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A Novel Carbohydrate-Glycosphingolipid Interaction between a β-(1–3)-Glucan Immunomodulator, PGG-glucan, and Lactosylceramide of Human Leukocytes*

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The immunomodulator Betafectin® PGG-glucan is a homopolymer of glucose derived from yeast cell walls which has been demonstrated to enhance leukocyte anti-infective activity *in vitro* and *in vivo*, without the induction of proinflammatory cytokines. We report here the purification of a PGG-glucan-binding element from human leukocytes and its identification as lactosylceramide, a major glycosphingolipid of neutrophils, which includes the CDw17 epitope. The binding of radiolabeled PGG-glucan to purified lactosylceramide was saturable, specific, and time- and temperature-dependent. Lactosylceramides from human leukocytes were fractionated by high performance liquid chromatography in order to analyze the effect of ceramide structure on binding. A variety of fatty acid chain lengths with varying degrees of unsaturation were found to support binding to radiolabeled PGG-glucan. However, DL-lactosylceramides containing dihydrosphingosine did not bind. Radiolabeled PGG-glucan bound several other neutral glycosphingolipids with a terminal galactose, including galactosylceramide, globotriaosylceramide, and gangliotetraosylceramide. The binding of radiolabeled PGG-glucan to lactosylceramide was not inhibited by glycogen, dextran, mannan, pustulan, laminarin, or a low molecular weight β-(1–3)-glucan, but was inhibited by high molecular weight β-(1–3)-glucans and by a monoclonal antibody to lactosylceramide. Although this glycosphingolipid has been shown in numerous reports to bind various microorganisms, this represents the first report of lactosylceramide binding to a macromolecular carbohydrate.

Crude preparations of yeast β-glucans have been known for over 40 years to stimulate animal defense mechanisms, and it is now generally believed that the active component in these preparations is β-(1–3)-glucans. β-(1–3)-Glucans are major components of yeast and fungal cell walls, therefore, this stimulation may reflect a natural defense in response to breakdown products of the fungal cell wall. Both soluble and particulate β-glucans have numerous biological activities including stimulation of the reticuloendothelial system, induction of hematopoiesis, activation of the complement and/or cytokine system, inhibition of tumor cell growth, and induced resistance to infections (for reviews, see Ref. 1 and 2). The various activities of β-(1–3)-glucans may reflect the presence of multiple cellular targets or receptors. Several different receptors for β-glucans have in fact been identified on leukocytes, including 160- and 180-kDa proteins on human monocytes and U937 cells (3) and the leukocyte complement receptor 3 protein (4). Other cellular receptors that recognize β-(1–3)-glucans have been described but not yet defined biochemically (5–8). Most glucan preparations that have been studied directly activate cells and induce cytokines, limiting their potential clinical use. In addition, intravenous administration of particulate glucans is toxic, resulting in embolization and the formation of granulomas (9).

PGG-glucan (Betafectin®) is a highly purified, neutral, water-soluble glucan that by contrast does not directly activate cells or induce the production of proinflammatory cytokines (10, 11) (reviewed in Ref. 12). It is a glucose homopolymer of 170,000 ± 20,000 Da derived from the cell wall of *Saccharomyces cerevisiae*, and is comprised of a β-(1–3)-glucan backbone containing β-(1–6)-linked, β-(1–3) branches. It has been shown to have immunomodulatory effects in animals and in humans. For instance, in mice PGG-glucan rapidly mobilized peripheral blood progenitor cells (10). In a rat model for periodontitis, PGG-glucan reduced infection-mediated periapical bone resorption, enhanced the number of circulating neutrophils and monocytes, and increased neutrophil phagocytic activity (13). *In vitro*, PGG-glucan has recently been shown to activate a transcription factor heteromer in a murine monocytic cell line (14) and, when immobilized, to elicit respiratory burst activity and secretion of tumor necrosis factor-α by rat macrophages (15). Immunomodulatory effects in humans were shown in phase II clinical trials, in which PGG-glucan was shown to reduce postoperative infection rates and to shorten the length of hospitalization (16, 17). PGG-glucan has also recently been shown to elicit functional responses in human neutrophils, including enhancement of oxidative burst and microbicidal activity, and induction of nuclear transcription factors.[1] In efforts to characterize the receptor responsible for these functions, it was also shown that binding of radiolabeled PGG-glucan could be detected in membranes from human leukocytes, and that the binding was due primarily to the neutrophil content. This binding activity was shown to be distinct from other reported glucan receptors.

We identify here the binding site on human leukocytes as lactosylceramide and describe the binding characteristics of radiolabeled PGG-glucan to immobilized LacCer.[2] LacCer con-

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[2] The designation of glycolipids is according to the recommendations of the Nomenclature Committee of the International Union of Pure and Applied Chemistry (18). Other abbreviations used are: HBSS, Hanks' balanced salt solution; HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry.

tains the CDw17 epitope, a cellular differentiation antigen expressed on the surface of mature myeloid cells (19). This glycolipid is especially abundant in human neutrophils where it makes up two-thirds of the total glycolipids, with approximately 20% found in the cell membrane (20, 21). Nevertheless, its function in these cells remains largely unknown.

EXPERIMENTAL PROCEDURES

Materials—Lactosylceramide was purified from human leukocyte membranes as described below, unless otherwise specified. All other sphingolipids, ceramides, phospholipids, and enzymes were from Sigma, except porcine lactosylceramide, which was from Matreya, Inc. (Pleasant Gap, PA) and jack bean β-galactosidase, which was from Oxford GlycoSystems Inc. (Rosedale, NY). Polystyrene 96-well plates were from Corning (New York). High performance TLC plates, 0.2-mm thickness, were from E. M. Science (Gibbstown, NJ). Silica gel 60 (200–400 mesh) was from Aldrich. PGG-glucan (170 ± 20 kDa), low molecular weight glucan (18 kDa), and high molecular weight glucan (1,000 kDa) were manufactured by Alpha-Beta Technology, Inc., and resuspended in 0.9% NaCl to the indicated concentration. Other macromolecular carbohydrates were obtained as follows: pustulan (average 20 kDa, Calbiochem), oyster glycogen (ICN, Cleveland, OH), dextran (average 70 kDa), laminarin, and mannan (Sigma). The control ascites fluid was a mouse IgM directed against *Cryptosporidium parvum* (Biodesign International, Kennebunk, ME). Solvable™ was from NEN Life Science Products. Protein concentration was determined with the BCA reagent (Pierce) using bovine serum albumin as a standard.

