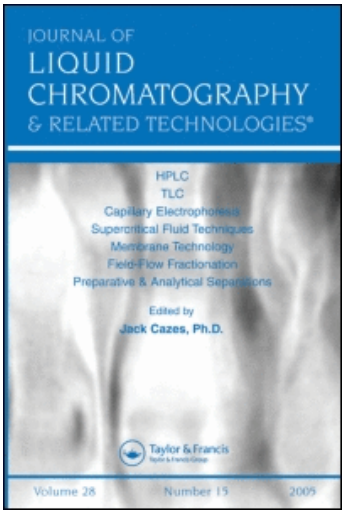

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Separation of Phospholipids and Glycolipids Using Analytical Toroidal-Coil Counter-Current Chromatography. II. Comparison of the Hydrophobicity Between *Mycoplasma fermentans* and Human-Brain Lipids

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ABSTRACT

Previously, we reported the successful separation of human-brain lipids by toroidal-coil countercurrent chromatography (TC-CCC) avoiding

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emulsification and optimizing the solvent systems. In this study, the TC-CCC technique was applied for the analysis of phosphocholine-containing glycoglycerolipids (GGPL-I and GGPL-III) of *Mycoplasma fermentans*, which is thought to be one of the causative microorganisms of rheumatoid arthritis (RA). Neutral lipids of *M. fermentans* were separated and their elution profile compared with that of human-brain lipids using a hexane : ethyl acetate : ethanol : 0.1% TFA (5 : 5 : 5 : 4, v/v/v/v) solvent system. In this solvent system, the hydrophobicities of GGPL-III and GGPL-I were similar to those of lysophosphatidylcholine and sphingomyelin (SPM II), respectively. Glycoglycerolipid III was isolated, and further separated into at least two molecular species, using an optimized solvent system composed of hexane : ethyl acetate : ethanol : 0.1% TFA (3 : 5 : 3 : 4, v/v/v/v). The TC-CCC technique is a powerful tool for the separation of lipids of microorganisms, and more importantly, it may become a useful tool for the analysis of a host-pathogen interaction or, in other words, a lipid–protein interaction at lipid microdomains.

Key Words: Counter-current chromatography; Phospholipid; Glycolipids; Lipid microdomain; *Mycoplasma fermentans*.

INTRODUCTION

Counter-current chromatography (CCC) is a liquid partition chromatography utilizing two immiscible solvent phases to eliminate the use of a solid support.[1–5] The partition process takes place in an open column in which one phase (the stationary phase) is retained, while the other phase (the mobile phase) continuously equilibrates solutes with the stationary phase. Because the method is free from the influence of a solid phase or spacers, it may be useful for studying the hydrophobicity and the behavior of various kinds of lipids. Previously, we have shown that high-speed CCC (HSCCC) separates alkali-labile glycolipids (ALGLs) isolated from the human brain into several groups.[6] In that experiment, HSCCC could finely separate the molecular species in the final step of purification. However, the method failed to separate the crude samples due to severe emulsification resulting in the subsequent loss of the stationary phase from the column. To overcome this problem, we used an analytical-scale toroidal-coil counter-current chromatography TC-CCC instrument, which takes advantage of radial acting strong centrifugal force to minimize emulsification and provide stable retention of the stationary phase.[7]

In the TC-CCC system, retention of the stationary phase can be improved by increasing the revolution speed (centrifugal force) and/or reducing the flow-rate of the mobile phase, thus, allowing the universal application of the

two phase solvent systems for separation of a variety of compounds. In this study, this TC-CCC system was applied for the separation of phospholipids and glycolipids. By optimizing the composition of the two-phase solvent systems, we succeeded in separation of major brain lipids.[8] Mycoplasmas have been suspected as a pathogen of chronic inflammatory diseases. *Mycoplasma fermentans* invades more frequently in tissues compared with other mycoplasmas, and it has been reported as a causative pathogen of rheumatoid arthritis (RA).[9–11] We found unique phosphocholine-containing glycoglycerolipids (GGPLs) as major antigens in *M. fermentans*,[12] and determined their structures.[13–17] Glycoglycerolipids have residues which are important for their biofunctions and give molecular similarity to the second messengers of cell signal transduction.[18,19] We proposed the hypothetical role of *M. fermentans* in the pathogenesis of RA.[20–23] We made specific anti-GGPL III monoclonal antibodies and examined the existence of GGPL-III in synovial tissues in RA. Glycoglycerolipids significantly increased IL-6 and TNF-α production in peripheral blood monocytes from healthy volunteers and proliferation of RA synovial fibroblasts.

