lipid constituents of several Gram-positive bacteria, but their structures are bacterial species-characteristic, i.e. Glcα1-2Glcα1-3′DG for *Streptococcus* and Acholeplasma, Glcβ1-6Glcβ1-3′DG for *Staphylococcus* and Mycoplasma, Galβ1-2Galβ1-3′DG for Bifidobacterium, Galα1-2Glcα1-3′DG for *Lactobacillus*, Glcβ1-4GlcUα1-3′DG for Pseudomonas, Galα1-4GalUα1-3′DG for Streptomyces, Manα1-3Manα1-3′DG for Micrococcus, and Galβ1-6Galβ1-3′DG for Arthrobacter (*5*, *7*). Antibodies produced on immunization of rabbits with bacteria have been shown to frequently react with bacterial specific DH-DG, and has been applied as a probe to discriminate bacterial species for serological classification, indicating that glycolipids are exposed on the bacterial cell wall and function as bacterial antigens, probably for mutual recognition among bacteria and immune recognition by the host animals (*8*, *9*). In this connection, an immune reaction toward symbiotic or infecting bacteria in humans has been shown to contribute to the production of natural antibodies including anti-blood group ones in human sera, as well as to the onset of diseases, such as multiple sclerosis (*9*, *10*). The anti-LJ antisera, as described above, also reacted with DH-DG Galα1-2Glcα1-3′DG, as a *Lactobacillus*-characteristic glycolipid, in the lipid fraction of LJ, but additional glycolipids, which migrated to positions corresponding to tri- and tetrahexaosyl diacylglycerols (TH-DG and TetH-DG), were found to react more strongly than DH-DG (*1*, *2*). As to glycolipids from *Lactobacillus* species, Glcβ1-6Galα1-2Glcα1-3′DG and Glcβ1-6Glcβ1-6Galα1-2Glcα1-3′DG have been isolated from *L. casei*, as reported in the literature (*11*, *12*), but TH-DG and TetH-DG in LJ detected by antisera were found to be different from them and to have novel structures, Galα1-6Galα1-2Glα1-3′DG and Galα1-6Galα1-6Galα1-2Glcα1-3′DG, respectively. In addition, we examined whether or not antibodies toward them are generated in human sera as a result of immune reaction to symbiotic bacteria.

Materials and Methods

Bacteria

LJ (JCM No. 1022), *L. intestinalis* (JCM No. 7548), *L. reuteri* (JCM No. 1112), *L. casei* (JCM No. 1134), *L. fermentum* (JCM No. 1137), *L. plantarum* (JCM No. 1550), *L. rhamnosus* (JCM No.1561), *S. epidermidis* (JCM No. 2414), and *S. salivalis* (JCM No. 5707) were purchased from the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center (Wako, Saitama, Japan). *S. aureus* (860) and *S. epidermidis* (866) and *Escherichia coli* (JM109) were obtained from the American Type Culture Collection (Rockville, MD, USA) and Invitrogen (Carlsbad, CA, USA), respectively. The culture media for bacteria were as follows: MRS broth (Beckton-Dickinson, Sparks, MD, USA) for lactobacilli, tryptic soy broth (Beckton-Dickinson) for *S. epidermidis*, heart infusion broth (Beckton-Dickinson) for *S. salivalis*, and LB broth (Nacalai Tesque, Kyoto) for *E. coli*.

Materials

Glycolipids, GlcCer, GalCer, LacCer, Gb_3Cer, Gb_4Cer, Gg_4Cer and IV^2Fucα-Gg_4Cer (*13*), were purified in our laboratory (*1*, *2*). Rabbit polyclonal antisera toward *LJ* (*1*) and murine monoclonal anti-human blood group B antibodies (HEB-29; Exbio, Praha, Czech) were used in this experiment. Phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), cardiolipin (CL), and II^6NeuAcα-lactose were purchased from Sigma (St Louis, MO, USA). The concentrations of standard phospholipids in chloroform/methanol (1:1, v/v) were determined by the phosphomolybdate procedure after decomposition of the lipids with 70% $HClO_4$ and H_2O_2 (*14*).

Human blood

Normal human blood, blood groups A, B, AB and O, was obtained from Keio University Hospital, and used according to the guidelines of the Committee for Informed Consent. Blood was separated into erythrocytes and plasma by centrifugation at 2,000 rpm for 10 min. Erythrocytes were haemolysed with 0.1% acetic acid in water and then centrifuged at 6,000*g* for 20 min to obtain the membrane fraction, which was washed with water until haemoglobin had been reduced sufficiently. While the plasma was left at 4°C for 6 h and the fibrin produced was removed to obtain serum.

Quantitation of bacterial lipids

The lipid extracts from bacteria were partitioned by Folch's procedure (*15*), and then, aliquots corresponding to 0.5–1.0 mg dry weight, were separated on glass-coated (Merck, Darmstadt, Germany) and plastic-coated (Macherey-Nagel, Düren, Germany) TLC plates with chloroform/methanol/water (65:25:4. v/v/v). Detection was performed with cupric acetate–phosphoric acid reagent for all lipids, Dittmer's reagent for phospholipids, ninhydrin reagent for aminolipids and orcinol-sulphuric acid reagent for glycolipids, and by TLC-immunostaining with rabbit anti-LJ antisera (1:500), murine anti-B antibodies (1:500) and human sera (1:100).

Enzyme-linked immunosorbent assay

A purified glycolipid from LJ was dissolved in ethanol by sonication (0.5 μg/100 μl) and then put in each well of a microtitre plate, which was left at room temperature until the ethanol had completely evaporated off. The plate was blocked with 100 μl of bovine serum albumin (BSA) in PBS (1%) at 4°C overnight, and then to the plate, 100 μl of human serum diluted 1:100 and 1:200 with 1% BSA in PBS was added, followed by reaction at room temperature for 2 h. After washing the plate with 0.1% Tween-20 in PBS 5×, the antibodies bound on the plate were detected by reaction with 100 μl of peroxidase-conjugated anti-human IgG, A and M antibodies diluted 1:2,000 with 1% BSA in PBS at room temperature for 2 h, followed by reaction with *o*-phenylenediamine (4.6 mM) and H_2O_2 (0.015%) in 25 mM citrate–phosphate buffer (pH 5.0) as the substrates for peroxidase at room temperature for 15 min. The reaction was terminated by the addition of 4 M sulphuric acid (20 μl), and then the optical density at 490 nm was determined. The background values obtained on reaction with human sera in wells without glycolipids under the same conditions, were subtracted from the values obtained above.

Purification of glycolipids

The neutral and acidic lipid fractions by DEAE-Sephadex (A-25, acetate form; GE Healthcare Bioscience, Piscatway, NJ, USA) column chromatography contained glycolipids, and CL and PG, respectively, and after Folch's partitioning, they were purified using a silica gel (Iatrobeads 6RS8060: Iatron Laboratory, Tokyo) column by gradient elution with chloroform/methanol/water (85:15:0.2, 70:30:4 and 10:90:4, v/v/v). The purity of purified lipids was examined by TLC as described above.

