Defective Lymphocyte Glycosidases in the Macrophage Activation Cascade of Juvenile Osteopetrosis

By Nobuto Yamamoto, Venkateswara R. Naraparaju, and Paul J. Orchard

Generation of macrophage-activating factor requires a precursor protein, Gc protein (serum vitamin D3-binding protein), as well as participation of β-galactosidase of inflammation-primed B lymphocytes and sialidase of T lymphocytes. The treatment of human peripheral blood mononuclear cells with an inflammatory lysophospholipid induced β-galactosidase and sialidase activity of lymphocytes, leading to the generation of macrophage-activating factor and activation of monocytes/macrophages. However, lysophospholipid treatment of peripheral blood mononuclear cells from three infantile patients with osteopetrosis resulted in no significant activation of monocytes/macrophages. The lysophospholipid-inducible β-galactosidase activity of B lymphocytes as well as that of the sialidase of T lymphocytes was found to be defective in these patients.

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Materials and Methods

Chemicals, reagents, and media. Lysophosphatidylcholine, o-nitrophenyl β-D-galactopyranoside and sodium 2-(4-methylumbelliferyl)-α-D-N-acetylneuraminate were purchased from Sigma Chemical Co (St Louis, MO). Lymphoprep (similar to Ficoll) was obtained from Polysciences, Inc (Warrington, PA). Phosphate-buffered saline (PBS), composed of 1 mmol/L sodium phosphate (pH 7.0) and 0.15 mol/L NaCl, was prepared without addition of Ca2+ and Mg2+ ions (PBS). Human sera were obtained from healthy children and OP children. Human Gc protein is found in three forms in the serum: Gc1f, Gc1s, and Gc2. The Gc1f and Gc1s subtypes of Gc1 have a sialic acid residue, whereas Gc2 does not. Gc1 protein, which contains both the Gc1f and Gc1s types, was purified by vitamin D-affinity chromatography and was used for the present study. β-Galactosidase and sialidase were purchased from Sigma Chemical Co and Boehringer Mannheim Biochemicals (Indianapolis, IN). Treatment of human Gc protein with immobilized β-galactosidase and sialidase generates an extremely high titered MAF.
Cultivation of mouse peritoneal macrophages for 3 hours with the presence of 50 pg GcMAF/mL generated about a 10-fold increased superoxide-generating capacity above that of untreated macrophages.

For manipulation in vitro and cultivation of peripheral blood cells (lymphocytes and monocytes/macrophages), 0.1% egg albumin-supplemented RPMI 1640 medium (EA medium) and 0.1% human serum-supplemented RPMI 1640 medium were used. Using the Limulus amebocyte lysate assay,\(^2\) we routinely tested for freedom of lipopolysaccharide contamination in the stock solution of enzyme suspension, human Gc protein, and culture media.

*Juvenile OP patients.* Three patients were tested. Patient OP-1 is a two-year-old child who had received an unrelated bone marrow transplant (BMT) but rejected the graft, with no significant change in manifestations of the disorder. Patient OP-2 received a matched sibling BMT 7 years previously at 7 months of age, and despite the presence of a minimal graft (approximately 5% circulating mononuclear cells were of donor origin at the time of testing) previous hepatosplenomegaly resolved, with moderate improvement in the bone marrow cavity. A 2-month-old infant (OP-3) had not received a BMT at the time of testing. Before transplantation, B lymphocytes of patients OP-1 and OP-2 were transformed with Epstein-Barr virus (EBV).

Peripheral blood samples from OP and healthy children. Blood samples were collected in purple-top tubes that contained EDTA to prevent coagulation. A 5-mL blood sample and 5 mL saline (0.9% NaCl) were mixed and gently laid on Lymphoprep (3 mL) contained in a 15-mL centrifuge tube and were centrifuged at 1,000g for 15 minutes. The peripheral blood mononuclear cell (PBMC) layer allowed adherence of phagocytes to the plastic substrata. Lymphocytes (B and T cells) was collected, washed twice with 10 mmol/L sodium phosphate buffer containing 0.15 mol/L NaCl, suspended in a 0.1% EA medium, and placed in 16-mm wells. Incubation for 45 minutes in a 5% CO\(_2\) incubator at 37°C allowed adherence of phagocytes to the plastic substrate. Lymphocytes (nonadherent cells) were removed. Phagocytes (2 × 10\(^5\) cells per well) of individual patients were cultured in EA medium supplemented with 100 pg GcMAF/mL for 3 hours at 37°C and assayed for superoxide generation of the phagocytes to determine the capacity of patient phagocytes to be activated.