These findings suggest that GGPLs from *M. fermentans*, as a persistent pathogen, play a role in initiating and perpetuating synovitis of RA. There were reports indicating that GGPLs have prostaglandin or IL-6 inducing activity[24] and MHC class II suppression activities.[25] Therefore, considering the pathogenesis of *M. fermentans*, it is important to isolate and examine the hydrophobicities of GGPLs. In this study, we used the TC-CCC centrifuge to separate the lipids of *M. fermentans*, and compared them with human brain lipids. Finally, we suggest that the TC-CCC technique may be a useful tool for analyzing lipid–protein interaction, or a host–pathogen interaction at lipid microdomains.

EXPERIMENTAL

Apparatus

The present studies employ a commercial model of the toroidal coil centrifuge (TC-CCC 1000) purchased from Pharma-Tech Research Corporation (Baltimore, MD). The apparatus is a compact, table-top unit measuring 30 cm × 30 cm × 40 cm. It is equipped with a flow-through device without the use of rotary seals, according to the principle described previously.[3] The rotation speed is continuously adjustable up to 3000 rpm with a speed regulator equipped with a digital display. The toroidal coil separation column was prepared by winding a 0.4 mm I.D., 60 m long polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ) onto a nylon pipe of

1.5 mm O.D., making a right-handed coil. Then, the coiled tube was affixed to the inner wall of the cylindrical centrifuge bowl (12 cm in diameter and 5 cm in height), thus forming a doughnut-shaped configuration (toroidal coil) consisting of two to three coiled layers. This toroidal coil measures about 6 m in length (made from 60 m long PTFE tubing) and consists of 12,000 helical turns with a total capacity of about 8 mL.

The inlet and outlet flow lines were made from thick-wall PTFE tubes (0.35 mm I.D.) that withstand constant flexing movements. A chromatographic metering pump (model 515 HPLC pump, Waters, USA) was used for pumping the mobile phase, and a fraction collector (Ultrorac, LKB Instruments, Stockholm, Sweden) was used to collect the eluate into test tubes.

Reagents

Phospholipid standards (Phospholipid kit) were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). Cerebroside (bovine) was purchased from Sigma Chemical Co. (St. Louis, MO). Water, hexane, and ethyl acetate, all of reagent grade, were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Ethanol was purchased from Pharmaco Products Inc. (Brookfield, CT). Trifluoroacetic acid (TFA) was purchased from Pierce Chemical Co. (Rockford, IL). Ammonium hydroxide was purchased from J. T. Baker (Philipsburg, NJ).

Human-Brain Lipid Extraction and Purification

Human brain tissue (200 g in wet weight) was homogenized, and total lipids were extracted successively with 3 L each of mixtures of chloroform : methanol, 2 : 1, 1 : 1, and 1 : 2, by volume. The total lipid extract was evaporated to dryness in a rotary evaporator, suspended, dialyzed against distilled water, and then lyophilized. Unbound (neutral) lipids and bound (acidic) lipids fractions were separated with a column packed with DEAE Sephadex A-25 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) (bed volume: 200 mL), as described elsewhere.[26]

Mycoplasma fermentans Cell Culture and Lipid Extraction

Mycoplasma was grown at 37°C in PPLO broth (Difco Laboratories, Detroit, USA) supplemented with 10% (v/v) fetal bovine serum, 5% yeast extract (Flow Laboratories, McLean, VA), 1000 U/mL of penicillin, and 0.002% (w/v) phenol red. One percent dextrose (final concentration) was added for the growth of *M. fermentans*, and 0.2% *L*-arginine monohydrochloride (final concentration) for the growth of the *L*-arginine-utilizing

mycoplasmas. The mycoplasma was harvested by centrifugation at $10{,}000 \times g$ for 30 min, and washed twice with phosphate-buffered saline (PBS), pH 7.2.

The procedures used for lipid extraction and purification were described previously.[14–16]

Preparation of Two-Phase Solvent System and Sample Solution

Each solvent system was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use. The sample solution was prepared by dissolving 1–5 mg of lipids in 0.05 mL each of the upper and lower phases.