Preparation of Human Leukocyte and U937 Membranes—Leukocytes were sedimented from human blood (Red Cross, Dedham, MA) by the addition of an equal volume of 3% dextran. The following steps were carried out at 4 °C. Red blood cells were removed by hypotonic lysis, and leukocytes were resuspended in 3–4 volumes of phosphate-buffered saline containing protease inhibitors, then lysed by sonication with a probe tip (50 watts, 30 × 1-s pulses). Following removal of nuclei and remaining intact cells by low speed centrifugation (700 × *g*, 7 min), membranes were collected from the supernatant by ultracentrifugation (180,000 × *g*, 60 min), and resuspended in HBSS to 5 mg of protein/ml.

Membranes were prepared from the human monocytic cell line U937 by the lysis procedure described above following growth of the cells in RPMI medium (Life Technologies, Inc.).

Preparation of ^{3}H-Labeled PGG-glucan—The polyaldehyde of PGG-glucan was prepared by oxidation with a 20-fold molar excess of $NaIO_4$ for 72 h in the dark. Following dialysis, the oxidized PGG-glucan was reductively labeled by NEN Life Science Products with 100 mCi of [^{3}H]$NaBH_4$, then dialyzed to give [^{3}H]PGG-glucan, specific activity 3.1 μCi/μg hexose.[1]

Assay for [^{3}H]PGG-glucan Binding to Membranes—Membranes (700 μg of protein; 140 μl) were incubated for 1.5 h at 37 °C with [^{3}H]PGG-glucan (1 μg/ml) and with unlabeled PGG-glucan (1 mg/ml) to determine nonspecific binding or an equal volume of 0.9% NaCl to determine total binding. Assays were brought to a total volume of 350 μl with HBSS. For assays containing ascites fluid, membranes (525 μg of protein; 105 μl) were incubated with [^{3}H]PGG-glucan and 0.9% NaCl or unlabeled PGG-glucan, as above. Aliquots (100 μl) of each assay were centrifuged (5 min, 12,000 × *g*), and the resulting pellets were rinsed with HBSS and dissolved in Solvable™, and radioactivity was determined. Specific binding is presented as the mean of triplicate samples ± S.D. and was calculated as (total binding) − (nonspecific binding).

Preparation of Reconstituted Membrane Lipids—Human leukocyte or U937 membranes (5 mg of protein; 1 ml) were extracted with chloroform and methanol (3:2:1 chloroform/methanol/membranes, by volume) essentially as described previously (22). The resulting upper and lower layers were separated from the proteinaceous interphase. Reconstituted membrane lipids were prepared by drying the combined layers under a stream of argon, followed by resuspension in HBSS (to the original volume of starting membranes) with brief sonication. 140 μl of the lipid suspension was assayed for [^{3}H]PGG-glucan binding activity as described above in a total volume of 350 μl. Where indicated, the upper (or lower) layer from an extraction of human leukocyte membranes was combined with the lower (or upper) layer of a U937 membrane extraction before drying.

Purification of the [^{3}H]PGG-glucan Binding Moiety from Human Leukocyte Membranes—The lower layer from an extraction of membranes (100 mg of protein) prepared as described above was dried under a stream of argon, dissolved in chloroform/methanol (10:1), then applied to a silica gel column (1 × 0.8 cm) equilibrated in chloroform. The column was eluted successively with chloroform, acetone, acetone/methanol (9:1), and methanol (22). Fractions were concentrated to dryness, and the acetone/methanol fraction was redissolved in chloroform/methanol (10:1) then applied to a DEAE-Sephadex A-25 column as described previously (23). The column was eluted with chloroform/methanol/water (30:60:8), then chloroform/methanol/0.8 M NaOAc (30:60:8). The chloroform/methanol/water fraction was dried, redissolved in chloroform/methanol (5:1), then applied to a second silica gel column (25 × 0.8 cm) equilibrated in chloroform. The column was eluted with 60 ml each of chloroform, chloroform/methanol (7.5:1), chloroform/methanol (5:1), chloroform/methanol (2:1), then methanol.

Aliquots of fractions from each column were dried and resuspended in ethanol for use in the 96-well plate assay described below.

TLC Analysis—Samples and standards were analyzed using high performance TLC silica gel plates run in chloroform/methanol/water (80:20:2) for separation of glycosphingolipids, or in 1-butanol/ethanol/water (5:5:4) for separation of mono- and disaccharides. Glycosphingolipids and saccharides were visualized with orcinol spray reagent or by iodine vapors (23).

Enzymatic Degradation of LacCer—LacCer from human leukocyte membranes was treated with jack bean β-galactosidase or ceramide glycanase (23). To test enzyme-treated fractions in the binding assay, detergent and enzyme were removed by passing the reaction over a Sep-Pak C_{18} cartridge (Waters Corp., Milford, MA) (23).

HPLC Fractionation of LacCer from Human Leukocyte Membranes—Fractionation of LacCer into individual components was carried out using a modification of a described procedure (24) on a Hewlett Packard 1090 HPLC using a 5-μm Symmetry C_{18} column (3.9 × 150 mm, Waters Corporation) and a mobile phase of 3.5% 0.2 M ammonium acetate in methanol at a flow rate of 1 ml/min. Peaks with UV absorbance at 206 nm were collected, dried down by vacuum, and analyzed by TLC to identify fractions containing LacCer. Isolated fractions were quantitated using a S.E.D.E.R.E. Sedex 55 evaporative mass detector (Alfortville France) at 45 °C, using high purity nitrogen at 2.1 bar as effluent.