Structural analysis of glycolipids

The purified glycolipids were analysed by positive ion FAB–MS (JMS-700TKM; JEOL, Tokyo) with triethanolamine (TEA) as the matrix and proton magnetic resonance spectroscopy (JNM-ECP700, JEOL) with dimethyl sulphoxide-d6/D_2O (98:2, v/v). For determination of the compositions of fatty acids and carbohydrates, they were methanolized with 5% HCl in methanol at 80°C for 16 h. The resulting fatty acid methyl esters were extracted with *n*-hexane, and 1-*O*-methyl hexoses in the methanol phase were converted to trimethylsilyl derivatives with pyridine/hexamethyl disilazane/trimethylchlorosilane (10:2:1, v/v/v) at 60°C for 5 min, followed by analysis with a GC–MS (GP5050: Shimadzu, Kyoto) equipped with a DB-1 column (0.25 mmø × 30 m) from 150°C to 250°C at 10°C/min. Also, linkage analysis of carbohydrates was carried out by conversion of glycolipids to partially methylated aldohexitol acetates, followed by analysis with a GC–MS with a DB-1 column from 150°C to 210°C at 4°C/min (*16*). Standard partially methylated

aldohexitol acetates were obtained from the following sources: terminal Glc from GlcCer, 2-linked Glc from kojibiosyl glycerol, 3-linked Glc from nigerosy glycerol, 4-linked Glc from LacCer and Gb_4Cer, 6-linked Glc from 6-glucosyl dextran, terminal Gal from GalCer, 3- and 4-linked Gal from Gb_4Cer, 2-linked Gal from $IV^2Fuc\text{-}Gg_4Cer$, and 6-linked Gal from II^6NeuAc lactose. In addition, for determination of the carbohydrate sequences and anomeric configurations, glycolipids were hydrolysed with the following glycosidases: α-glucosidase (rice: Sigma) in 50 mM citrate buffer (pH 4.5), β-glucosidase (almond: Wako, Tokyo) in 50 mM citrate buffer (pH 6.5), and α-galactosidase (coffee bean: Sigma) in 50 mM citrate buffer (pH 4.5). Glycolipids (~2 μg) together with 100 μg/μl sodium taurocholate were hydrolysed by incubation with the enzyme at 37°C for 1–16 h in a final volume of 100 μl, and the products were recovered by solvent partitioning with 100 μl of chloroform and 50 μl methanol, followed by with 100 μl of methanol/water (1:1, v/v), and were examined by TLC with chloroform/methanol/water (65:25:4, v/v/v) and orcinol-sulphuric acid reagent for glycolipids, and with *n*-hexane/diethyl ether/acetic acid (80:30:4, v/v/v) and cupric acetate–phosphoric acid reagent for diacylglycerol.

Results

Bacterial lipids

As shown in Fig. 1, the total lipid extracts from several bacteria were examined by TLC and TLC-immunostaining. In accord with previous reports (*17*, *18*), the amounts of PE (4.8 ± 0.8 μg/mg dry weight) and CL plus PG (3.2 ± 0.7 μg/mg dry weight) in *E. coli*, as a Gram-negative bacterium, were exceedingly higher than those in the other Gram-positive bacteria, and glycolipids were absent in *E. coli*. This was the reason why glycolipids in Gram-negative bacteria have been characterized as lipooligosaccharides with lipid A as the hydrophobic moiety in the outer membrane of the cell wall, and why they can not be extracted with chloroform/methanol (*19*). In contrast, the major phospholipids in Gram-positive bacteria, i.e. *Lactobacillus*, *Staphylococcus* and *Streptococcus* species, were CL and PG, and DH-DG was present in significantly high amounts (0.34–2.05 μg/mg dry weights), indicating that the cytoplasmic membranes of Gram-positive bacteria are mainly composed of glycerophospholipids and glyceroglycolipids.

Although PG and DH-DG were present in all Lactobacilli examined, CL, TH-DG and TetH-DG were present in relatively high concentrations in the following strains: CL in LJ, *L. reuteri*, *L. fermentum* and *L. plantarum*, and TH-DG in LJ, *L. intestinalis* (LI), *L.casei* and *L. rhamnosus*, and TetH-DG in LJ and LI (Table I). On TLC-immunostaining with anti-LJ antisera, DH-DG, TH-DG and TetH-DG in *Lactobacillus* species exhibited a positive reaction, and TH-DG and TetH-DG were detected with sensitivity of more than 10 times higher than that in the case of DH-DG, but DH-DG in *Staphylococcus* and *Streptococcus* species were not reactive with the antisera at all (Fig. 1). DH-DG was confirmed to be a common antigenic glycolipid in *Lactobacillus* species, and TH-DG and TetH-DG were revealed to exhibit a restricted distribution in *Lactobacillus* species.

Glycolipids in LJ

The major glycolipids, DH-DG, TH-DG and TetH-DG, in LJ were purified by means of DEAE-Sephadex and Iatrobeads column chromatographies

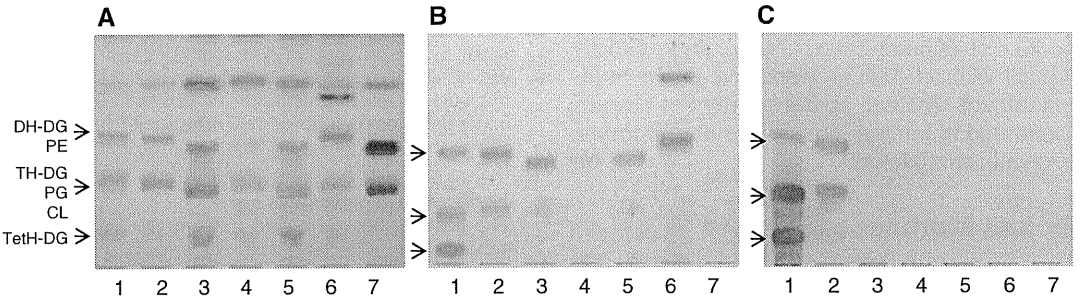

Fig. 1 TLC and TLC-immunostaining of lipids from several bacteria. Lipids, corresponding to 0.5 mg dry weight, were developed on glass-coated (A and B) and plastic-coated (C) TLC plates with chloroform/methanol/water (65:25:4, v/v/v), and were detected with cupric acetate–phosphoric acid (A), orcinol-sulphuric acid (B) and anti-LJ antisera (1:500 dilution) (C). 1, LJ; 2, *L. casei*; 3, *S. aureus* (ATCC 860); 4, *S. epidermidis* (JCM2414); 5, *S. epidermidis* (ATCC866); 6, *S. salivalis*; 7, *E. coli*. Arrows beside each TLC plate indicate the positions of DH-DG, TH-DG and TetH-DG, from the top, respectively.

Table I. Amounts of lipids in several lactobacilli. (μg/mg dry weight).

	Lactobacillus johnsonii	*Lactobacillus intestinalis*	*Lactobacillus reuteri*	*Lactobacillus casei*	*Lactobacillus fermentum*	*Lactobacillus plantarum*	*Lactobacillus rhamnosus*
Phospholipids							
CL	1.14	0.18	1.11	0.10	1.45	0.35	0.05
PG	0.69	0.11	0.58	1.91	0.54	0.30	0.72
Glycolipids							
MH-DG	0.15						
DH-DG	1.36	0.22	0.99	2.22	1.54	0.93	0.81
TH-DG	1.10	0.17	0.31	0.96			0.63
TetH-DG	0.93	0.15	tr				

tr, trace amount, <0.01 μg/mg dry weight.