Counter-Current Chromatographic Procedure

In each separation, the toroidal coil was first entirely filled with a stationary phase (either the upper or the lower phase), and a sample solution was injected into the coil. Then, the other phase was pumped into the column while the column was rotated at the desired rate. The effluent from the outlet of the column was collected into test tubes at a rate of 0.2 mL/tube, at a flow rate of 0.1 mL/min. After the desired peaks eluted, the centrifuge run was terminated and the column contents were fractionated into test tubes at 0.5 mL/tube by eluting the column with the solvent initially used as the stationary phase, at a flow rate of 0.25 mL/min.

High-Performance Thin-Layer Chromatography

Lipids were separated on a high-performance thin-layer chromatography (HPTLC) plate. The developing solvent was a mixture of chloroform, methanol, and 0.2% aqueous $CaCl_2$. Orcinol reagent[27] and Dittmer's reagent[28] were used for the detection of glycolipids and phospholipids.

RESULTS

Separation of Neural Phospholipids and Glycolipids of Human Brain

The elution profiles of neutral human-brain phospholipids and glycolipids by TC-CCC are shown in Fig. 1 where 5 mg of the neutral lipid fraction from human brain lipids was separated using hexane/ethyl

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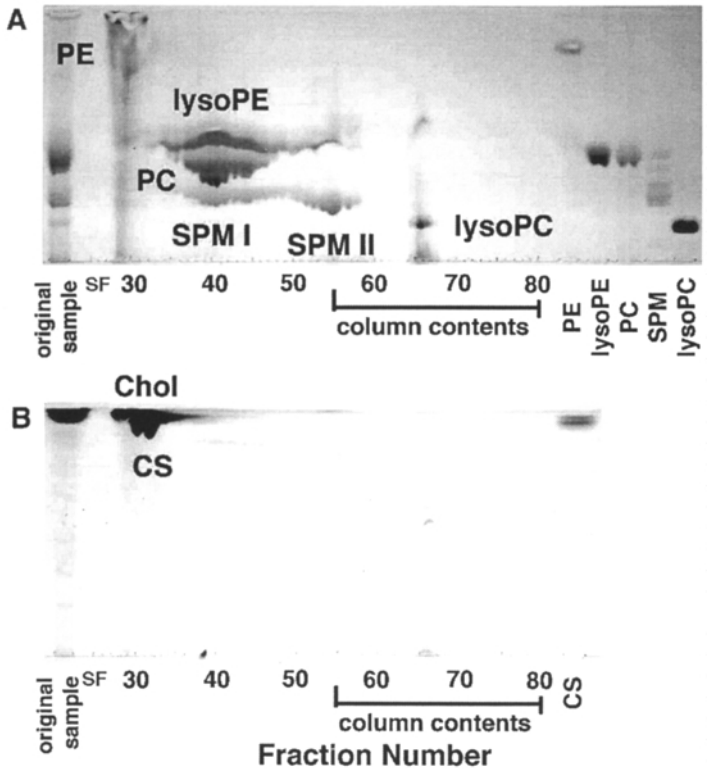

Figure 1. Elution profiles of neural phospholipids (A) and glycolipids (B) of human brain. The solvent system used for TC-CCC was hexane/ethyl acetate/ethanol/0.1% TFA (5 : 5 : 5 : 4, v/v/v/v). Upper phase (organic phase) was mobile. Revolution speed was controlled from 1500 rpm to 700 rpm. The highest column pressure was 350 psi. Five milligrams of neutral fraction of human brain lipids were loaded. Each fraction was spotted, and developed on HPTLC using chloroform : methanol : 0.2% $CaCl_2$ (60 : 32 : 4). *Key:* PC, phosphatidylcholine; SPM, sphingomyelin; PE, phosphatidylethanolamine; lysoPE, lysophosphatidylethanolamine; lysoPC, lysophosphatidylcholine; Chol, cholesterol; CS, cerebrosides; SF, solvent front.

acetate/ethanol/0.1% aqueous ammonia (5 : 5 : 5 : 4). As previously reported,[8] we could separate phospholipids and glycolipids using the same solvent system. As for phospholipids [Fig. 2(A)], phosphatidylethanolamine (solvent front), lysophosphatidylethanolamine (frs. 33–56), phosphatidylcholine (frs. 33–47), sphingomyelin (SPM I, frs. 33–48; SPM II, frs. 47–58), and lysophosphatidylcholine (frs. 66–68) were eluted successively.

