Binding of glycosylglycerolipid derived from membranes of *Acholeplasma laidlawii* PG8 and synthetic analogues to lymphoid cells

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A component that binds to human lymphoid cells was isolated from the membranes of *Acholeplasma laidlawii* PG8. The component was extracted using the Bligh–Dyer method and purified using a silica-gel column and TLC. The active component was identified as 3-O-[2'-O-(α -D-glucopyranosyl)-6'-O-acyl- α -D-glucopyranosyl]-1,2-di-O-acyl-sn-glycerol (GAGDG) using ¹H- and ¹³C-NMR and GC-MS. The compositions of the major saturated fatty acids were nC₁₄ (17.8%), isoC₁₄ (10.7%) and nC₁₆ (34.9%) as determined by GC-MS. The amounts of unsaturated species were less than 10% of those of the corresponding saturated acids. GAGDGs which have three tetradecanoyl groups were synthesized. These synthetic GAGDGs, as well as GAGDGs derived from *A. laidlawii* membranes, had a high binding affinity for MOLT-4 and HUT-78 (human T cell lines), Raji (a B cell line), HL-60 (a monoblastoid cell line) and primary cultured human T cells. The binding affinities of GAGDGs with an isoC₁₄ acyl group was higher than those with nC₁₄ and nC₁₆ acyl groups. The binding to lymphoid cells reveals a novel biological activity of GAGDGs.

Keywords: *Acholeplasma laidlawii*, adherence, glycosylglycerolipids, lymphoid cells

INTRODUCTION

The first stage in many bacterial infections is adherence and formation of microcolonies on mucous membranes. Various components on the outer surface of bacteria, such as protein, polysaccharides and teichoic acid, are known to contribute to adherence (Espersen & Clemmensen, 1982; van Houte 1983; Elleman *et al.*, 1986; Courtney *et al.*, 1986). However, β -lactam antibiotics, which inhibit cell wall synthesis, can induce the formation of L-form variants (Allan *et al.*, 1993). Glycosylglycerolipids are found not only in the cell membranes of mycoplasmas, but also in those of Gram-positive and Gram-negative bacteria (Kates, 1990). Although there have been many investigations into the role of microbial glycosylglycerolipids in the membrane physiology of prokaryotic cells (Shaw, 1970; Silvius *et al.*, 1980; Boggs 1987), we have no knowledge of interactions of glycosylglycerolipids with eukaryotic cells. It is

important in investigating the pathogenesis of bacteria lacking cell walls that such factors which may influence attachment to host cells be studied.

The aim of the work described in this paper was to examine the capacity of glycosylglycerolipids isolated from the membranes of *Acholeplasma laidlawii*, and of chemically synthesized glycosylglycerolipids, to bind to human lymphoid cells.

METHODS

Cells. Two human T-cell lines, MOLT-4 and HUT-78, a human B cell line, Raji, and a human monoblastic cell line, HL60, were maintained in exponential growth in RPMI1640 medium containing 10% foetal calf serum (FCS; Mitsubishi Chemical), 2 mM L-glutamine, 100 μ g penicillin G ml⁻¹ and 100 μ g streptomycin ml⁻¹ (RPMI/FCS). To exclude the possibility of mycoplasma infections in the cell cultures, these cell suspensions were inoculated onto Hayflick's mycoplasma agar medium once a week.

Peripheral mononuclear cells from healthy donors were isolated using Lymphoprep (Nycomed Pharma AS Diag-

Abbreviation: GAGDG, 3-O-[2'-O-(α -D-glucopyranosyl)-6'-O-acyl- α -D-glucopyranosyl]-1,2-di-O-acyl-sn-glycerol.

Table 1. Binding activity of fractions of *A. laidlawii* membrane extracts separated by silica-gel chromatography

MOLT-4 cells (2×10^5) were incubated at 37 °C for 4 h in the presence of a coverglass with 10 µg of each fraction. Values shown are means $\pm$ SD of five assays from one representative experiment.

CHCl ₃ /MeOH (400 ml)	Recovery (%)*	$10^{-4} \times$ Number of adherent cells per coverglass
CHCl ₃	19.9	0.3 ± 0.3
95:5	9.0	0.3 ± 0.2
90:10	30.2	2.8 ± 0.6
80:20	9.1	0.8 ± 0.2
70:30	2.8	0.8 ± 0.4
60:40	3.5	0.5 ± 0.4
MeOH	15.5	0.3 ± 0.2
No sample		0.2 ± 0.2

* [Eluted amounts (dry wt) in each fraction divided by applied chloroform/methanol phase (50 mg)] $\times$ 100.