with monitoring of their reactivities with orcinol-sulphuric acid and anti-LJ antisera (lanes 1, 2 and 3 in Fig. 2). Their carbohydrate compositions, as determined by GC–MS of 1-*O*-methyl 2,3,4,6-tetraTMS derivatives, indicated that DH-DG, TH-DG and TetH-DG in LJ contained Glc and Gal in the ratios of 1:1, 1:2 and 1:3, respectively (Table II). Hydrolysis of them with α-galactosidase (coffee bean) yielded MH-DG, whose carbohydrate was proven to be Glc by GC–MS, and which was further converted to DG by subsequent treatment with α-glucosidase (rice) (Fig. 3). These results indicated that both Glc and Gal were of the α-configurations, and α-Gal was sequentially linked with Glc-DG in DH-DG, TH-DG and TetH-DG. Then, linkage analysis of the carbohydrate moieties was performed by GC–MS of partially methylated aldohexitol acetates prepared from them. As shown in Fig. 4, partially methylated aldohexitol acetates of TetH-DG from LJ gave three peaks, whose retention times and mass spectra were identical with those of 1,5-di-*O*-acetyl, 2,3,4,6-tetra-*O*-methyl galactitol (terminal Gal) for peak a, 1,2,5-tri-*O*-acetyl 3,4,6-tri-*O*-methylglucitol (2-linked Glc) for peak b, and 1,5,6-tri-*O*-acetyl 2,3,4-tri-*O*-methyl galactitol (6-linked Gal) for peak c, and their peak ratio compared to that of 2-linked Glc was 1:1:2, respectively (Table II). Thus, the structure of TetH-DG from LJ was concluded to be Galα1-6Galα1-6Galα1-2Glcα1-3′DG. In a similar way, TH-DG and DH-DG from LJ were determined to be Galα1-6Galα1-2Glcα1-3′DG and Galα1-2Glcα1-3′DG, respectively (Table II and Fig. 3). Concerning the fatty acid compositions (Table III), 11-octadecenoic acid (18:1, cis-baccenic acid) and 11,12-methylene-octadecanoic acid (cyclopropane-19:0, c19:0, lactobacillic acid) were found to be the major ones in all glycolipids from LJ, and the abundant molecular ions of DH-DG, TH-DG and TetH-DG were 18:1 and c19:0-containing ones, m/z 982 for $[DH\text{-}DG+Na]^+$, m/z 1,108 for $[DH\text{-}DG+TEA]^+$, m/z 1,144 for $[TH\text{-}DG+Na]^+$ and m/z 1,306 for $[TetH\text{-}DG+Na]^+$, respectively (Fig. 5).

Accordingly, glycolipid antigens detected on TLC-immunostaining with anti-LJ antisera were found to carry a common α-Gal residue as the antigenic determinant at the non-reducing terminals of glycolipids, because no reaction of antisera with Glcα1-3′DG (MH-DG) was observed. Also, the binding capacities of antibodies as to TH-DG and TetH-DG were significantly higher than that as to DH-DG, and >2 ng of TH-DG and TetH-DG, corresponding to 1×10^6 cells of LJ, could be detected on TLC-immunostaining (Fig. 2) (*2*).

Glycolipids in other bacteria

The major glycolipids in Fig. 1 were also purified as described above, and their structures were elucidated by permethylation analysis and exoglycosidase treatment (Table II). DH-DG, TH-DG and TetH-DG from LI were identical with those from LJ, and DH-DGs from *L. reuteri, L. fermentum, L. plantarum, L. rhamnosus* and *L. casei* were also proven to be Galα1-2Glcα1-3′DG as a common glycolipid in *Lactobacillus* species (Table II). However, DH-DG from *L. casei* was separated into two bands, one exhibiting identical mobility with that of DH-DG from LJ and the other migrating above DH-DG, it being slightly less polar than DH-DG. The less polar glycolipid was demonstrated to be an esterified derivative of TH-DG with 16:0, 18:0 and c19:0 as the fatty acids and with terminal non-esterified hexose by positive ion FABMS (data not shown). As shown in Table II, permethylation analyses of TH-DG and esterified TH-DG revealed that they were Glc1-6Gal1-2Glc1-3′DG and Glc1-6Gal1-2Glc(6-fatty acid)1-3′DG, respectively, which were identical with those reported in the literature (*10, 11*).

On the other hand, glucose was the sole carbohydrate in the DH-DGs from *S. epidermidis* and *S. salivalis*, whose oligosaccharide structures were shown to be Glc1-6Glc1-3′DG and Glc1-2Glc1-3′DG, respectively, by permethylation analysis (Table II). The carbohydrate moieties of DH-DGs from *S. salivalis* and *S. epidermidis* were susceptible to α-glucosidase (rice) and β-glucosidase (almond), DG being yielded, indicating that the anomers of the two glucose residues in DH-DG are of the α- and β-configurations, respectively. In addition, ^1H NMR spectra of DH-DG from *S. epidermidis* contained doublet signals at $\delta=4.26$ ppm ($J=7.8$ Hz) and $\delta=4.02$ ppm ($J=7.7$ Hz) due to gentibiose. Thus, in accord with previous reports, the major glycolipids from *S. epidermidis* and *S. salivalis* were Glcβ1-6Glcβ1-3′DG, gentibiosyl DG, and Glcα1-2Glcα1-3′DG, kojibiosyl DG, respectively (*5, 7*).

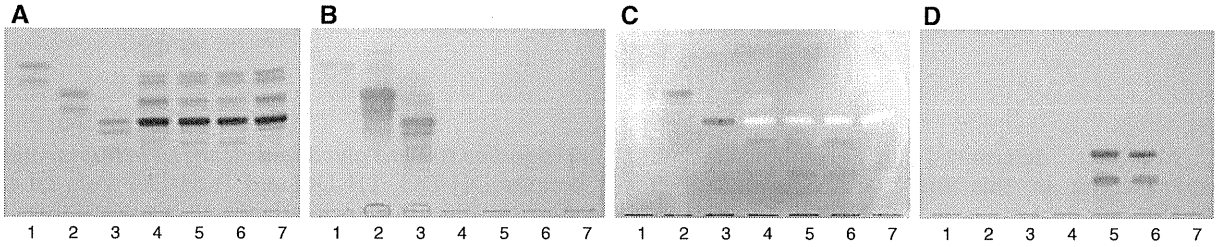

Fig. 2 TLC and TLC-immunostaining of purified glycolipids from LJ and neutral glycolipids from human erythrocytes. DH-DG (*1*), TH-DG (*2*) and TetH-DG (*3*) purified from LJ, 0.5 μg of each, and neutral glycolipids from human erythrocyte membranes, blood groups A (*4*), B (*5*), AB (*6*) and O (*7*), corresponding to 0.5 mg of dried membranes, were developed on plastic-coated TLC plates with chloroform/methanol/water (65:25:4, v/v/v), and were detected with orcinol-sulphuric acid (A) and anti-LJ antisera (1:500 dilution) (B), human blood group O serum (1:100 dilution)(C) and anti-blood group B antibodies (1:500 dilution) (D). The two bands of DH-DG, TH-DG and TetH-DG, respectively, represent molecular species with different fatty acids.

Table II. Carbohydrate compositions determined by GC–MS of TMS derivatives of carbohydrates and partially methylated aldohexitol acetates of glycolipids from several bacteria.

	TMS derivatives		Partially methylated aldohexitol acetates					
	Glc	Gal	t-Glc	t-Gal	2-Glc	6-Glc	2,6-Glc	6-Gal
Lactobacillus johnsonii								
DH-DG	1	0.9	nd	1.1	1	nd	nd	nd
TH-DG	1	1.9	nd	1.2	1	nd	nd	1.1
TetH-DG	1	2.8	nd	0.9	1	nd	nd	2.2
Lactobacillus intestinalis								
DH-DG	1	1.1	nd	1.1	1	nd	nd	nd
TH-DG	1	1.8	nd	0.9	1	nd	nd	1.1
TetH-DG	1	2.8	nd	0.9	1	nd	nd	2.0
Lactobacillus casei								
DH-DG	1	1.2	nd	1.2	1	nd	nd	nd
TH-DG	1	0.5	1.1	nd	1	nd	nd	1.2
Esterified TH-DG	1	0.5	1.1	nd	nd	nd	1	1.2
Staphylococcus epidermidis								
DH-DG	1	nd	1	nd	nd	0.8	nd	nd
Streptococcus salivalis								
DH-DG	1	nd	1	nd	1.1	nd	nd	nd

Peak areas of TMS-Gal were compared to that of TMS-Glc. Peak areas of partially methylated aldohexitol acetates were compared to those of 2-linked Glc for *Lactobacillus* glycolipids, 2,6-linked Glc for esterified TH-DG from *L. casei*, and terminal Glc for glycolipids from *S. epidermidis* and *S. salivalis*. Terminal Gal and 2-linked Glc in DH-DGs from *L.reuteri, L.fermentum, L.plantarum and L.rhamnosus* were also detected in the ratio of 1:1. t-Glc, terminal Glc (1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-methylglucitol); t-Gal, terminal Gal (1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-methylgalactitol); 2-Glc, 2-linked Glc (1,2,5-tri-*O*-acetyl 3,4,6-tri-*O*-methylglucitol); 6-Glc, 6-linked Glc (1,5,6-tri-*O*-acetyl 2,3,4-tri-*O*-methylglucitol); 2,6-Glc, 2,6-linked Glc (1,2,5,6-tetra-*O*-acetyl 3,4-di-*O*-methylglucitol); 6-Gal, 6-linked Gal (1,5,6-tri-*O*-acetyl 2,3,4-tri-*O*-methylgalactitol); nd, not detected.