*Roles of lymphocytes and plasma Gc protein in activation of phagocytes.* PBMC (mixture of lymphocytes and phagocytes) of individual patients and control subjects were incubated with 1 \(\mu\)g lyso-Pc/mL in EA medium at 37°C for 30 minutes. Phagocytes and lymphocytes were separately washed with PBS, admixed, and cultured for 3 hours at 37°C in a medium supplemented with Gc protein (1 ng/mL) or the patient's own plasma (0.1%), and assayed for activation of phagocytes as determined by superoxide generation.\(^{21,23}\)

Briefly, phagocytes were washed with PBS and incubated in 1 mL PBS containing 20 \(\mu\)g cytochrome c for 10 minutes. About 30 minutes after addition of phorbol-12-myristate acetate (5 \(\mu\)g/mL), the superoxide-generating capacity of the phagocytes was determined by cytochrome-c reduction, measured spectrophotometrically at 550 nm. The data were expressed as nanomoles of superoxide produced per minute per 10\(^5\) cells. The values from this assay with Gc protein and patient PBMC determined the MAF-generating capacity of patient lymphocytes. The assay with healthy PBMC and patient plasma determined the precursor activity of plasma Gc protein.

*Assay of \(\beta\)-galactosidase on B-cell surface.* Peripheral B cells were purified by the previously described procedure\(^{24,25}\) and were incubated with 1 \(\mu\)g lyso-Pc/mL in EA medium at 37°C. After a 30-minute incubation, the cells were washed two to three times in Hanks' balanced salt solution and once in 1 mmol/L PBS (pH 7.2) containing 0.15 mol/L NaCl. The cell pellet was then resuspended in 1 mmol/L PBS containing 0.15 mol/L NaCl at a concentration of 5 × 10\(^6\) cells/mL. Isotonicity of media for preparation of cell suspension was maintained to prevent cell lysis. Because B-cell \(\beta\)-galactosidase is a cell surface bound enzyme, the enzyme assay was carried out by the successive addition of 200 \(\mu\)L of cell suspension (5 × 10\(^6\) cells/mL) and 200 \(\mu\)L of \(\alpha\)-nitrophenyl-\(\beta\)-galactopyranoside (4 mg/mL or 0.5 mmol/L) in Eppendorf tubes. The reaction mixture was incubated at 37°C for 30 minutes by tumbling motion to ensure efficient contact between the substrate and the membrane-bound enzyme. The reaction was stopped by adding 200 \(\mu\)L of a 1 mol/L Na\(_2\)CO\(_3\) solution. The reaction mixture was centrifuged. Production of \(\alpha\)-nitrophenol was determined by the absorbance of the supernatant at 420 nm using Beckman DU-640 Spectrophotometer. This assay was also used to determine the lyso-Pc inducible \(\beta\)-galactosidase activity of EBV-transformed B lymphocytes of patients OP-1 and OP-2 as well as EBV-transformed donor B cells.

*Assay of sialidase (neuraminidase) on T-cell surface.* The assay\(^{25,26}\) was performed by the successive addition of 100 \(\mu\)L of PBS (pH 7.0), 200 \(\mu\)L of cell suspension (5 × 10\(^6\) cells/mL), and 100 \(\mu\)L of 0.4 mmol/L sodium 2-(4-methylumbelliferyl)-o-N-acetylneuraminic acid in small glass tubes. The reaction mixture was incubated at 25°C for 30 minutes by tumbling motion. Cleavage of the substrate by sialidase yields the fluorescent product, 4-methylumbellifere. Substrate without cell suspension served as a blank to determine the nonspecific degradation of the substrate. The reaction was terminated by the addition of 2 mL of absolute ethanol. The reaction mixture was centrifuged at 1,000g for 15 minutes. The supernatants were transferred into new tubes. One hundred microliters of 1 mol/L NaOH was added to each sample before reading fluorescence (excitation, 365 nm; emission, 450 nm) with a Perkin-Elmer (Norwalk, CT) 650–105 Fluorescence Spectrophotometer.