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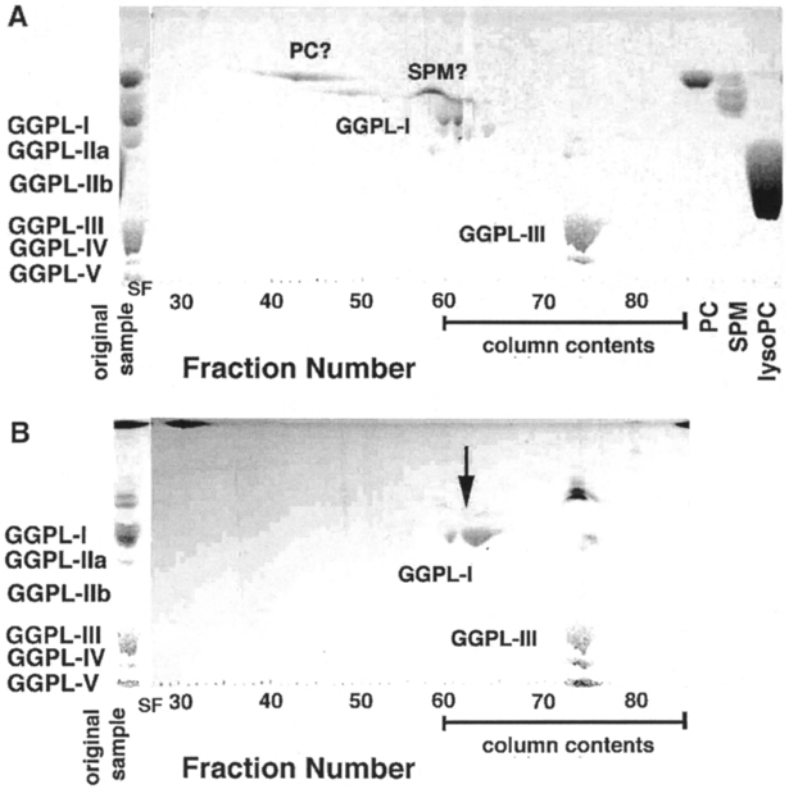

Figure 2. Elution profiles of neural phospholipids (A) and glycolipids (B) of *M. fermentans.* The solvent system used for TC-CCC was hexane/ethyl acetate/ethanol/0.1% TFA (5 : 5 : 5 : 4, v/v/v/v). Upper phase (organic phase) was mobile. Revolution speed was controlled from 1500 rpm to 700 rpm. The highest column pressure was 350 psi. Two milligrams of neutral fraction of *M. fermentans* lipids were loaded. Each fraction was spotted, and developed on HPTLC using chloroform : methanol : 0.2% $CaCl_2$ (55 : 45 : 10). *Key:* PC, phosphatidylcholine; SPM, sphingomyelin; lysoPC, lysophosphatidylcholine; Chol, cholesterol; SF, solvent front.

Spingomyelin, which is usually difficult to separate into two groups with a silica bead column, was completely separated into two groups (SPM I and SPM II). Some other minor phospholipids were eluted at the solvent front or remained in the column. Cerebroside (frs. 28–36) and some other neutral glycolipids (frs. 35–43 and frs. 55–63) were visualized with orcinol staining. Cholesterol was eluted at the solvent front (frs. 28–32) and stained non-specifically in a brown color.

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Elution Profiles of Neural Phospholipids (A) and Glycolipids (B) of *Mycoplasma fermentans*

Neutral lipids of *M. fermentans* were separated with the same solvent system, which was used for the separation of neutral human-lipids in Fig. 1. Figure 2 shows the elution profiles of neutral phospholipids (A) and glycolipids (B) of *M. fermentans* separated using hexane/ethyl acetate/ethanol/0.1% TFA (5 : 5 : 5 : 4). Phospholipids, which correspond to phosphatidylcholine (frs. 36–50) and sphingomyelin (SPM I, frs. 43–53; SPM II, frs. 54–64) were eluted as shown in Fig. 2. Glycoglycerolipids (GGPL-I, IIa, IIb, III, IV, and V), which are stained as both phospholipids and glycolipids, are species-specific main antigens of *M. fermentans.*[12] Glycoglycerolipid-I (frs. 59–67) was separated from other GGPLs (frs. 72–76), and was further separated into two bands (arrow) [Fig. 2(B)].