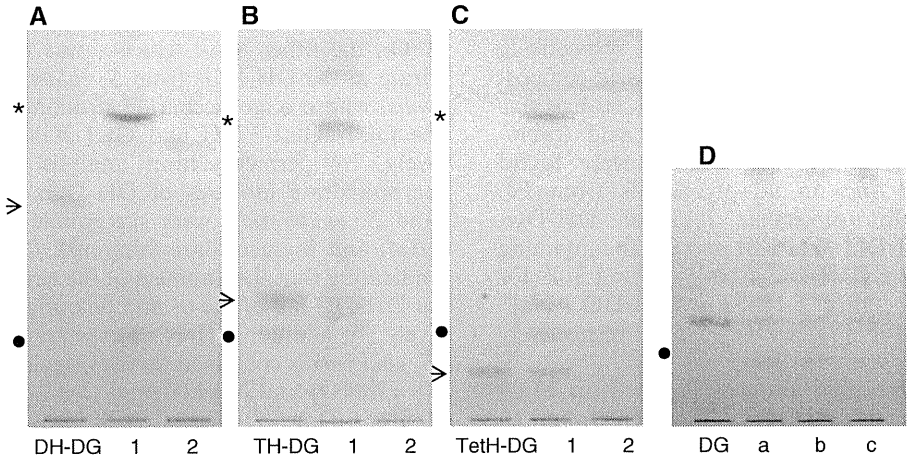

Fig. 3 TLC of the products on treatment with α-galactosidase and α-glucosidase. The products on hydrolysis of DH-DG (A), TH-DG (B) and TetH-DG (C) by α-galactosidase (coffee bean) (*1*), followed by α-glucosidase (rice) (*2*), as described in the text, were developed on TLC plates with chloroform/methanol/water (65:25:4, v/v/v), and were visualized with orcinol-sulphuric acid reagent. The products of DH-DG (a), TH-DG (b) and TetH-DG (c) on hydrolysis with α-galactosidase and α-glucosidase were also developed with *n*-hexane/diethyl ether/acetic acid (80:30:4, v/v/v), and were visualized with cupric acetate–phosphoric acid reagent (D). Arrows besides plates A–C indicate the positions of DH-DG, TH-DG and TetH-DG, respectively. Asterisk, MH-DG; filled circle, sodium taurocholate-derived spots.

The structures determined of glycolipids from several bacteria in this study are summarized in Table IV.

On the other hand, the fatty acid compositions of DH-DG from several bacteria were also found to be bacterial species-characteristic, c19:0 being preferentially present in those from *Lactobacillus* species, and anteiso15:0 and anteiso17:0 in DH-DG from *S. epidermidis* (Table III).

Antibodies toward glycolipids from LJ in human sera

Because DH-DG, TH-DG and TetH-DG from LJ exhibited strong antigenicities, antibodies toward them in human sera were examined by enzyme-linked immunosorbent assay (ELISA) and TLC-immunostaining. On ELISA, all human sera (36 cases) were revealed to contain antibodies toward them, with various titres, and the antibody titer as to TetH-DG in individual sera was constantly higher than those as to DH-DG

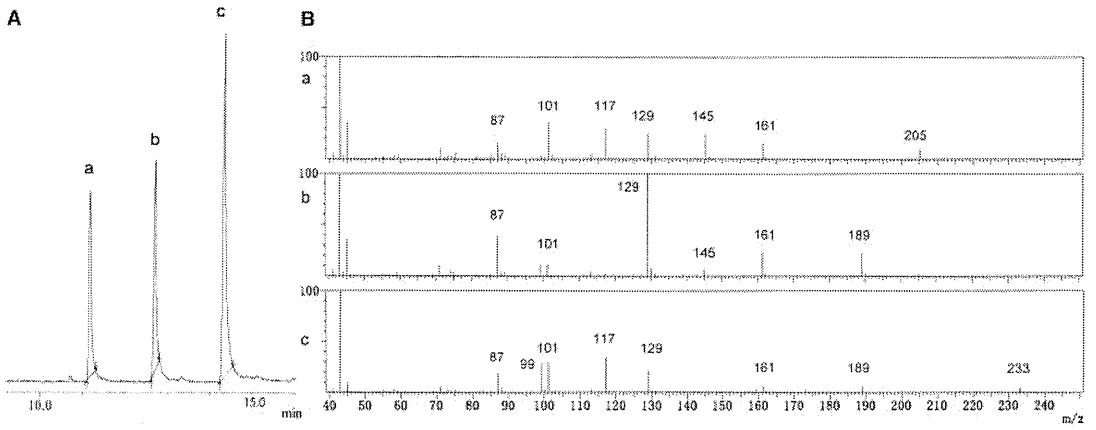

Fig. 4 GC–MS chromatogram (A) and mass spectra (B) of partially methylated aldohexitol acetates prepared from TetH-DG of LJ. Partially methylated aldohexitol acetates were prepared from TetH-DG of LJ and were analysed by GC–MS. Mass spectra a–c correspond to peaks a–c, which were characterized as terminal Gal, 2-linked Glc and 6-linked Gal, respectively.

Table III. Fatty acid compositions of DH-DG from several bacteria.

	Lactobacillus johnsonii	*Lactobacillus casei*	*Staphylococcus epidermidis*	*Streptococcus salivalis*
14:0	0.5	–	2.6	10.9
14:1	–	–	0.8	–
15:0	–	–	–	–
a15:0	–	–	66.8	–
16:0	2.2	15.5	5.1	50.6
16:1	–	–	1.1	9.7
a17:0	–	–	19.4	–
18:0	–	7.5	4.2	5.4
18:1	57.7	36.6	–	23.4
18h:0	10.1	7.8	–	–
c19:0	29.5	32.6	–	–

a, anteiso; c, cyclopropane; h, hydroxy; 18:1, 11-octadecenoic acid; c19:0, 11,12-methylene-octadecanoic acid.

and TH-DG, irrespective of the blood group, A, B, AB or O. The optical densities on ELISA of sera at 1:200 dilution were 0.25 (0.01–0.68) for DH-DG, 0.39 (0.1–1.14) for TH-DG, and 0.64 (0.08–1.96) for TetH-DG. On TLC-immunostaining with blood group O serum at 1:100 dilution, antibodies were found to bind with TH-DG, TetH-DG, and blood group A- and B-glycolipids, and the density of TetH-DG was three times higher than that of TH-DG (Fig. 2C). Since rabbit anti-LJ antiserum reacted with DH-DG, TH-DG and TetH-DG, but not with B-glycolipids (Fig. 2B), and Gb_3Cer and $IV^3Gal\alpha\text{-}nLc_4Cer$ (*1*), and reversely anti-blood B antibodies reacted with B-glycolipids, but not all glycolipids from LJ (Fig. 2D), the α-Gal residues in glycolipids from LJ seemed not to cross-react with that of B-glycolipids containing α-Gal (Fig. 2). Similar to in the cases of A and B-glycolipids, TetH-DG after reaction with human sera more intensively stained with peroxidase-conjugated anti-human IgM antibodies than with anti-human IgA and anti-human IgG ones, indicating that human antibodies against TetH-DG are mainly of the IgM isotype. Thus, an immune reaction against antigenic glycolipids in lactobacilli was shown to occur in the human body, probably as a result of bacterial symbiosis.