GC-MS Analysis—Isolated fractions from HPLC were analyzed for sugar, fatty acid, and sphingosine composition following methanolysis (25) as follows. An aliquot was methanolyzed and the methanol solution extracted with hexane. The extracted fatty acid methyl esters were analyzed by GC-MS on a Hewlett Packard 5890 series II gas chromatograph with a Hewlett Packard 5971A mass selective detector. The methanol layer was dried, trimethylsilylated, and dissolved in hexane, and the resulting trimethylsilylated methyl glycosides were analyzed by GC-MS. A second aliquot was treated with ceramide glycanase and freeze dried. The resulting acylated sphingosine and oligosaccharide were trimethylsilylated, dissolved in hexane, and analyzed by GC-MS on a HT-5 aluminum-clad capillary column.

Binding Assay in 96-Well Plates—The indicated glycolipid/sphingolipid or purification fraction was suspended in ethanol, and aliquots were applied in triplicate to the wells of a 96-well plate then dried under a stream of argon. The following components were added to each well: PGG-glucan (1 mg/ml) or an equal volume of 0.9% NaCl, [^{3}H]PGG-glucan (1 μg/ml), and HBSS (80 μl) in a final volume of 100 μl. Plates were incubated at 37 °C for 1.5 h unless indicated otherwise, then supernatants were removed from each well and discarded. Wells were rinsed twice with HBSS (200 μl), then Solvable™ (100 μl) was added, and the plate was incubated at 60 °C for 5 min, after which supernatants were transferred to vials containing scintillation fluid, and radioactivity was determined. To verify that the glycolipids/sphingolipids were not dissociating from the plate during the incubation, mock reactions were run without [^{3}H]PGG-glucan, incubated, and rinsed as above, then chloroform/methanol (1:2) was added to wells. The liquid was transferred from the wells, concentrated, and analyzed by TLC. Following orcinol and/or iodine visualization, no detectable decrease in staining intensity was seen compared with the amount coated. Specific binding was determined as described for the membrane binding assay.

Preparation of a Monoclonal Antibody to LacCer—A mouse monoclonal antibody to LacCer was developed using antigen comprised of a LacCer/bovine serum albumin mixture prepared as described for globoside (26). Female BALB/c mice were inoculated subcutaneously with 0.1 ml of the antigen mixed in an equal volume of TiterMax Adjuvant (Vaxcel, Inc., Norcross, GA). Hybridomas and ascites fluid were prepared following the procedure and schedule described (27), except that TiterMax was used as adjuvant throughout the procedure. Antibodies were detected by enzyme-linked immunosorbent assay using LacCer immobilized as described above, with alkaline-phosphatase-conjugated goat anti-mouse IgG/IgM (Tropix Inc., Bedford, MA) as a secondary antibody. One LacCer-specific hybridoma was isolated, 8D12, which secreted specific IgM antibody.

RESULTS

Binding Activity from Human Leukocyte Membranes Copurifies with LacCer—Extraction of human leukocyte membranes with chloroform/methanol results in two phases (upper and lower layers) and a proteinaceous interphase. When reconstituted, the upper plus lower layers showed significant specific binding to [^{3}H]PGG-glucan, indicating that reconstituted membrane lipids retained binding capacity (Fig. 1*A*). In fact, a 4-fold increase in binding activity was recovered from the extraction as compared with the starting membranes. As a control, U937 membranes, which do not show appreciable binding, were extracted in the same manner and found not to demonstrate binding. We found that the upper or lower layers of human leukocyte membrane extractions did not pellet efficiently by microcentrifugation when assayed for binding alone. Therefore, we combined each of these layers with the opposite layer from a U937 membrane extraction to determine where the binding activity resided. This combination revealed that the activity apparently resided in the lower layer of the human leukocyte membrane extraction. In addition, the organic-extractable binding activity was essentially quantitatively recovered following silica and DEAE chromatography of the lower layer (Fig. 1*B*). Following a previously described purification scheme known to separate the general classes of non-lipids, gangliosides, phospholipids, neutral lipids, and glycosphingolipids (28) we found that the activity in the lower layer purified as a neutral glycosphingolipid which co-migrated on TLC with a standard LacCer doublet (Fig. 1*C*). This compound was not detected in extracts from U937 membranes. The purified compound also represented a major orcinol-reactive species in the starting leukocyte membrane extract, and LacCer is known to be the major neutral glycosphingolipid in human neutrophils (29). By TLC analysis, we found that the purified material produced compounds co-migrating with GlcCer and galactose when treated with β-galactosidase, and a compound co-migrating with lactose upon treatment with ceramide glycanase, consistent with a LacCer (Galβ1–4GlcCer) structure (Fig. 1*C*). 1 mg of LacCer was purified from ~1×10^9 human leukocytes (100 mg of leukocyte membrane protein).

The Binding of [^{3}H]PGG-glucan to Immobilized LacCer Is Time- and Temperature-dependent—To characterize the binding of [^{3}H]PGG-glucan to LacCer, we examined several aspects of this interaction using the 96-well plate assay. This assay was found to be linear to 1.6 μg/well LacCer (Fig. 2*A*). The binding of [^{3}H]PGG-glucan to immobilized LacCer was time-dependent, with equilibrium binding reached at approximately 60 min (Fig. 2*B*). Temperature dependence of the binding was observed with no detectable binding of [^{3}H]PGG-glucan at 25 °C or lower, but significant binding at 37 °C (Fig. 2*C*).

Competition for [^{3}H]PGG-glucan Is Specific for β-(1–3)-Glucan of Defined Structure—[^{3}H]PGG-glucan binding to LacCer was reversible and was inhibited by unlabeled PGG-glucan (IC_{50} ~9 μg/ml) in the 96-well plate binding assay (Fig. 3*A*). We tested other glucans and mannan at concentrations close to this value for their ability to compete with [^{3}H]PGG-glucan binding to LacCer. Only β-(1–3)-glucan structures were found to compete for binding, since dextran, glycogen, pustulan, or mannan did not compete at 15 μg/ml (Fig. 3*B*). Competition by β-(1–3)-glucan was found with PGG-glucan and a high molecular weight β-(1–3)-glucan, while lower molecular weight β-(1–3)-glucan (and laminarin) did not compete efficiently for [^{3}H]PGG-glucan binding. These β-(1–3)-glucans differ not only in their size but in some cases their degree of branching and length of branches. Therefore β-(1–3)-glucan of specific structure is necessary to compete [^{3}H]PGG-glucan binding to LacCer.