It is interesting to note, that PE, lysoPE, and other minor phospholipids components, which could be detected around solvent front in the case of human-brain lipids (Fig. 1), were absent in the neutral lipids of *M. fermentans.* Cerebroside also could not be detected.

It is noteworthy that most of the glycolipids of *M. fermentans* are phospholipids, so that the relative amount of glycolipids is apparently higher than that in the human brain.

Purification of Glycoglycerolipid-III of *Mycoplasma fermentans* with Optimized Solvent System by Toroidal-Coil Counter-Current Chromatography

Optimizing the solvent system, we could isolate GGPL-III (fr. 69–82) from other GGPLs of *M. fermentans*, human brain and *M. fermentans* lipids (Fig. 3). A more polar solvent system (hexane/ethyl acetate/ethanol/0.1% TFA at 3 : 5 : 3 : 4, v/v/v/v) was suitable. The GGPL-III was further separated into at least three bands (arrow in Fig. 3). Because the polarity of the solvent system was increased, most of the neutral phospholipids of human brain moved close to the solvent front fraction (data not shown).

DISCUSSION

In this study, we could separate GGPL-I or GGPL-III into two or three bands. These bands may be corresponding to the major molecular species. We have determined the structures of GGPL-I and GGPL-III.[13–17] Both GGPLs contained two moles of saturated stearic acid (C_{18} : 0) and palmitic acid

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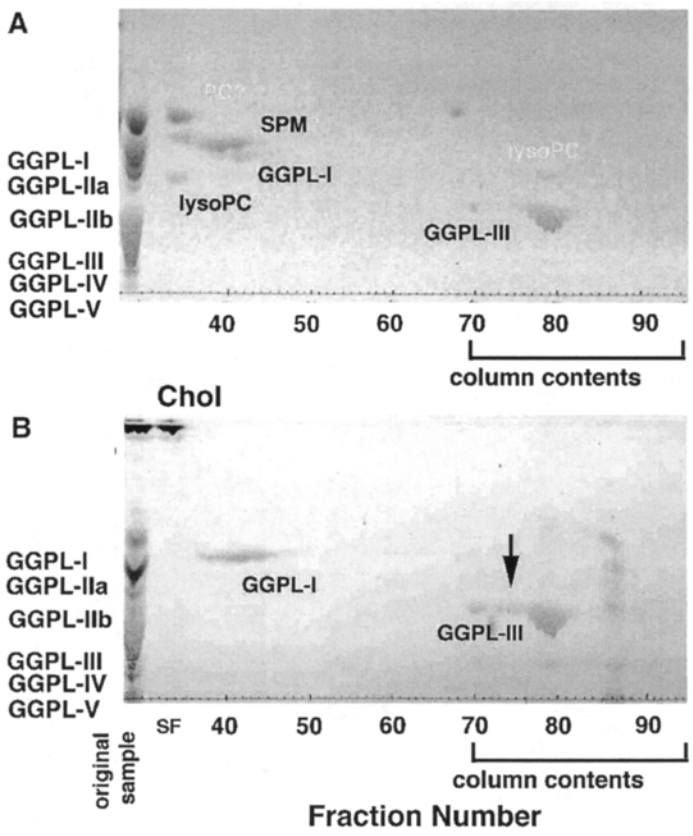

Figure 3. Elution profile of neural phospholipids (A) and glycolipids (B) of *M. fermentans* using more polar solvent system. The solvent system used for TC-CCC was hexane/ethyl acetate/ethanol/0.1% TFA (3 : 5 : 3 : 4, v/v/v/v). Upper phase (organic phase) was mobile. Revolution speed was controlled from 1500 rpm to 700 rpm. The highest column pressure was 350 psi. Two milligrams of neutral fraction of *M. fermentans* lipids were loaded. Each fraction was spotted, and developed on HPTLC using chloroform : methanol : 0.2% $CaCl_2$ (55 : 45 : 10). *Key:* PC, phosphatidylcholine; SPM, sphingomyelin; lysoPC, lysophosphatidylcholine; Chol, cholesterol; SF, solvent front.

(C_{16} : 0). The minor components consisted of saturated myristoic acid, stearic acid, and palmitic acid, or the mixtures of these.