**RESULTS**

*Functions of patient phagocytes and Gc protein.* A deficiency in the macrophage activation cascade could result from (1) an inadequate response of monocytes/macrophages (phagocytes) to MAF, (2) a deficiency in the production or function of Gc protein, or (3) an inability of lymphocytes to convert Gc protein to MAF. The defect in these mediators can be ascertained by in vitro analyses using various combinations of the individual mediators\(^{26,27}\) from OP and healthy children. To investigate the ability of OP phagocytes to be activated by MAF, phagocytes of OP patients or a control were incubated for 3 hours in EA medium supplemented with 100 pg GcMAF/mL, and activation was determined by superoxide production. Both patient and control phagocytes were efficiently activated as shown in Fig 1A. Subsequently, the sera of OP patients were tested for the precursor activity of Gc protein using control lymphocytes to convert Gc protein to MAF. Functional Gc protein was shown to be present in all three patients in concentrations comparable with that of controls (Fig 1B). These studies confirmed that these OP patient phagocytes were capable of activation by MAF and that the functional precursor protein (Gc protein) required for the generation of MAF was present in the OP patient sera.

**Lymphocyte glycosidases.** The capacity for OP patient lymphocytes to generate MAF from serum Gc protein was tested by incubating lyso-Pc–treated PBMC from patients or controls in media supplemented with purified Gc protein (1 ng/mL), and the production of MAF was determined by superoxide generation of the phagocytes. As shown in Fig 2, the superoxide-generating activity of phagocytes after 3-
A

![Graph A](image)

**Fig 1.** Activation of phagocytes with GcMAF or with serum Gc protein. (A) Superoxide-generating capacity of phagocytes treated with GcMAF. (B) Superoxide-generating capacity of phagocytes activated by the incubation of lyso-Pc-treated PBMC with patient or control sera. Healthy human PBMCs were treated with 1 μg lyso-Pc/mL in EA medium for 30 minutes. Lymphocytes and adherent phagocytes were washed separately, admixed, and cultured in EA media supplemented with 0.1% patient or control sera for 3 hours. Phagocytes were assayed for superoxide-generating capacity.

hour cultivation of lyso-Pc–treated healthy PBMC in medium supplemented with Gc protein (4.6 nmol superoxide produced/10⁶ phagocytes) was increased 19-fold above the control value of 0.24 nmol superoxide produced/10⁶ phagocytes with medium alone (see Fig 1B). In contrast, lyso-Pc treatment of all three OP patient PBMCs resulted in no significant activation of phagocytes. Because the production of MAF is dependent on the enzyme levels of both lyso-

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Pc–inducible β-galactosidase of B cells and sialidase of T cells, it was desirable to examine the individual glycosidase activities of the lymphocytes from these patients. To characterize the β-galactosidase function of patient B cells, Gc protein was incubated in medium with lyso-Pc–treated patient lymphocytes for 2 hours. The conditioned culture medium was then treated with immobilized sialidase (0.1 U) for 1 hour. The resultant medium was added to cultures of healthy control phagocytes for 3 hours. As shown in Fig 2, no significant activation of phagocytes was demonstrated, indicating that the B lymphocytes of all three patients are defective in lyso-Pc–inducible β-galactosidase.

To evaluate the sialidase function of patient T lymphocytes, Gc protein was first treated with immobilized β-galactosidase (0.1 U) for 1 hour. β-Galactosidase–treated Gc protein was added to the lyso-Pc–treated patient lymphocyte culture for 2 hours. The resultant conditioned media were used for cultivation of healthy phagocytes. No significant activation of phagocytes could be demonstrated (Fig 2), indicating that the T lymphocytes of all three patients are defective in lyso-Pc–inducible sialidase. These findings are therefore consistent with defects in both the lyso-Pc–inducible β-galactosidase of B lymphocytes and sialidase of T lymphocytes.