Fig. 1. Chemical structure of glycosylglycerolipid isolated from membranes of *A. laidlawii*.

nostics) as described previously (Arai *et al.*, 1983). Five million cells ml^{-1} were incubated in a Petri dish at 37 °C for 1 h to remove adherent cells. Nonadherent cells were collected and incubated with M-450 CD4 Dynabeads (Dynal) for 3 h at 37 °C, and CD4⁺ cells were then collected according to the manufacturer's instructions. The purified CD4⁺ cells were cultured in RPMI1640 with 12% FCS, 100 units human recombinant interleukin-2 (Becton Dickinson Labware) ml^{-1} and 100 units of human recombinant interleukin-4 (Genzyme Diagnostics) ml^{-1} in a 24-well cell culture plate (Costar) coated with anti-human CD3 antibody (Serotec).

Cell binding assay. Extracts from the membranes of *A. laidlawii* and synthetic 3-O-[2'-O-(α -D-glucopyranosyl)-6'-O-acyl- α -D-glucopyranosyl]-1,2-di-O-acyl-sn-glycerols (GAGDGs) were dissolved in chloroform and 50 µl samples

were spread on individual 10 mm diameter coverglasses (Matsunami) to make thin films of GAGDGs. To avoid nonspecific adherence of cells, each coverglass was immersed in RPMI/FCS medium. After 1 h, the medium was removed by suction and replaced with serum-free tissue culture medium (Cosmo medium; Cosmo Bio). Cells were suspended in Cosmo medium at a concentration of 4×10^5 cells ml^{-1} and 0.5 ml of the suspension was added to each well of a 24-well cell culture plate with a GAGDG-coated coverglass. The plate was then incubated at 37 °C in a CO₂ incubator for 4 h. The coverglass was then removed with forceps and gently dipped twice in warmed PBS (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH₂PO₄, 8.1 mM Na₂HPO₄). The adherent cells on the coverglass were scraped off with a rubber policeman. The cell suspensions from five coverglasses were centrifuged at 170 g for 5 min and the cells were resuspended in 0.2 ml RPMI/FCS. The cells were counted with a haemocytometer. For detection of adherence to *A. laidlawii*, 0.1 ml of a 1×10^7 c.f.u. ml^{-1} suspension of *A. laidlawii* was inoculated into each well of a cell culture plate and incubated in 1 ml RPMI/FCS medium for 3 d at 37 °C in a CO₂ incubator. The medium was then removed and suspensions of eukaryotic cells in Cosmo medium were added to different wells. The adherent cells were counted as described above. An alternative method for estimation of cell numbers was also employed. Briefly, after removing nonadherent cells, residual cells on the coverglasses were incubated for 4 h at 37 °C in a CO₂ incubator in the presence of 1 µCi (37 kBq) [6-³H]thymidine [specific activity 16.3 Ci (603 GBq) mmol^{-1} ; Dupont NEN]. The cells were lysed, DNA was precipitated onto glass filters and the amount of [6-³H]thymidine incorporation was determined. Both methods for estimation of numbers of binding cells were used in five independent experiments.

Fractionation of *A. laidlawii* membranes. Membranes of *A. laidlawii* PG8 ATCC 23206 were isolated by osmotic lysis as described previously (Iyama *et al.*, 1996). The membranes of *A. laidlawii* were extracted twice using the Bligh–Dyer method with 40 vols water/chloroform/methanol (0.8:2:1, by vol.). Extracted lipids were dried on a rotary evaporator. Fifty milligrams of the lipid was resuspended in chloroform for application to a column of 10 g activated silica gel (Silica gel 60; Merck) prepared in chloroform. Sequential elution of the lipids from the column was performed with a discontinuous gradient starting with 400 ml of chloroform and followed by 400 ml each of the following mixtures of chloroform/methanol (v/v): 95:5, 90:10, 80:20, 70:30, 60:40, and finally 400 ml methanol. The fraction showing binding activity was further purified by TLC (Silica gel 60, 1.2 mm in thickness) using chloroform/methanol/acetic acid (85:15:0.5, by vol.).