In addition, binding of [^{3}H]PGG-glucan to LacCer was not competed with lactose, galactose, glucose, or the corresponding methyl glycosides at 100 mM, or with the corresponding *p*-nitrophenyl glycosides at 11 mM (data not shown).

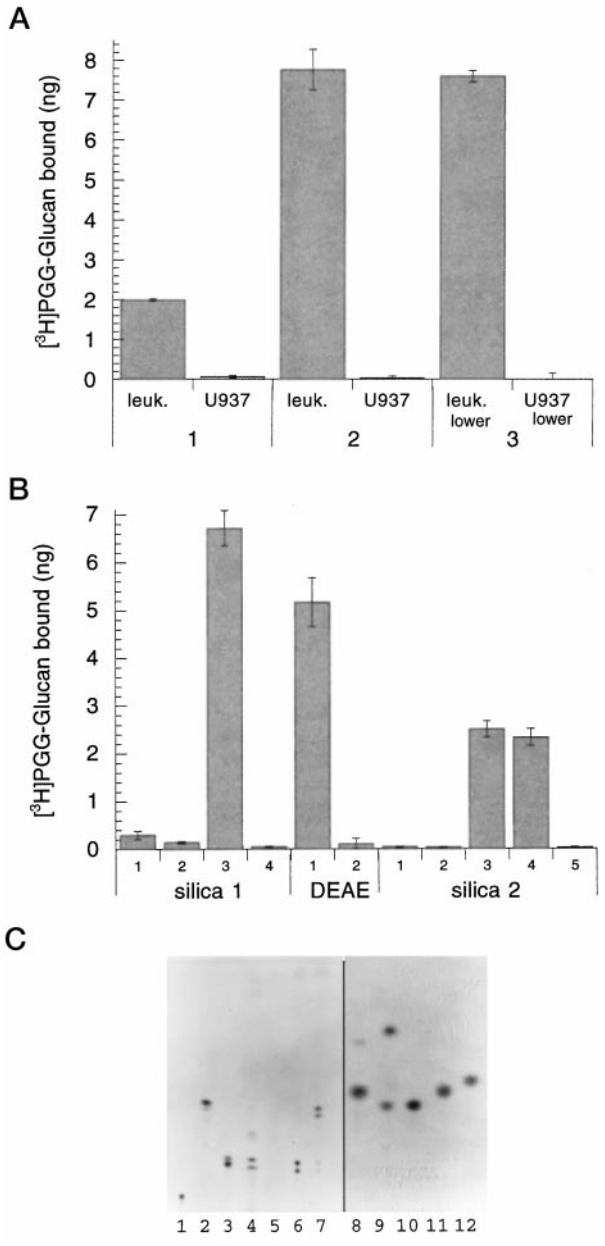

FIG. 1. **Purification of the binding moiety from human leukocyte membranes.** *A*, specific binding of [^{3}H]PGG-glucan to *1*, leukocyte or U937 membranes; *2*, reconstituted lipids from leukocyte or U937 membranes; or *3*, combined leukocyte plus U937 layers from an organic extraction of membranes was determined using the membrane or reconstituted membrane lipid binding assays described under "Experimental Procedures." *B*, the lower layer of extracted leukocyte membranes was fractionated by silica and DEAE and then silica chromatography with the following solvents; silica 1: *1*, chloroform; *2*, acetone; *3*, acetone/methanol (9:1); *4*, methanol; DEAE-Sephadex: *1*, chloroform/methanol/water (30:60:8); *2*, chloroform/methanol/0.8 M sodium acetate (30:60:8); silica 2: *1*, chloroform; *2*, chloroform/methanol (7.5:1); *3*, chloroform/methanol (5:1); *4*, chloroform/methanol (2:1); *5*, methanol. Total binding of [^{3}H]PGG-glucan to 0.2% of each fraction is shown and was determined using the 96-well plate assay described under "Experimental Procedures." *C*, TLC was carried out with the solvent systems 80:20:2 (chloroform/methanol/water, *lanes 1–7*) or 5:5:4 (1-butanol/ethanol/water, *lanes 8–12*), and chromatograms were visualized with orcinol reagent. *1*, Gb3; *2*, GlcCer; *3*, LacCer (bovine); *4*, lower layer of leukocyte membrane extract; *5*, lower layer of U937 membrane extract; *6*, silica 2, combined fractions 3 and 4 (*panel B*, above); *7* and *8*, β-galactosidase-treated LacCer; *9*, ceramide glycanase-treated LacCer; *10*, lactose; *11*, galactose; and *12*, glucose.