Discussion

As reported in this article, glycolipids in both bacteria and the hosts were revealed to play a role in mutual recognition. That is, glycolipids in LJ provided the antigenic determinant for the immune recognition of host animals, and those in the tissues and cells of animals provided the receptor for bacterial attachment. In general, molecular functions of glycolipids are the anchoring of functional carbohydrate chains on biomembranes through hydrophobic ceramides or diglycerides, and the exposure of their oligosaccharide moieties to the extracellular circumstances, and they have been localized in the raft structure, being assembled together with several physiological proteins and intracellular signal transduction systems (*20*). In contrast, glycolipids in Gram-positive bacteria had been thought to be in the cytoplasmic membrane covered with a thick cell wall, and to play a role by anchoring teichoic acids to the wall as lipoteichoic acid without their expression on the surface of the cell wall (*4–6*).

However, in the lipid extract from LJ, we were not able to detect lipoteichoic acid, probably due to the different extraction and structural characterization procedures from those described in the literature (*4–6*), or due to removal of hydrophilic materials from the lipid extract on Folch's partitioning (*15*). As shown in Fig. 1, the major lipids in the Folch's lower phase from LJ were phospholipids and glycolipids. In our previous study (*1*), we determined the amounts of the major phospholipids, CL and PG, by TLC-densitometry after visualization with cupric acetate–phosphoric acid, but the densities of CL and PG from LJ were found to be significantly lower than those of standard CL and PG in the same molar amounts, probably due to the difference in their fatty acid compositions. Accordingly, in this study, we used Dittmer's reagent for determination of CL and PG by TLC-densitometry, for which the plates, after spraying of the reagent, were kept at 4°C for more than 4 h to reduce the background. In the Folch's lower phase, the amounts of glycolipids,

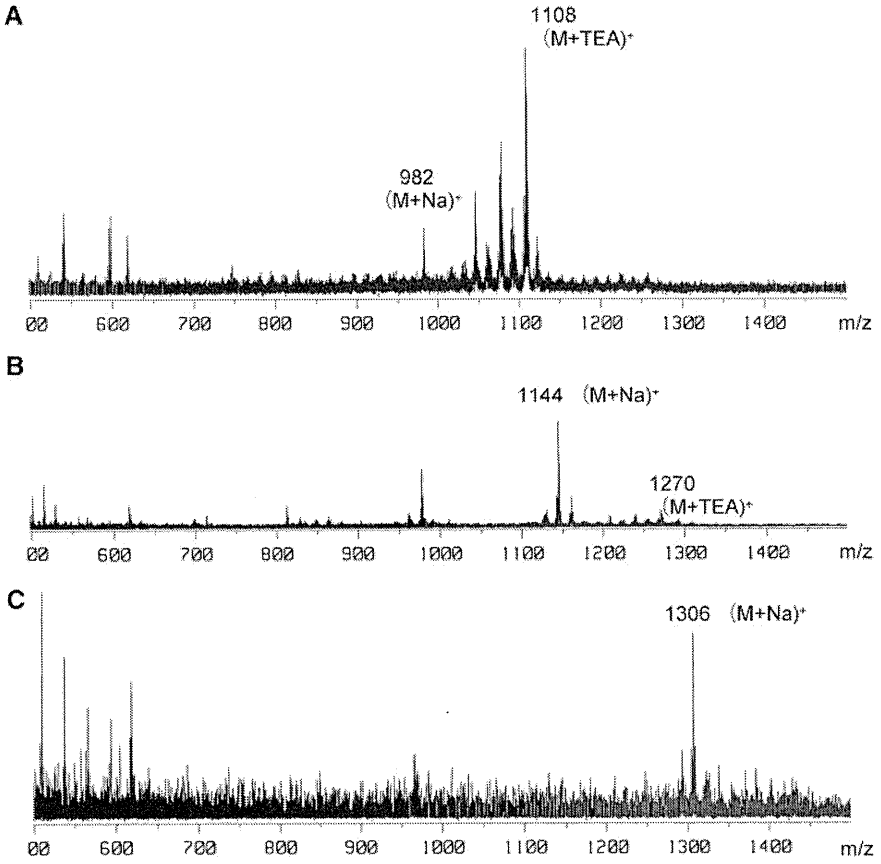

Fig. 5 Positive ion FABMS spectra of DH-DG (A), TH-DG (B) and TetH-DG (C) from LJ. Purified glycolipids (5 μg) in 5 μl C/M (1:1, v/v) were mixed with TEA (~5 μl) on a target plate and then analysed by FABMS.

Table IV. Structures of glycolipids from several bacteria.

Bacteria	Glycolipids	Structures
Lactobacillus johnsonii	DH-DG	Galα1-2Glcα1-3′DG
Lactobacillus intestinalis	TH-DG	Galα1-6Galα1-2Glcα1-3′DG
	TetH-DG	Galα1-6Galα1-6Galα1-2Glcα1-3′DG
Lactobacillus reuteri	DH-DG	Galα1-2Glcα1-3′DG
Lactobacillus fermentum		
Lactobacillus plantarum		
Lactobacillus rhamnosus		
Lactobacillus casei	DH-DG	Galα1-2Glcα1-3′DG
	TH-DG	Glc1-6Galα1-2Glcα1-3′DG
		Glc1-6Galα1-2Glc(6-fatty acid)α1-3′DG
Staphylococcus epidermidis	DH-DG	Glcβ1-6Glcβ1-3′DG
Streptococcus salivalis	DH-DG	Glcα1-2Glcα1-3′DG

DH-DG, TH-DG and TetH-DG, were equivalent to those of phospholipids, indicating that the majority of glycolipids are present without teichoic acids as the membrane constituents in LJ. Also, since anti-glycolipid antisera have been shown to react with the respective bacteria, and can be used as probes for bacterial classification (*9*, *10*), glycolipids seemed to be partly expressed on the surface of the cell wall. In fact, bacterial agglutination with anti-LJ antisera was inhibited by half of the activity after absorption of antisera with TetH-DG-containing liposomes according to the procedure reported previously (data not shown) (*21*).

A common structure of glycolipid antigens in LJ is α-Gal at their non-reducing terminals, and the extended structures in DH-DG, TH-DG and TetH-DG exhibited strong antigenicities in that order. To a lesser extent, TH-DH and esterified TH-DG, whose non-reducing terminals are Glc, in *L. casei* also reacted, but Glcα1-2Glcα1-3′DG in *S. salivalis* and Glcβ1-6Glcβ1-3′DG in *S. epidermidis* did not react with anti-LJ antisera at all, indicating that Galα1-2Glcα is a minimal structure for recognition by anti-LJ antisera and its modification with Glc at the non-reducing terminal does not significantly affect the reactivity. The non-reducing terminal Gal extended by an α1-6

linkage in TH-DG and TetH-DG from LJ was found to enhance the antigenicity of DH-DG, and therefore the Galα1-6 group is an antigenic determinant for LJ.

As to the glycolipid composition in LI, the relative amounts of TH-DG and TetH-DG were significantly higher than those reported previously (Fig. 2C) (*1*). Although the glycolipid composition of the original LI was 92% DH-DG, 6% TH-DG and <2% TetH-DG, immunization of rabbits with the original LI generated antibodies that reacted strongly with TetH-DG in LJ as well as with TetH-DG in LI, indicating that the original LI has TetH-DG. On structural characterization, DH-DG and TH-DG from the original LI, and DH-DG, TH-DG and TetH-DG from LI in this study were proven to have the same structures as those from LJ. Continuous subculture of LI for more than 1 year seemed to result in a change in glycolipids, 40.7% DH-DG, 31.5% TH-DG and 27.8% TetH-DG, but the change in the lipid compositions in other bacteria on continuous subculture was estimated to be <10%. To clarify the genetic background of the differences in glycolipid composition between the original and long-term cultured LI, and among various bacteria, analysis of sugar transferase genes is now in progress in our laboratory. In conclusion, TetH-DG was detected in intestinal lactobacilli, i.e. LJ, LI, *L. reuteri* and LJ isolated from the cecal and colonal contents of mice (*2*).

