocerebroside required to reproduce suppression of the oxygen burst in normal monocytes, approximately 1,000 times the concentration of glucocerebroside in plasma of Gaucher disease patients, underlines the role of glucocerebrosidase in the regulation of intracellular glucocerebroside concentration in normal phagocytes. The 12-hour lag necessary to produce the abnormality in the normal monocytes (Fig 4) suggests that the specific involvement of monocytes on the one hand, and sparing of granulocytes on the other hand, could be ascribed to differences in life span in the circulation.

Table 3. Individual Results of Granulocyte Superoxide Generation, NBT Reduction, Phagocytosis, Chemotaxis, and Staphylococcal Killing in Nine Gaucher Disease Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>PMA (% positive)</th>
<th>OZ (% positive)</th>
<th>FMLP (% positive)</th>
<th>Chemotaxis (IAR)</th>
<th>Killing (%)</th>
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<td>98</td>
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</table>

Total* 106 ± 5 101 ± 3 103 ± 12 74 ± 4 64 ± 2 2.1 ± 3 89 ± 4

Control 78 ± 4 66 ± 3 3.4 ± 3 90 ± 10

* Results are the mean ± SE.

Fig 3. Effect of glucocerebroside concentration on stimulated superoxide generation rate in normal monocytes. Peripheral blood monocytes were incubated with various concentrations of glucocerebroside for 48 hours. At the end of the incubation period, superoxide generation rate was determined after stimulation by either PMA, OZ, or FMLP. Cell viability was confirmed by trypan blue exclusion and MTT staining. Results are the means ± SE of five separate experiments.
MONOCYTE DYSFUNCTION IN GAUCHER DISEASE

The three recognized cytosolic components are p47, p67, and rac2, a ras-related guanosine triphosphate (GTP)-binding protein. On activation, p47 undergoes phosphorylation, and the three cytosolic components translocate to the membrane to form the active enzyme complex. Available evidence implies an important role for the activation and translocation from the cytosol to the cell membrane of protein kinase C (PKC) in the process of phosphorylation of the p47 component and stimulation of the NADPH oxidase complex.

Since granulocytes from Gaucher disease patients display normal activity of the NADPH oxidase system, as do monocytes following SDS treatment (Fig 5) and in a cell-free system (Fig 6), it is unlikely that components of the NADPH oxidase system are inherently, quantitatively or qualitatively, abnormal in Gaucher disease.

OZ and FMLP activate the NADPH oxidase complex through a receptor-coupled response, while PMA does so by direct activation of PKC. Since superoxide generation in response to all three stimulators was equally affected by glucocerebroside, it is most likely that glucocerebroside interferes with the signal transduction at, or distal to, PKC. The precise mechanism remains to be defined.

Compared with chronic granulomatous disease (CGD), which is characterized by reduced respiratory burst and increased susceptibility to bacterial infections, the extent of monocyte abnormality in Gaucher disease is smaller and, unlike CGD, granulocytes are not affected. These differences account for the lower incidence of infection in Gaucher disease patients.

Despite occasional reports describing the occurrence of recurrent or fulminant infections in Gaucher disease pa-

and to the relatively prolonged exposure of monocytes, compared with granulocytes, to elevated plasma glucocerebroside concentrations.

Superoxide generation, or the respiratory burst, is produced by an enzymatic complex known as NADPH oxidase, in response to various microbial pathogens and by soluble inducers. It constitutes the principal mechanism responsible for the killing of invading microbial pathogens. In resting cells, the various components of the enzyme complex are separated between the cell membrane and the cytosol. The membrane component is a flavocytochrome b558 that contains heme, flavin, and NADPH binding sites. The three recognized cytosolic components are p47, p67, and rac2, a ras-related guanosine triphosphate (GTP)-binding protein. On activation, p47 undergoes phosphorylation, and the three cytosolic components translocate to the membrane to form the active enzyme complex. Available evidence implies an important role for the activation and translocation from the cytosol to the cell membrane of protein kinase C (PKC) in the process of phosphorylation of the p47 component and stimulation of the NADPH oxidase complex.

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Despite occasional reports describing the occurrence of recurrent or fulminant infections in Gaucher disease pa-

![Graph](image-url)

**Fig 4.** Time course of glucocerebroside effect on stimulated superoxide generation in normal monocytes. Peripheral blood monocytes were incubated with glucocerebroside (25 µg/mL) for various periods. At the end of each incubation period, superoxide generation rate was determined after stimulation by either PMA, OZ, or FMLP. The results are expressed as percent of parallel incubations without glucocerebroside. Cell viability was confirmed by trypan blue exclusion and MTT staining. Results are the means ± SE of three separate experiments.

![Graph](image-url)

**Fig 5.** Effect of PMA or SDS on superoxide generation rates in monocytes from a Gaucher patient and a healthy control. Superoxide generation was stimulated by either PMA (50 ng/mL) or SDS (100 µmol/L) in the presence of FAD (1 µmol/L) and NADPH (100 µmol/L). FAD and NADPH did not alter PMA-induced superoxide generation. The results are the means ± SE of a representative experiment. There were three other experiments with similar results.
without glucocerebroside (20 pg/mL). (B) Superoxide generation in a cell-free system. Cytosol (1106 cell-equivalents) and membranes (5 x 10^5 cell-equivalents) from normal or from glucocerebroside-treated monocytes were mixed. Monocytes used in these experiments were obtained from the same donor. The results are the means ± SE expressed as superoxide dismutase-inhibitable reduction of acetylferricytochrome c (Δ OD550) from a representative experiment of three identical experiments.

Fig 6. Kinetic data of superoxide generation in intact monocytes and in a cell-free system. (A) Superoxide generation, stimulated with PMA (50 ng/mL), in cultured normal monocytes incubated with or without glucocerebroside (20 μg/mL). (B) Superoxide generation in a cell-free system. Cytosol (10^6 cell-equivalents) and membranes (5 x 10^5 cell-equivalents) from normal or from glucocerebroside-treated monocytes were mixed. Monocytes used in these experiments were obtained from the same donor. The results are the means ± SE expressed as superoxide dismutase-inhibitable reduction of acetylferricytochrome c (Δ OD550) from a representative experiment of three identical experiments.

patients, gross susceptibility to infection in these patients is remarkably undocumented. Nevertheless, we strongly believe that osteomyelitis in Gaucher disease patients, particularly by anaerobic pathogens, could represent specific local failure of the reticuloendothelial system to eliminate invading pathogens. Susceptibility toward systemic infections is probably less likely due to the relative integrity of granulocytes function.

Until further data regarding infections in Gaucher disease patients become available, the possibility of impaired host defense in these patients should be considered. Since enzyme replacement therapy effectively decreases plasma glucocerebroside, and thus could ameliorate the adverse effects of glucocerebroside on macrophage activity, enzyme replacement should be considered as ancillary therapy in patients with Gaucher disease associated with recurrent or chronic infections.

REFERENCES


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Monocyte dysfunction in patients with Gaucher disease: evidence for interference of glucocerebroside with superoxide generation [see comments]

Y Liel, A Rudich, O Nagauer-Shriker, T Yermiyahu and R Levy