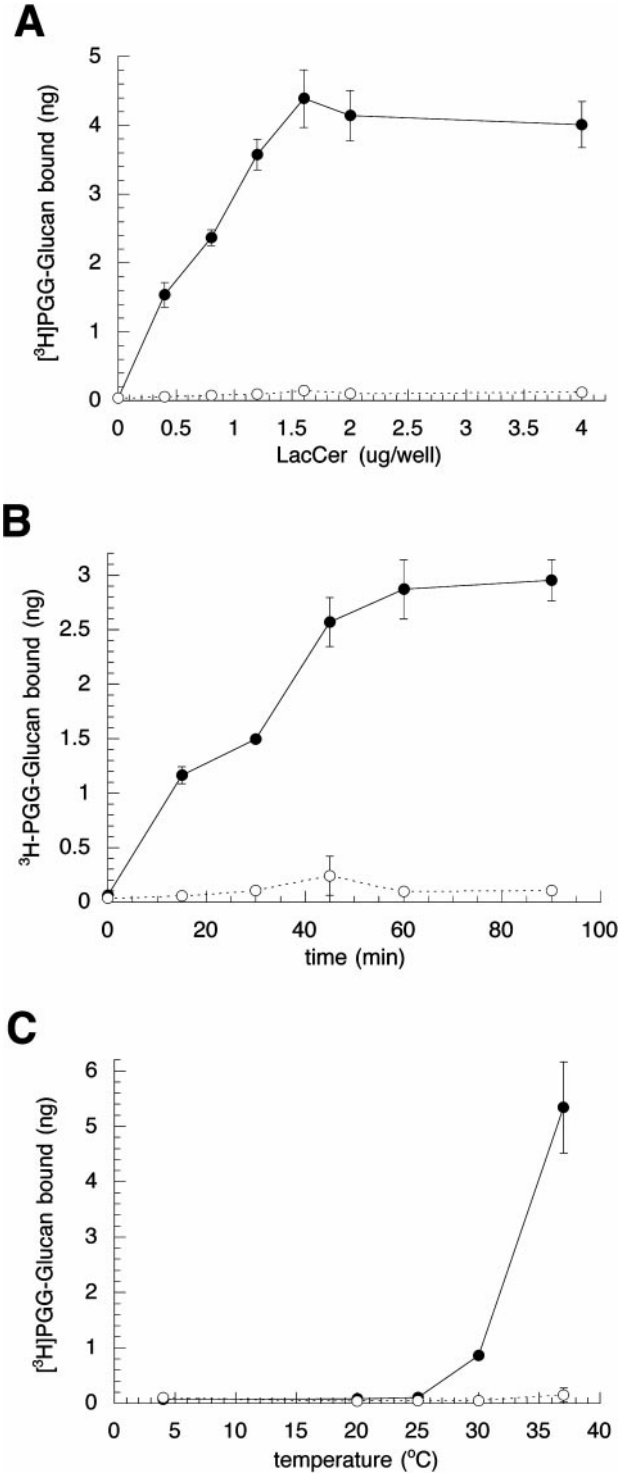

FIG. 2. **Concentration, time, and temperature dependence of [^{3}H]PGG-glucan binding to immobilized LacCer.** Assays were carried out at 37 °C in wells coated with *A,* the indicated amount of LacCer, or *B,* 1 μg/well LacCer for the indicated time. *C,* assays were carried out for 1.5 h at the indicated temperatures with 1 μg/well LacCer. *A–C*, assays were carried out in the absence (*closed circles*) or presence (*open circles*) of 1 mg/ml unlabeled PGG-glucan.

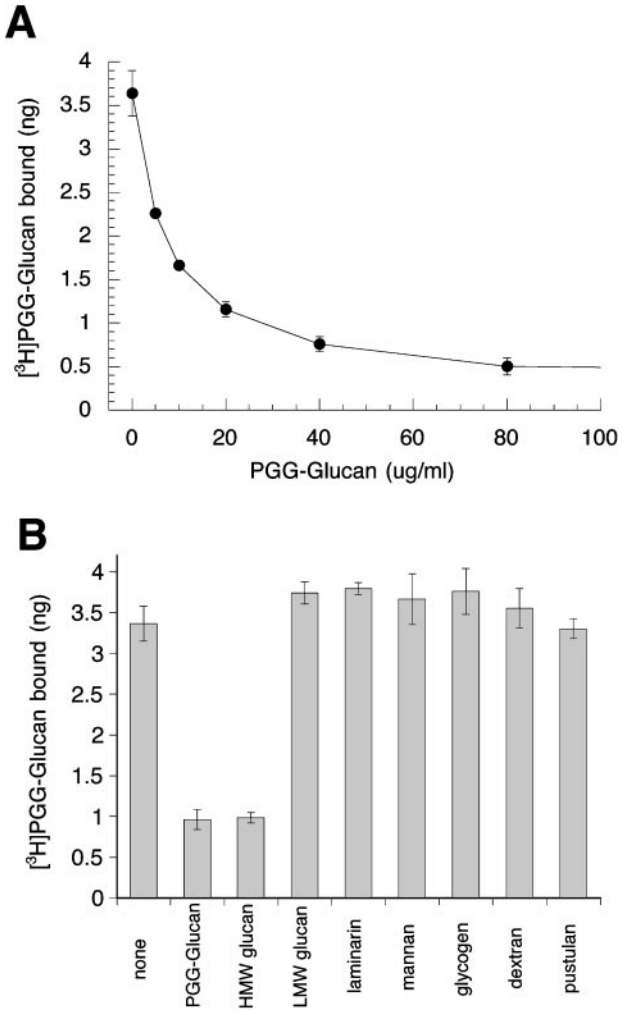

FIG. 3. **Competition of [^{3}H]PGG-glucan binding to LacCer by various glucans or mannan.** LacCer was coated at 1 μg/well, and assays were carried out in the 96-well plate assay at 37 °C in the presence of *A,* the indicated final concentration of unlabeled PGG-glucan, or *B,* indicated glucans or mannan at 15 μg/ml final concentration.

[^{3}H]PGG-glucan Binds to LacCer with a Variety of Fatty Acid Chain Lengths and Degrees of Unsaturation—Previous reports of bacteria or toxin interactions with glycosphingolipids have shown an effect of the ceramide structure on binding (30, 31). We were interested in whether this structure affected [^{3}H]PGG-glucan binding, and consequently isolated individual LacCer species by HPLC. The profile of LacCer content is shown by the evaporative mass trace in Fig. 4, representing HPLC fractionation of LacCer isolated from human leukocyte membranes as described above. The major peaks that contained LacCer by TLC analysis were analyzed by GC-MS to determine the fatty acid structure (denoted in Fig. 4) and the sphingosine structure. The peak at 13 min also contained LacCer by TLC analysis but, upon further separation by HPLC fractionated into three peaks, resulting in insufficient amounts for analysis by GC-MS.

Neutrophils account for ~90% of the binding of [^{3}H]PGG-glucan to human leukocytes.[1] Neutrophils also contribute the majority of LacCer found in whole blood cells, and reportedly contain two species of LacCer, with C16:0 and C24:1 fatty acids (32). Consistent with these data, we found that these were the major species in human leukocyte LacCer (see Fig. 4). The other species of LacCer are likely contributed by other blood cells, including monocytes, lymphocytes (33, 34), and platelets (35, 36).