The strong antigenicity of TetH-DG was revealed not only on characterization of antigens for rabbit anti-LJ antisera, but also from its reactivity with normal human sera. As described above, antibodies toward TH-DG and TetH-DG were readily generated on immunization of rabbits with LJ and LI, even with <0.01 μg/mg dry weight of the original LI, and they were frequently detected in normal human sera as a result of an immune reaction against them, probably for protection from bacterial invasion of human body. In Peyer's patches, bacteria bound with receptors were shown to be incorporated into the M cells in the follicle-associated epithelium and to be transported to gut-associated lymphoid tissues, where secretory IgA was produced and secreted into the mucosal layer of the digestive tract to prevent bacterial invasion and irregular diffusion of bacteria in the mucosal layer (*22*, *23*). Also, production of IgM as an event in the initial immune reaction was also supposed to occur for protection from bacterial invasion of the blood circulation system, because IgM is the major immunoglobulin with strong agglutination activity in human sera. Since anti-LJ antisera did not cross-react with α-Gal containing sphingoglycolipids, i.e. Gb_3Cer, $IV^3Gal\alpha$-nLc_4Cer and blood group B-antigen, antibodies toward TH-DG and TetH-DG in symbiotic lactobacilli seemed not to react with the tissues or cells of the host animal (*1*).

On the other hand, although the Gg_4Cer-like structure was detected in the protein fraction of LJ on Western blotting and antibodies against it were generated on immunization of rabbits with LJ (*1*, *2*), it was not detected in normal human sera even on dilution to 1:32. While, on infection with *Campylobacter jejuni* cells containing oligosaccharides mimicking Gg_4Cer and gangliosides, antibodies against them have been revealed to be produced occasionally and to be closely associated with the onset of autoimmune diseases such as Guilain–Barré syndrome (*24*, *25*). Probably, the whole oligosaccharides profile in individual bacteria is important for immunocytes to distinguish bacterial species, even if Gg_4Cer is a strong antigenic molecule. Accordingly, one can suggest that oligosaccharide profiles recognized by antibodies in normal human sera reveal the bacterial species with symbiosis or infection.

Since several bacteria, i.e. LJ, *L. reuteri, L. casei, Bifidobacterium bifidum, Pseudomonous aeruginosa, Actinomyces maeslundii* and *Neiseria gonorrhoeae,* utilize Gg_4Cer as a receptor, suppression of competitive or injurious bacteria through the structural modification of Gg_4Cer seems to be important for establishment of symbiosis or protection from infection, respectively. Gg_4Cer in the murine digestive tract is known to be structurally modified in a region-characteristic mode. Namely, Gg_4Cer in the stomach, cecum and colon is completely converted to its fucosylated derivative by α1,2-fucosyltransferase from the FUT-2 gene product, but >85% was expressed in the small intestine of conventionally breeding mice (*2*, *26*). The modification of Gg_4Cer to $IV^2Fuc\alpha$-Gg_4Cer has not been observed in germ-free mice, but has been revealed to occur on transcriptional induction of the FUT-2 gene on infection by indigenous filamentous bacteria and wild-type *Bacteroides thethaiotaomicron*, indicating that the expression of the FUT-2 gene is regulated epigenetically (*27*, *28*). Also, in FUT-2 gene null mice, although $IV^2Fuc\alpha$-Gg_4Cer is completely absent in the stomach, cecum and colon, its synthesis in the small intestine is maintained through alternative expression of the FUT-1 gene, indicating that modification of Gg_4Cer by fucosyltransferase in the small intestine is maintained in both FUT-1 and FUT-2 null mice, and is indispensable for regulation of the receptor activity of Gg_4Cer under conventionally breeding conditions in both FUT-1 and FUT-2 null mice (*26*). So far, it is unclear whether the epigenetic expression of the fucosyltransferase gene in the small intestine is due to bacteria or the immune response of the host.

In addition, Gg_4Cer, corresponding to ~20% of the total amount in the whole digestive tract, was present in the digestive tract contents and excreted into the faeces without degradation. The amounts of Gg_4Cer and LJ in the caecal and colonic contents were 37 μg versus 9.8×10^7 cells and 49 μg versus 1.4×10^8 cells, respectively (*2*). Consequently, Gg_4Cer in the caecal contents corresponded to 1.6×10^{16} molecules, which is sufficient for one-to-one binding of 9.8×10^7 cells of LJ, indicating that Gg_4Cer in the contents might facilitate the discharge of intestinal bacteria by becoming attached to them to prevent their irregular diffusion (*2*). Thus, glycolipids in the tissues of host animals were revealed to be actively metabolized, and to participate in the attachment of bacteria to the receptors and the discharge of bacteria from the body.

Conflict of interest
None declared.