The overall results of our studies showed that human brain contains more classes of lipids, and, also, have many more molecular species than *M. fermentans.* The molecular species of the lipids in *M. fermentans* was much simpler than human counterparts. The present TC-CCC system provides

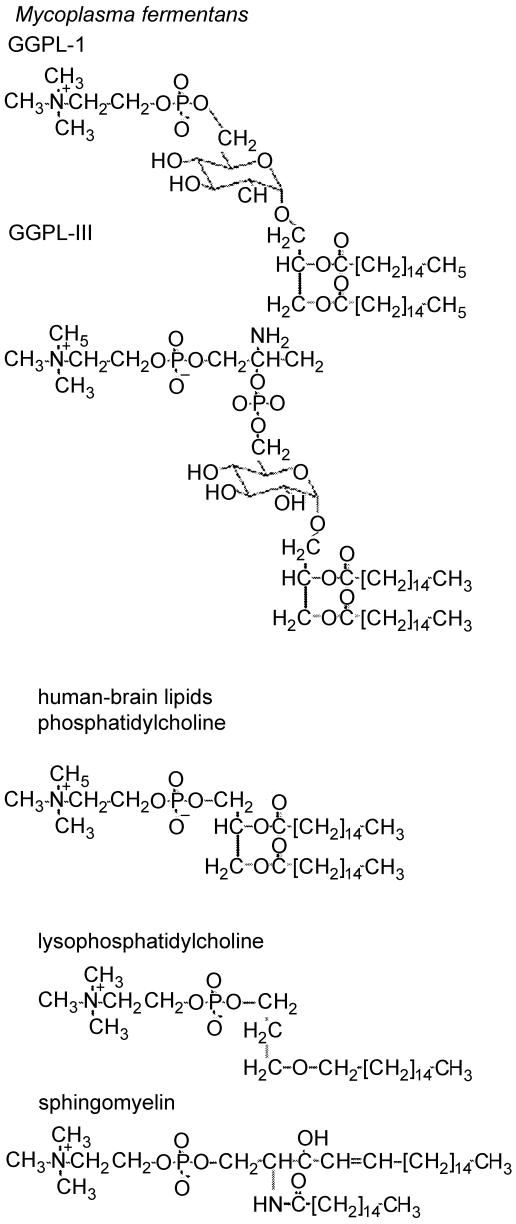

Figure 4. Structures of GGPL-I, GGPL-III, PC, lysoPC, SPM, and sphingosylphosphorylcholine.

a powerful tool for further study on a GGPL metabolic pathway. Combined with a mass-spectrometer, we can characterize the major molecular species, as well as the minor components. The TC-CCC has advantages in separation or purification of natural and chemically synthesized GGPLs. Running cost is much lower than a gel system, because it only needs organic solvents, which occupies 8 mL of the column space. By optimizing the composition of the solvent system, we can apply several milligrams of samples and recover them without loss by the adsorption of the samples to the solid support.

Glycoglycerolipids are choline-containing compounds, and have close similarity to choline-containing human lipids. The hydrophobicity of GGPLs is similar to that of human phosphocholine-containing lipids (PC, lysoPC and SPM). We are interested in the structural similarity between human and *M. fermentans* phosphocholine-containing lipids (Fig. 4). Glycoglycerolipid-I, with 1 mol of glucose, has a similar hydrophobicity to those of PC and SPM, free of sugar moiety. Glycoglycerolipid-III, with an additional phosphate and amino group, is more hydrophilic than PC. The hydrophobicity of GGPL-III is similar to that of lysoPC.

These observations clearly show the similarity of the hydrophobicity between GGPLs and cellular phospholipids, which play an important role in cell signaling transduction.[18,19] Our observations also suggests the possibility that GGPLs may be acting to these cellular molecules to exert the biological functions. The TC-CCC might be an interesting tool for the analysis of molecular interaction in biomembranes. The lipids, which have similar hydrophobicity, form lipid microdomains to play important roles in cell signal transduction. We showed[29] that the TC-CCC system visualized the dynamic change of hydrophobicity of the phospholipids of the human brain. We would like to propose that GGPLs, which is a causative pathogen of RA, affect the lipid microdomains where is a battle field of host–parasite interaction. Our next aim is to apply the TC-CCC for the analysis of molecular interaction in the lipid microdomains.

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