To determine the influence of LacCer fatty acid structure on [^{3}H]PGG-glucan binding, the isolated LacCer species were analyzed in the 96-well plate assay. As shown in Table I, all species were able to bind [^{3}H]PGG-glucan, with less than a 2-fold range in binding activity. Variations in length or degree of unsaturation of the fatty acid chain seemed to have little effect on the binding activity. In contrast to the isolated LacCer with a C18:1 long chain base, commercially available semisyn-

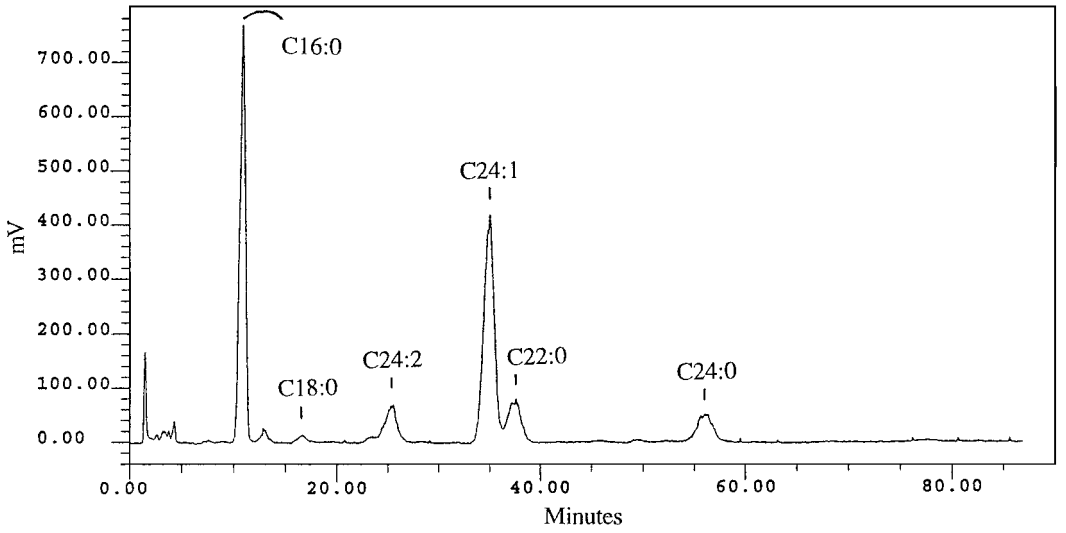

FIG. 4. **HPLC profile of LacCer isolated from human leukocyte membranes.** Evaporative mass trace (45 °C, N_2 effluent, gain 12) is shown for 10 μg of LacCer purified from leukocyte membranes as described in Fig. 1 and "Experimental Procedures." Separation was performed isocratically on a 5-μm C_{18} column using 3.5% ammonium acetate (0.2 M) in methanol at 1 ml/min. The injection mixture was comprised of roughly 100 μl of methanol:methylene chloride (1:1). GC-MS analysis of the indicated peak fractions showed that all contained glucose and galactose in a 1:1 ratio, C18:1 long chain base, and the fatty acid structure indicated over the peak. Quantitation by evaporative mass detection of the HPLC fractions gave the following results for indicated species (% of total): C16:0 (32.4), C18:0 (2.7), C24:2 (8.4), C24:1 (33.9), C22:0 (9.4), and C24:0 (8.1).

TABLE I
Binding of [³H]PGG-glucan to lactosylceramides

Binding of [³H]PGG-glucan to indicated LacCer was carried out in the 96-well plate assay as described under "Experimental Procedures." Specific binding is shown, and each value is a mean of triplicate determinations.

	Fatty acid	Long chain base	[³H]PGG-glucan bound
			ng/μg LacCer
LacCer	C16:0	C18:1	3.31 ± 0.27
	C18:0	C18:1	1.93 ± 0.13
	C24:2	C18:1	2.01 ± 0.23
	C24:1	C18:1	2.31 ± 0.37
	C22:0	C18:1	3.57 ± 0.77
	C24:0	C18:1	3.14 ± 0.54
N-Palmitoyl-DL-dihydrolactocerebroside	C16:0	C18:0	ND[a]
N-Stearoyl-DL-dihydrolactocerebroside	C18:0	C18:0	ND
N-Lignoceroyl-DL-dihydrolactocerebroside	C24:0	C18:0	ND

[a] ND, not detected.

TABLE II
Specific binding of [³H]PGG-glucan to various sphingolipids

1 μg of indicated sphingolipid was assayed in 96-well plates as described under "Experimental Procedures." Each value is a mean of triplicate assays ± S.D. Sphingolipids were demonstrated to bind to the wells throughout the assay period as described under "Experimental Procedures." β-Galactosidase-treated LacCer was prepared also as described under "Experimental Procedures."

	[³H]PGG-glucan bound
	ng/μg sphingolipid
LacCer	3.14 ± 0.29
β-Galactosidase-treated LacCer	0.04 ± 0.05
LacCer (bovine)	2.18 ± 0.39
LacCer (porcine)	2.35 ± 0.23
GalCer Sigma type I	1.02 ± 0.32
GalCer Sigma type II	11.8 ± 0.54
Globotriaosylceramide	0.84 ± 0.13
Gangliotetraosylceramide	0.50 ± 0.10
Gangliotriaosylceramide	0.00 ± 0.01
Ceramides	0.02 ± 0.01
Sulfatides	0.00 ± 0.01
Sphingosine	0.02 ± 0.00
GalSph	0.09 ± 0.00
Globotetraosylceramide	0.00 ± 0.01
GlcCer	0.12 ± 0.03
G_{M1}	0.00 ± 0.02
G_{M2}	0.00 ± 0.12
G_{M3}	0.00 ± 0.01
G_{D3}	0.06 ± 0.19

thetic (DL)-LacCer containing dihydrosphingosine (C18:0) gave no binding activity. The presence of stereoisomers in this preparation might be expected to have a diluting effect on binding activity, but instead complete abrogation was observed. This striking loss of binding activity is, therefore, most likely due to the saturation of the long chain base.