References

1. Iwamori, M., Shibagaki, T., Nakata, Y., Adachi, S., and Nomura, T. (2009) Distribution of receptor glycolipids for Lactobacilli in murine digestive tract and production of antibodies cross-reactive with them by immunization of rabbits with Lactobacilli. *J. Biochem.* **146**, 185–191
2. Iwamori, M., Iwamori, Y., Adachi, S., and Nomura, T. (2011) Excretion into feces of asialo GM1 in the murine digestive tract and Lactobacillus johnsonii exhibiting binding ability toward asialo GM1. A possible role of epithelial glycolipids in the discharge of intestinal bacteria. *Glycoconj. J.* **28**, 21–30
3. Kojima, N., Uchikawa, K., Araki, Y., and Ito, E. (1985) A common linkage saccharide unit between teichoic acids and peptidoglycan in cell walls of Bacillus coagulans. *J. Biochem.* **97**, 1085–1092
4. Fischer, W., Schuster, D., and Laine, R.A. (1979) Studies on the relationship between glycerophosphoglycolipids and lipoteichoic acids. IV. Trigalactosylglycerophospho-acylkojibiosyldiacylglycerol and related compounds from *Streptococcus lactis Kiel* 42172. *Biochim. Biophys. Acta* **575**, 389–398
5. Shaw, N. and Baddiley, J. (1968) Structure and distribution of glycosyl diglycerides in bacteria. *Nature* **217**, 142–144
6. Koch, H.U. and Fischer, W. (1978) Acyldiglucosyldiacylglycerol-containing lipoteichoic acid with a poly(3-O-galabiosyl-2-O-galactosyl-sn-glycero-1-phosphate) chain from *Streptococcus lactis Kiel* 42172. *Biochemistry* **17**, 5275–5281
7. Shaw, N. (1970) Bacterial glycolipids. *Bacteriol. Rev.* **34**, 365–377
8. Smith, P.F. (1972) Lipid composition of *Mycoplasma neurolyticum*. *J. Bacteriol.* **112**, 554–558
9. Sugiyama, T., Smith, P.F., Langworthy, T.A., and Mayberry, W.R. (1974) Immunological analysis of glycolipids and lipopolysaccharides derived from various mycoplasmas. *Infect. Immun.* **10**, 1273–1279
10. Hirsch, H.E. and Parks, M.E. (1976) Serological reactions against glycolipid-sensitised liposomes in multiple sclerosis. *Nature* **264**, 785–787
11. Nakano, M. and Fischer, W. (1978) Trihexosyldiacylglycerol and acyltrihexosyldiacylglycerol as lipid anchors of the lipoteichoic acid of *Lactobacillus casei* DSM 20021. *Hoppe Seylers Z. Physiol. Chem.* **359**, 1–11
12. Ntamere, A.S., Taron, D.J., and Neuhaus, F.C. (1987) Assembly of D-alanyl-lipoteichoic acid in *Lactobacillus casei*: mutants deficient in the D-alanyl ester content of this amphiphile. *J. Bacteriol.* **169**, 1702–1711
13. IUPAC-IUB Commission on Biochemical Nomenclature. (1977) The Nomenclature of Lipids. *Eur. J. Biochem.* **179**, 11–21
14. Bartlett, G.R. (1959) Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**, 466–468
15. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497–509
16. Iwamori, M., Takamizawa, K., Momoeda, M., Iwamori, Y., and Taketani, Y. (2008) Gangliosides in human, cow and goat milk, and their abilities as to neutralization of cholera toxin and botulinum type A neurotoxin. *Glycoconj. J.* **25**, 675–683
17. Shibuya, I., Yamagoe, S., Miyazaki, C., Matsuzaki, H., and Ohta, A. (1985) Biosynthesis of novel acidic phospholipid analogs in *Escherichia coli*. *J. Bacteriol.* **161**, 473–477
18. Asai, Y., Katayose, Y., Hikita, C., Ohta, A., and Shibuya, I. (1989) Suppression of the lethal effect of acidic-phospholipid deficiency by defective formation of the major outer membrane lipoprotein in *Escherichia coli*. *J. Bacteriol.* **171**, 6867–6869
19. Dzieciatkowska, M., Brochu, D., van Belkum, A., Heikema, A.P., Yuki, N., Houliston, R.S., Richards, J.C., Gilbert, M., and Li, J. (2007) Mass spectrometric analysis of intact lipooligosaccharide: direct evidence for O-acetylated sialic acids and discovery of O-linked glycine expressed by *Campylobacter jejuni*. *Biochemistry* **46**, 14704–14714
20. Füllekrug, J. and Simons, K. (2004) Lipid rafts and apical membrane traffic. *Ann. NY Acad. Sci.* **1014**, 164–169
21. Iwamori, M., Murata, M., Toyoda, M., and Iwamori, Y. (2009) Contribution of glycolipids to species-specific antigens on erythrocytes of several animal species as to recognition of antigens with rabbit anti-glycolipids and anti-erythrocyte antisera. *Glycoconj. J.* **26**, 467–476
22. Bockman, D.E. and Cooper, M.D. (1973) Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix, and Peyer's patches. An electron microscopic study. *Am. J. Anat.* **136**, 455–477
23. Owen, R.L. and Jones, A.L. (1974) Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. *Gastroenterology* **66**, 189–203
24. Jacobs, B.C., Rothbarth, P.H., van der Meché, F.G., Herbrink, P., Schmitz, P.I., de Klerk, M.A., and van Doorn, P.A. (1998) The spectrum of antecedent infections in Guillain-Barré syndrome: a case-control study. *Neurology* **51**, 1110–1115
25. Godschalk, P.C., Kuijf, M.L., Li, J., St Michael, F., Ang, C.W., Jacobs, B.C., Karwaski, M.F., Brochu, D., Moterassed, A., Endtz, H.P., van Belkum, A., and Gilbert, M. (2007) Structural characterization of *Campylobacter jejuni* lipooligosaccharide outer cores associated with Guillain-Barre and Miller Fisher syndromes. *Infect. Immun.* **75**, 1245–1254
26. Iwamori, M. and Domino, S.E. (2004) Tissue-specific loss of fucosylated glycolipids in mice with targeted deletion of alpha(1,2)fucosyltransferase genes. *Biochem. J.* **380**, 75–81
27. Lin, B., Hayashi, Y., Saito, M., Sakakibara, Y., Yanagisawa, M., and Iwamori, M. (2000) GDP-fucose: beta-galactoside alpha1,2-fucosyltransferase, MFUT-II, and not MFUT-I or -III, is induced in a restricted region of the digestive tract of germ-free mice by host-microbe interactions and cycloheximide. *Biochim. Biophys. Acta* **1487**, 275–285
28. Bry, L., Falk, P.G., Midtvedt, T., and Gordon, J.I. (1996) A model of host-microbial interactions in an open mammalian ecosystem. *Science* **273**, 1380–1383

J Cancer Res Clin Oncol (2011) 137:715–722
DOI 10.1007/s00432-010-0930-8

ORIGINAL PAPER

CRABP1-reduced expression is associated with poorer prognosis in serous and clear cell ovarian adenocarcinoma

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Received: 8 April 2010 / Accepted: 14 June 2010 / Published online: 23 June 2010

Abstract
Purpose CRABP1 is a modulator of retinoic acid function. The aim of the present study was to investigate CRABP1 expression and its clinical significance in ovarian carcinoma.
Methods Expression of CRABP1 protein was investigated by immunohistochemical analysis in 100 ovarian carcinomas of various histological sub-types, including serous and clear cell adenocarcinomas. Relationship of CRABP1 expression to clinical features, including prognosis, was analyzed.
Results Reduced expression of CRABP1 protein was detected especially frequently in the serous and clear cell adenocarcinomas sub-types, 50% (20 of 40) and 38% (10 of 26) of cases, respectively. We found that in both serous and clear cell adenocarcinomas overall survival was significantly poorer in the cases with reduced CRABP1 expression compared to similar cases where expression was maintained, irrespective of the disease stage ($P = 0.0073$ and 0.049, respectively). Disease-free survival of the serous and clear cell adenocarcinoma cases with reduced CRABP1 expression was significantly poorer, compared to the cases whose CRABP1 expression was maintained ($P = 0.024$). Multivariate analysis showed that reduced expression of CRABP1 was a significantly important prognostic factor (adjusted hazard ratio: 8.189 (95% CI, 2.186–30.672, $P = 0.0019$)).
Conclusions The present study is the first to demonstrate that the reduced expression of CRABP1 has a potential as a prognostic marker for serous adenocarcinoma which is the most frequent histological ovarian tumor type and also for clear cell carcinoma that often exhibits chemo-resistance. Further study is necessary to clarify how CRABP1 protein expression was altered and how CRABP1 affects ovarian carcinoma cells.

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Keywords CRABP1 · Ovarian carcinoma · Serous · Clear cell · Prognosis

Abbreviations

CRABP1	Cellular retinoic acid binding protein 1
RA	Retinoic acid

Introduction

Ovarian cancer is the ninth most common cancer in US women (not counting skin cancers, the fifth leading cause of cancer deaths after lung and bronchus, breast, colorectal, and pancreatic cancers) and causes more deaths than any other cancer of the female reproductive system. In 2005, the last year in which accurate statistics were compiled in

the US, 19,842 women were diagnosed with ovarian cancer and 14,787 died from the disease.

The prognosis of early stage ovarian carcinoma, compared to later stages, is relatively good, and it can be cured by appropriate therapy. The preferred primary management of early ovarian carcinoma is surgical debulking followed by multi-agent adjuvant chemotherapy (DiSaia and Creasman 2002). Unfortunately, in many cases of ovarian carcinoma the tumor has only subtle symptoms or is asymptomatic, and a useful screening test has yet to be established. Thus, three-fourths of all ovarian carcinomas are thus diagnosed at an advanced stage, and the prognosis for these women is generally very poor, with a 5-year survival rate of 23–41% for stage III and only 11% for stage IV (DiSaia and Creasman 2002).

The major histological sub-types of ovarian carcinoma are serous, endometrioid, mucinous, and clear cell adenocarcinomas. In the US, serous adenocarcinomas represent 40–75% of all the ovarian epithelial carcinomas and clear cell adenocarcinomas 5–10% (DiSaia and Creasman 2002; Kurman 1994; Berek 2002). However, we have recently demonstrated that in Japan, clear cell adenocarcinoma accounted for a larger proportion of ovarian carcinoma cases (23% vs 5–10% in US) (our unpublished data). This is an important difference, since clear cell adenocarcinoma has been shown to exhibit a higher resistance to platinum-based chemotherapy, leading to a poor prognosis (Sugiyama et al. 2000). On the other hand, we previously showed that serous adenocarcinoma responded to combined chemotherapy of paclitaxel and carboplatin significantly better than the other tumor sub-types (Ueno et al. 2006).