Structure determination by NMR and GC-MS. The structure of a component of the membranes of *A. laidlawii* with binding activity was determined by ¹³C-NMR, ¹H-NMR and GC-MS analysis. Detailed one- and two-dimensional NMR studies of lipid X {[α]_D²⁵ + 39 °C (c 0.1, CHCl₃)} by ¹H-¹H DQF-COSY, HOHAHA (τ_m = 60 ms) and HMQC experiments with a JEOL α -500 spectrometer in CHCl₃-CH₃OD (1:1, v/v) allowed sequential assignments of proton and carbon resonances. Molecular ion peaks of the fatty acid methyl esters from lipid X by GC-MS indicated that the fatty acyl groups of the lipid were a mixture of straight- and branched-chain fatty acids. The lipids (0.7 mg in 0.5 ml methanol) were treated with 15 µl 140 mM sodium methoxide in methanol and the resulting fatty acid methyl esters were analysed by GC-MS equipped with a 25 m PEGM capillary column and electron ionization-MS spectrometry (Huang & Anderson, 1995).

Fig. 2. Assignments for the compounds obtained by TLC (natural) and their synthetic analogues (synthesized). (a, c) ^1H -NMR, 500 MHz; (b) ^{13}C -NMR, 125 MHz.

Synthesis of GAGDGs. Synthesis of glycolipids essentially followed a method established by van Boeckel & van Boom (1985a, b, c).

RESULTS

Purification of the active component from *A. laidlawii* membranes

The adherence of MOLT-4 cells to coverglasses bearing colonies of *A. laidlawii* or glycolipids extracted from *A. laidlawii* using the Bligh–Dyer method was tested. The number of adherent cells was determined by both direct counting of cell numbers and $[6\text{-}^3\text{H}]\text{thymidine}$ incorporation by adherent cells. The numbers of cells binding in the presence of *A. laidlawii*, their membranes, the chloroform/methanol phase, the water/methanol phase and in the negative control were $1.5 \pm 0.9 \times 10^4$, $1.3 \pm 0.1 \times 10^4$, $4.5 \pm 0.5 \times 10^4$, $0.3 \pm 0.1 \times 10^4$ and less than 0.1×10^4 per coverglass, respectively. The amounts of $[6\text{-}^3\text{H}]\text{thymidine}$ incorporated by adherent cells in the presence of these fractions, were $2.5 \pm 0.07 \times 10^4$, $2.8 \pm 0.05 \times 10^4$, $12.9 \pm 0.09 \times 10^4$, $0.8 \pm 0.01 \times 10^4$ and $0.08 \pm 0.03 \times 10^4$ c.p.m., respectively. These results indicated that the binding activity was in the chloroform/methanol phase, but not in the water/methanol phase. Further purification of the active component in the chloroform/methanol phase was performed using silica gel column chromatography. As shown in Table 1, binding activity was clearly demonstrated in the fraction eluted with chloroform/methanol (90:10, v/v), but not in the other six fractions. The component showing the binding activity was further purified by TLC. Two spots of R_F 0.36 and R_F 0.64 were separated by development with chloroform/methanol/acetic acid (85:15:0.5, by vol.). Both spots were scraped off and eluted with chloroform/methanol (1:1, v/v). The activity was clearly found in the spot with R_F 0.36.

Chemical structure of active natural components and their synthetic compounds

The chemical structure of the active component separated by TLC was determined by ^1H -NMR, ^{13}C -NMR and GC-MS. The chemical structure of synthetic compounds was also examined. As shown in Figs 1 and 2(a), the two glucose units were readily assigned by sequential large axial-axial couplings for the pyranose methine protons of H2' to H5' (3.58, 3.74, 3.31, 3.75 p.p.m.) and H2'' to H5'' (3.40, 3.67, 3.29, 3.85 p.p.m.). Five spin systems for the glycerol protons were also easily recognized (Fig. 2a). HMQC spectra clearly showed ^{13}C assignments for the glucosyl and glycerol units, C-1 to C-3 (66.6, 70.8, 63.5 p.p.m., C-1' to C-6' (97.2, 77.4, 72.6, overlapped with methanol peak, 70.7, 64.4 p.p.m.) and C-1'' to C-6'' (97.7, 72.8, 74.3, overlapped with methanol peak, 73.2, 62.2 p.p.m.) (Fig. 2b). Also, ^1H resonance at 0.79–0.86 (m, Me), 1.17–1.31 (m, methylene), 1.54–1.62 (6H, m, $\text{CH}_2\text{CH}_2\text{CO}$) and 2.27–2.34 p.p.m. (6H, m, CH_2CO) (Fig. 2c) and ^{13}C