[³H]PGG-glucan Can Bind Other Glycosphingolipids with a Terminal Galactose—When LacCer was treated with jack bean β-galactosidase to give GlcCer, little binding was seen with [³H]PGG-glucan (see Table II). We therefore tested several other commercially available sphingolipids to determine the specificity for binding. As shown in Table II, several glycosphingolipids in addition to LacCer which contain a terminal galactose were found to bind [³H]PGG-glucan, namely, GalCer Sigma types I and II, gangliotetraosylceramide (Gal(β1–4)GalNAc(β1–4)Gal(β1–4)GlcCer), and globotriaosylceramide (Gal(α1–4)Gal(β1–4)GlcCer). GalSph, which contains a terminal galactose but no fatty acid chain, did not bind significantly. GalCer Sigma type I and GalCer Sigma type II (type I contains ~98% α-hydroxy fatty acids, while type II contains ~98% nonhydroxy fatty acids according to the description provided by Sigma) showed a 10-fold difference between them in binding to [³H]PGG-glucan. GalCer II-sulfate (sulfatides) did not bind, nor did the gangliosides G_{M1}, G_{M2}, G_{D3}, or G_{M3}. This finding is consistent with the fact that fractions shown in Fig. 1*B* that did not contain LacCer did not show binding, although since LacCer is the predominant neutral glycosphingolipid, it does not rule out the possibility that other glycosphingolipids could bind if higher levels were used in the 96-well plate assay. Neolactotetraosylceramide (Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)GlcCer), for instance, was not tested here but contains a terminal galactose and is found in granulocytes (32).

An Anti-LacCer Monoclonal Antibody Inhibits [³H]PGG-glucan Binding to Both Immobilized LacCer and Human Leukocyte Membranes—To provide evidence that [³H]PGG-glucan binds to LacCer in human leukocyte membranes, an anti-Lac-

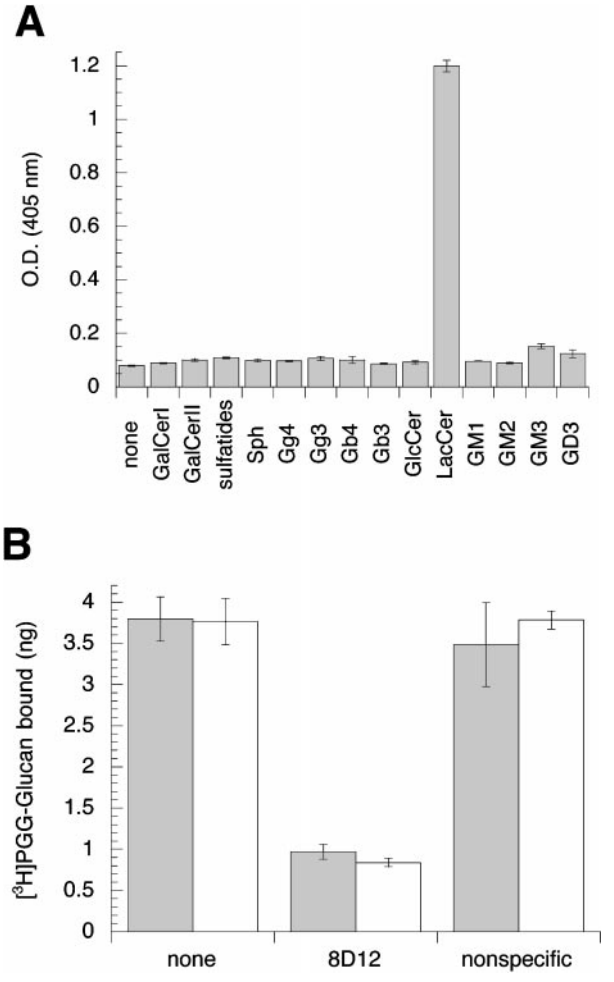

FIG. 5. **Inhibition of binding of [^{3}H]PGG-glucan to immobilized LacCer and to human leukocyte membranes by anti-LacCer antibody.** *A*, monoclonal antibody 8D12 was tested at a 10^4 dilution for specificity against several immobilized glycosphingolipids (coated at 1 μg/well). Detection of bound antibody was carried out as described under "Experimental Procedures." *B*, LacCer was coated at 1 μg/well, and the 96-well plate assay was carried out as described under "Experimental Procedures" in the presence or absence of a 1:2 dilution of 8D12 or nonspecific ascites fluid (*shaded bars*). Leukocyte membranes (*open bars*) were assayed as described under "Experimental Procedures" in the presence or absence of a 1:2 dilution of 8D12 ascites or nonspecific ascites.

Cer monoclonal antibody (8D12) was developed which was found to specifically recognize LacCer (see Fig. 5*A*). This antibody inhibited ~75% of [^{3}H]PGG-glucan binding to immobilized LacCer and to human leukocyte membranes (Fig. 5*B*). The 8D12 antibody did not inhibit [^{3}H]PGG-glucan binding to GalCer Sigma type II (data not shown).

DISCUSSION

PGG-glucan is a macromolecular carbohydrate that has been shown to elicit several functional responses in human neutrophils, presumably through cell surface receptors. The purpose of this study was to identify compounds from human leukocyte membranes that bind this carbohydrate immunomodulator. After a significant effort failed to identify binding proteins, we turned our attention to other membrane components. Reported here, binding activity was found in an organic extract of human leukocyte membranes. Following organic extraction of these membranes, we found a 4-fold increase in binding activity and after purification found that this activity corresponded to the glycosphingolipid LacCer. The increase in activity indicates that some of the LacCer in human leukocyte membranes is inaccessible to PGG-glucan, but is exposed when lipids are extracted and reconstituted. A population of LacCer on human neutrophils, in fact, has been suggested to be "cryptic" based on its inaccessibility to a monoclonal antibody (37).

To characterize the binding of radiolabeled PGG-glucan to LacCer, we employed the common technique of immobilization of glycosphingolipids on an insoluble matrix. Many reports exist which describe the immobilization of glycosphingolipids to silica TLC plates, or 96-well polyvinylchloride or polystyrene plates, in order to measure binding to microorganisms, toxins, viral coat proteins, or receptors (30, 31, 38). Using the 96-well plate assay described here, we characterized the binding of [^{3}H]PGG-glucan to LacCer purified from human leukocytes and found that these characteristics closely resemble those for [^{3}H]PGG-glucan binding to human leukocyte membranes.[1] Also, a monoclonal antibody to LacCer inhibited binding to approximately the same extent with human leukocyte membranes or immobilized LacCer.