**Lack of β-galactosidase in patient B lymphocytes.** To confirm these findings, specific assays for β-galactosidase of B lymphocytes and sialidase of T lymphocytes were performed. As has been described in mouse and rat B cells, two β-galactosidase isozymes, constitutive (designated,
and lyso-Pc-inducible (designated, BgL), are present in healthy human B-cell membranes and can be distinguished on the basis of lyso-Pc inducibility. The membranous \( \beta \)-galactosidase activity of healthy-child B cells was examined by using \( o \)-nitrophenyl \( \beta \)-d-galactopyranoside as the substrate. Because B-cell \( \beta \)-galactosidase is cell-surface bound, washed lyso-Pc-treated or -untreated B cells (10° cells/200 \( \mu \)L) were incubated with \( o \)-nitrophenyl \( \beta \)-d-galactopyranoside at pH 7.0 for 30 minutes under isotonic conditions, and production of \( o \)-nitrophenol was determined by absorption at 420 nm. When healthy human B cells were treated with lyso-Pc, the total \( \beta \)-galactosidase activity level increased approximately 2.8 times above the constitutive isozyme activity level of the untreated control as shown in Fig 3A. In contrast, the lyso-Pc-treated B cells of OP patients exhibited no significant increased level of membranous \( \beta \)-galactosidase above that of the untreated patient B cells, confirming that the lyso-Pc-inducible isozyme is absent in the B cells of these patients. The \( \beta \)-galactosidase activity of lyso-Pc-treated B cells of patient OP-2 is slightly higher than that of patients OP-1 and OP-3, which may relate to the presence of a small proportion of donor-derived mononuclear cells. To further substantiate this observation, EBV-transformed B lymphocytes of patients OP-1 and OP-2 established before transplantation, as well as transformed cells from their donors, were tested for lyso-Pc-inducible \( \beta \)-galactosidase activity. Neither of the transformed OP patient B-cell lines showed an increase in \( \beta \)-galactosidase activity after lyso-Pc stimulation, whereas transformed donor B cells exhibited an approximately twofold increase, confirming that the lymphocytes established before BMT were deficient in inducible \( \beta \)-galactosidase activity (Fig 3B).

**Lack of sialidase in patient T lymphocytes.** Similar to the lyso-Pc inducibility of the mouse and rat Neu-1 gene-encoded sialidase, 72 hours after treatment of healthy human T lymphocytes with lyso-Pc (1 \( \mu \)g/mL) a 2.2-fold increase in sialidase activity above that of untreated T lymphocytes was observed, as shown in Fig 4. Untreated T cells express equal amounts (approximately 3.5 nmol) of two sialidases, noninducible (designated Neu,) and inducible (designated Neu,) isozymes. The OP patient T cells appear to retain activity of the Neu, sialidase (3.5 nmol) but not the Neu, isozyme.

**DISCUSSION**

MAF is a protein, with N-acetylgalactosamine, produced by sequential removal of galactose and sialic acid residues from Gc protein by membranous \( \beta \)-galactosidase and sialidase of the lymphocytes. These enzymes are induced by inflammation or inflammatory lipid metabolites such as lyso-Pc. MAF increases phagocytic capabilities of monocytes/macrophages, which can be measured by production of superoxide. From the present study we conclude that the OP patients lack both lyso-Pc-inducible \( \beta \)-galactosidase of B lymphocytes and sialidase of T lymphocytes, leading to an inability to generate MAF. A small level of lyso-Pc-inducible \( \beta \)-galactosidase and sialidase activity present in patient OP-2’s lymphocytes appears to be a reflection of the presence of 5% circulating donor cells. Determination of \( \beta \)-galactosidase activity in EBV-transformed B cells isolated from patient samples before the BMT confirmed that no significant inducible \( \beta \)-galactosidase activity was present in B cells of patient OP-2. The absence of both the inducible \( \beta \)-galactosidase and sialidase of lymphocytes in these patients is unique and consistent.

In the murine oplop mutation, the development of osteoclasts, monocytes, and macrophages is severely affected. Osteopetrosis in the oplop mouse is thought to result from a frameshift mutation within the coding region of the M-CSF gene. Daily administration of purified recombinant
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Fig 4. Time course of specific sialidase activity of T cells after treatment of osteopetrotic and control T cells with 1 μg lyso-Pc/mL.

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