Table 2. Fatty acid composition of the lipid side chain as determined by GC-MS

Retention time in GC (min)	Proportion (%)	Assignment (fatty acid methyl esters)*
11.05	0.4	NA
12.2	4.6	nC ₁₂
14.6	5.6	isoC ₁₃
15.0	1.3	NA
19.7	10.7	isoC ₁₄
21.4	17.8	nC ₁₄
23.8	4.2	isoC ₁₅
24.1	8.4	C ₁₅ †
25.0	1.3	nC ₁₅
25.2	0.5	NA
26.8	1.4	isoC ₁₆
27.9	34.9	nC ₁₆
29.4	0.7	isoC ₁₇
29.7	0.4	NA
30.3	0.4	nC ₁₇
31.3	0.7	NA
32.2	2.3	nC ₁₈
32.5	4.3	NA

NA, Not assigned.

* nC_m, $\text{nCH}_3(\text{CH}_2)_m\text{CO}_2\text{CH}_3$; isoC_m, $(\text{CH}_3)_2\text{CH}(\text{CH}_2)_m\text{CO}_2\text{CH}_3$.

† Structure not known.

resonance at 174.3, 174.8 and 175.0 p.p.m. (Fig. 2b) indicated three fatty acids attached to the diglucosyl glycerol. Downfield shift of the five proton resonance at H-2 (5.18–5.23 p.p.m.) and H-3 (4.21, 4.35 p.p.m.) of the glycerol moiety as well as the hydroxymethyl resonance H-6' (4.21, 4.35 p.p.m.) of one glucosyl unit implied that the three acyl groups were located on the C-2, C-3 and C-6 hydroxyl groups. A ROESY spectrum ($\tau_m = 200$ ms) showed a signal cross peak between the two sugar moieties [H-2' (3.76 p.p.m.) and H-1'' (4.92 p.p.m.)]. The coupling constant of the anomeric proton H-1'' was 4.0 Hz. These observations indicated that the two glucosyl units had an α -glycoside linkage between C-2' and C-1''. Another anomeric proton H-1' (4.96 p.p.m.) also showed a small coupling constant (3.7 Hz). Therefore, the anomeric carbon (C-1') also had an α -linkage. The structure of the lipid was determined as 3-O-[2'-O-(α -D-glucopyranosyl)-6'-O- α -D-glucopyranosyl]-1,2-di-O-acyl-sn-glycerol (GAGDG, Fig. 1). The spectra of the natural compound were identical to the spectra of the synthetic compound except for that for the acyl groups. As shown in Table 2, the compositions of the major saturated fatty acids nC₁₄, isoC₁₄, nC₁₆ were 17.8, 10.7 and 34.9%, respectively, as determined by GC-MS. The amounts of the unsaturated species were less than 10% of those of the corresponding saturated acids.

Fig. 3. (a) Effect of GAGDG concentration on binding to MOLT-4 cells. Cells (20×10^4) were incubated in a culture plate in the presence of a coverglass with synthetic GAGDGs containing isoC₁₄ (●), nC₁₄ (○) or nC₁₆ (□), or GAGDGs of *A. laidlawii* membranes (■) for 4 h at 37 °C. Values shown are means $\pm$ SD of five assays. (b) Cell attachment over time. MOLT-4 cells ($20 \times 10^4 \text{ ml}^{-1}$) were incubated in a culture plate in the presence of a coverglass coated with 10 μg of synthetic GAGDGs containing isoC₁₄ (●), nC₁₄ (○) or nC₁₆ (□), GAGDGs of *A. laidlawii* membranes (■) or an uncoated control (Δ). Values shown are means $\pm$ SD of five assays from one representative experiment.

Table 3. Binding of GAGDGs to various lymphoid cells

Various cell lines (2×10^5 cells) were incubated in the presence of coverglasses with 20 or 10 μg of the natural GAGDGs of *A. laidlawii* and synthetic GAGDGs at 37 °C for 4 h in a CO₂ incubator. Values shown ($10^{-4} \times \text{no. of adherent cells per coverglass}$) are means $\pm$ SD of five assays from one representative experiment.