The epitopes on LacCer and PGG-glucan that interact have yet to be determined. The fact that galactose, glucose, or lactose did not inhibit binding may indicate that a multivalent interaction between the glucan and LacCer is occurring. This interpretation is supported by the observation that low molecular weight β-(1–3)-glucans did not compete for PGG-glucan binding while the high molecular weight β-(1–3)-glucan did compete. The reducing and nonreducing ends of PGG-glucan are modified upon radiolabeling the molecule, indicating that intact ends are not required for the interaction. The importance of the galactose of LacCer was shown by the diminution of binding following the enzymatic removal of this monosaccharide. Furthermore, all other glycosphingolipids that demonstrated binding to PGG-glucan contained a terminal galactose. However, other factors clearly play a role, since G_{M1} and GalSph both have terminal galactoses but do not bind. Interpretation of PGG-glucan binding by the glycosphingolipids (other than the β-galactosidase treated LacCer) given in Table II is complicated by the fact that each contains a mixture of ceramide structures that are possibly different from those of leukocyte membrane LacCer. The sphingosine contribution to binding, however, was clearly demonstrated with the dihydrolactosylceramides shown in Table I. These compounds differ from corresponding LacCer species isolated from leukocyte membranes only in their sphingosine structure, but did not bind [^{3}H]PGG-glucan.

The glycosphingolipids other than LacCer that were shown to bind [^{3}H]PGG-glucan (Table II) have not been reported to be present in neutrophils, but have been shown in other cell types to bind a range of molecules. Globotriaosylceramide, for instance, is found in erythrocytes (32), endothelial cells, and monocytes (39). This glycosphingolipid has been reported to be the relevant receptor for verotoxins of *Escherichia coli* in the pathogenesis of hemolytic uremic syndrome (31, 40, 41). GalCer, which is one of the major glycosphingolipids of myelin, has been shown to interact with cerebroside sulfate in a Ca^{2+}-mediated manner, and this interaction was proposed to be involved in the adhesion of extracellular surfaces of myelin (42). GalCer has also been shown to interact with HIV-1 surface envelope glycoprotein gp120, and to act as an alternate receptor for viral entry into $CD4^-$ cells of neural and colonic origin (43–45). Whether PGG-glucan interacts with globotriaosylceramide, GalCer, or gangliotetraosylceramide in these other cell types remains to be determined.

PGG-glucan is derived from the cell wall of *S. cerevisiae*. Interestingly, several yeast including *S. cerevisiae* were previously shown to bind to LacCer immobilized on a TLC plate or in polyvinylchloride microdilution wells (46). The binding of [^{3}H]PGG-glucan to LacCer shows several similarities to that previously reported binding. For example, the yeast/LacCer

interaction showed temperature dependence (binding was seen at 37 °C, not at 4 °C), was not inhibited by lactose, and was abolished upon β-galactosidase treatment of the LacCer. In the same report, yeast did not bind a synthetic LacCer (DL-dihydrolactocerebroside containing palmitoyl fatty acid), and this lack of binding was attributed to the short chain fatty acid, although our results indicate that the sphingosine portion may be involved. While the binding component(s) on yeast cells for LacCer has not been isolated thus far, our data suggests that cell wall β-glucans could be involved.

Several bacteria have also been shown to bind LacCer (for review, see Ref. 47). Bacterial colonization and subsequent infection is generally thought to depend in part on adherence of the bacteria to host cell-surface glycosphingolipids. LacCer is widely distributed in epithelial tissues where colonization often occurs, and may even be generated by the action of bacterial enzymes (sialidases, for example) on some of the host's larger cell-surface glycolipids (47). In a rat model for intra-abdominal sepsis brought on by the implantation of cecal contents, PGG-glucan treatment was found to reduce mortality and result in a lower bacterial load in the blood (48). The cecal contents were composed of a semidefined set of microorganisms, and some overlap exists in this set and the set of bacteria demonstrated to bind LacCer. In addition to its leukocyte immunomodulatory activities, PGG-glucan may therefore interfere with bacterial colonization and/or entry into the blood through binding to LacCer.

LacCer has been shown to give a biological response under certain circumstances. It has been demonstrated to be involved in cell proliferation (49) and signal transduction (50) in human aortic smooth muscle cells. In human granulocytes, Lund-Johansen *et al*. (51) demonstrated that anti-LacCer monoclonal antibodies, when bound to LacCer on the cell surface, could activate respiratory burst in those cells following cross-linking of the antibodies with a secondary antibody. Enhanced degranulation and increased calcium flux were also seen under these circumstances. The authors suggested, therefore, that LacCer can act as a receptor in granulocytes by mediating a response when aggregated, as may occur by recognition molecules on bacteria. A correlation may exist between this finding and the one reported here. Thus PGG-glucan, which has been shown to have an oxidative priming effect on human neutrophils (12), may bind LacCer in a multivalent fashion, aggregating the glycolipid to some degree. In fact, we have recently demonstrated that the anti-LacCer antibody 8D12 will bind to the cell surface of human neutrophils by fluorescein-activated cell sorting analysis, and that the antibody will block a signal transduction event mediated by PGG-glucan in these cells.[1] The lactose epitope in cells is found solely in glycosphingolipids, not in glycoproteins (37, 52), and although the antibody may recognize epitopes on human leukocyte membranes that were not tested here, the combined data suggest that [^{3}H]PGG-glucan binds to LacCer on human leukocytes.

In conclusion, we have demonstrated a specific carbohydrate-glycosphingolipid interaction between a β-(1–3)-glucan from the cell wall of yeast and LacCer from human leukocytes. This glucan has been shown to have anti-infective properties, which may involve its interaction with LacCer. We are currently investigating the role of LacCer and other molecules that may be involved in the PGG-glucan-mediated response in human neutrophils, and trying to better understand the nature of the interaction between LacCer and this macromolecular carbohydrate.

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