Cell line	GAGDG of <i>A. laidlawii</i>		Synthetic GAGDGs				None
			isoC ₁₄		nC ₁₆		
	20 μg	10 μg	20 μg	10 μg	20 μg	10 μg	
Raji	28.0 ± 2.7	18.7 ± 2.0	27.5 ± 3.0	16.7 ± 3.1	15.0 ± 1.2	5.6 ± 2.0	0.9 ± 0.5
HL-60	24.7 ± 2.5	15.3 ± 1.8	29.6 ± 3.0	12.4 ± 2.5	13.8 ± 2.0	8.5 ± 1.5	0.2 ± 0.2
MOLT-4	6.4 ± 1.4	4.5 ± 0.7	26.0 ± 4.0	16.3 ± 1.8	8.3 ± 2.2	5.2 ± 1.2	0.1 ± 0.3
HUT-78	12.5 ± 1.8	2.8 ± 0.2	22.4 ± 1.8	14.2 ± 2.0	5.3 ± 0.8	3.5 ± 1.0	0.2 ± 0.8
CD4 ⁺ cells*	5.0 ± 0.6	3.0 ± 0.4	15.3 ± 4.0	10.5 ± 1.5	8.2 ± 0.4	3.7 ± 1.0	0.1 ± 0.1

* CD4⁺ cells purified using magnetic beads were cultured in the presence of 100 U IL-2 and IL-4 ml^{-1} .

The binding activity of synthetic GAGDGs

As shown in Fig. 3(a), the number of adherent cells was directly related to the concentration of GAGDGs. However, GAGDGs with nC₁₄ and nC₁₆ had a lower binding activity compared with those with isoC₁₄. As shown in Fig. 3(b), the adherence of cells began within 30 min and reached a maximum at 6 h.

Adherent cells were not observed in the absence of GAGDG. Table 3 shows one set of representative results from five experiments on the adherence of various cells

to GAGDGs. The number of adherent cells was highest for Raji cells, but the difference between the other types of cells was not significant.

DISCUSSION

In this study we have shown that glycosylglycerolipids obtained from *A. laidlawii* membranes and synthetic glycosylglycerolipids bind to both human lymphoid cell lines and human peripheral T cells. Our results provide new information on the interaction between glyco-

glycerolipids of prokaryotic cell membranes and eukaryotic cells.

Glycoglycerolipids are not only found in the cell membranes of mycoplasmas, but also in those of Gram-negative and Gram-positive bacteria, and their L-forms (Kates, 1990). However, their function in the membranes of prokaryotic cells is not clear. Several investigators have reported that glycolipids in the membranes of *A. laidlawii* are mono- and di-glycosyldiacylglycerol (Bhakoo *et al.*, 1987; Dahlqvist *et al.*, 1995; Gross & Rottem, 1979; Shaw 1968) and that they might play an important role in physiological functions such as membrane viscosity (De Kruyft *et al.*, 1973; McElhaney *et al.*, 1970; McElhaney, 1975; Rottem *et al.*, 1973). Several reports suggest that the fusion of mycoplasmas with eukaryotic cells may result in the delivery of mycoplasma components into the host cells (Toole & Lowdell, 1990; Franzoso *et al.*, 1992). Furthermore, Rottem (1980) reported that the presence of cholesterol in small unilamellar vesicles is required to allow their fusion with mycoplasmas. These findings suggest that GAGDGs, a major component of *A. laidlawii* membranes (Shaw, 1970; Silvius *et al.*, 1980), might play an important role not only in the membrane fluidity of prokaryotic cells, but also in interactions between micro-organisms lacking cell walls and eukaryotic cells.

Our results strongly suggest that glycoglycerolipids in *A. laidlawii* membranes might participate in the adhesion of bacterial cells to eukaryotic cells. The role of glycoglycerolipids in the pathogenesis of *A. laidlawii* infections is unknown. The induction of conformational changes in the membranes of eukaryotic cells as well as in those of prokaryotic cells by glycoglycerolipids may be responsible for these biological activities. In this study, we have clearly demonstrated that glycoglycerolipids possess the ability to bind to certain kinds of eukaryotic cells. However, we need to clarify the initial binding sites and the significance of this phenomenon in infections.

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