In 80–85% of serous adenocarcinoma cases, the tumor cells are already disseminated to other pelvic tissues and the peritoneum, or metastasized to regional lymph nodes, at the time of the initial diagnosis; however, up to 60% of clear cell adenocarcinomas are in stage I at diagnosis (Kurman 1994). Thus, it would contribute immensely to the care of patients with serous ovarian adenocarcinoma (which is the most frequent histological type, most wide spread at diagnosis, and yet most responsive to combined chemotherapy of paclitaxel and carboplatin) and those with clear cell adenocarcinoma (which demonstrate the poorest prognosis) to clarify additional diagnostic and prognostic factors for these diseases.

It was recently shown that metabolism of vitamin A, and its active cellular catabolite retinoic acid (RA), was impaired in human ovarian cancer (Williams et al. 2009). RA has the potential to alter the growth and differentiation of a wide range of cell types and was shown to induce the differentiation of many murine teratocarcinoma cell lines (Means et al. 2000). Aldehyde dehydrogenase 1 (ALDH1) that participates in retinoic acid metabolism was shown to be related to prognosis of ovarian carcinoma cases (Chang et al. 2009).

Cellular retinoic acid-binding protein 1 (CRABP1) is a small, well-conserved member of a family of cytosolic lipid-binding proteins; it has a high affinity for RA and is an important modulator of RA signaling (Poulain et al. 2009). Homozygous deletion of the *crabp1* gene was demonstrated to result in decreased intracellular RA concentrations (Boylan and Gudas 1992; Liu et al. 2005). Silencing of *crabp1* by methylation of its promoter CpG island has long been associated with colorectal tumors, and it is one of a small battery of genes often screened in colorectal tumors for indications of the 'CpG island methylator phenotype' (CIMP). *Crabp1* hypermethylation is associated with poor patient survival in thyroid and hepatocellular tumors (Huang et al. 2003; Lee et al. 2009).

A recent study showed that promoter hypermethylation of the *crabp1* gene was detected in 2 of 3 ovarian clear cell adenocarcinomas, but none of 19 serous, 4 mucinous and 16 endometrioid adenocarcinomas (Wu et al. 2007), suggesting that *crabp1* hypermethylation might be an additional potential marker for ovarian clear cell adenocarcinomas. Whether the promoter region hypermethylation was reflected in loss of CRABP1 protein expression, and how this might be linked to patient outcome has yet to be established. Toward that end, in our current study expression of *crabp1* was analyzed in 80 clinical samples of ovarian carcinoma to investigate, first, whether altered expression of *crabp1* was specific to clear cell adenocarcinoma, as suggested, and second, to determine the relationship of loss of *crabp1* expression to clinical features of ovarian carcinomas, including prognosis, which has not yet been analyzed.

Materials and methods

Materials

One hundred cases of ovarian carcinoma were randomly picked from cases diagnosed during 1997 to 2008 at the Department of Obstetrics and Gynecology of the Osaka University Hospital in Osaka, Japan. The 120 cases included 50 serous, 29 clear cell, 26 endometrioid, 12 mucinous, and 3 undifferentiated cases. After asking for informed consent, the tissues from only 100 of these 120 patients were available for our study. They included 40 cases of serous adenocarcinoma, 26 cases of clear cell adenocarcinoma, 24 cases of endometrioid adenocarcinoma, and 10 cases of mucinous adenocarcinoma. The patient age at surgery ranged from 25 to 90 years (median: 54 years). The tumor stages diagnosed following surgery were stage I

in 41 cases, stage II in 19 cases, stage III in 38 cases, and stage IV in 2 cases.

In our institution, for primary ovarian carcinomas, we typically perform a surgical removal of the ovaries, fallopian tubes, uterus, omentum, and the retroperitoneal lymph nodes, followed by giving combination chemotherapy using taxane and platinum. These cases were carefully followed post-operatively with regular exams that included pelvic examinations and tumor marker and radiological tests. The median follow-up period was 42 months (range 1–133 months). Salvage chemotherapy, with or without surgical removal, was performed for recurrent diseases.

Immunohistochemical staining

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue blocks from a total of 100 cases of ovarian cancer, using a LSAB+/HRP kit (Dako, Cambridge, UK) following the manufacturer's instructions. Briefly, after removing the paraffin, the antigens were retrieved by microwave pretreatment in target retrieval solution at 95°C for 5 min. After blocking in peroxidase reagent, the tissues were incubated with an anti-human-CRABP1 primary antibody (Sigma–Aldrich, Saint Louis, MO) at room temperature for 1 h. After washing, the tissues were incubated with secondary antibody, followed by incubation with peroxidase. Visualization was performed with diaminobenzidine with Mayer's hematoxylin. Squamous epithelium of normal uterine cervix was used as a positive control, and tissue sections incubated with only antigen-dilution-reagent were used as negative controls. These controls were used for each staining.

Evaluation of immunohistochemical staining

The slides were observed by light microscopy, with review of the entire histological section from each case, to evaluate for possible tumor microheterogeneity in antigen distribution. Immunohistochemical staining was scored on a 3-tiered scale for both intensity of cytoplasmic staining (grade 1: absent/weak, grade 2: moderate, and grade 3: strong) and extent (grade 1: percentage of positive cells is <10%, grade 2: 10–50%, and grade 3: >50%). The intensity and the extent were then multiplied to give a composite score of 1–9 for each tumor, as described in a previous study (Greenspan et al. 1997). The composite scores of 1–4 were defined as a reduction of CRABP1 protein expression, compared to those of 6–9 (composite scores resulting in the prime numbers 5 and 7 can't mathematically occur). The evaluation of immunohistochemical staining was carried out by two independent pathologists who were unaware of the patient outcomes.

Analysis of patient prognosis

Patient clinical records were reviewed, including histology and surgical records. Overall survival was defined as the time from initial surgery until death or, if still alive, to the date of the last follow-up. Disease-free survival was defined as the time from complete remission of the disease by surgery with/without chemotherapy until documentation of recurrence or, if still free of recurrence, to the date of the last follow-up.

Statistical analysis

The χ^2 test was used for comparison of the distribution of stages between the cases in which CRABP1 expression was reduced and those in which it was maintained. Overall and disease-free survivals were calculated using the Kaplan–Meier method. Univariate and multivariate Cox proportional hazards models (step-wise method) for the factors including age, histology, initial stage, and CRABP1 expression were calculated to evaluate whether reduced expression of CRABP1 was a significantly important factor on OS. A P value <0.05 was considered to be statistically significant.

Approval of the study

This study was approved by our Institutional Review Board and Ethics Committee.

Results

Reduced expression of CRABP1 in various types of ovarian cancer

Staining specific for CRABP1 protein was found in the cytoplasm of all the histological sub-types of ovarian tumor cells. Examples of the immunohistochemical study of CRABP1 expression are shown in Fig. 1. Reduced expression of CRABP1 was observed in 33 (33%) of 100 ovarian cancer cases, and especially frequently in serous and clear cell adenocarcinomas, 20 (50%) of 40 and 10 (38%) of 26 cases, respectively; however, in endometrioid and mucinous adenocarcinomas, only 2 (8%) of 24 and 1 (10%) of 10 cases showed reduced expression. In serous adenocarcinomas, CRABP1 expression was reduced in 4 (44%) of 9 stage I cases, in 4 (57%) of 7 stage II cases, in 11 (48%) of 23 stage III cases, and 1 (100%) of 1 stage IV case. In clear cell adenocarcinomas, CRABP1 expression was reduced in 7 (41%) of 17 stage I cases, in 1 (25%) of 4 stage II case, 1 (25%) of 4 stage III case, and 1 (100%) of 1 stage IV case. The distribution of the stages between the cases in which

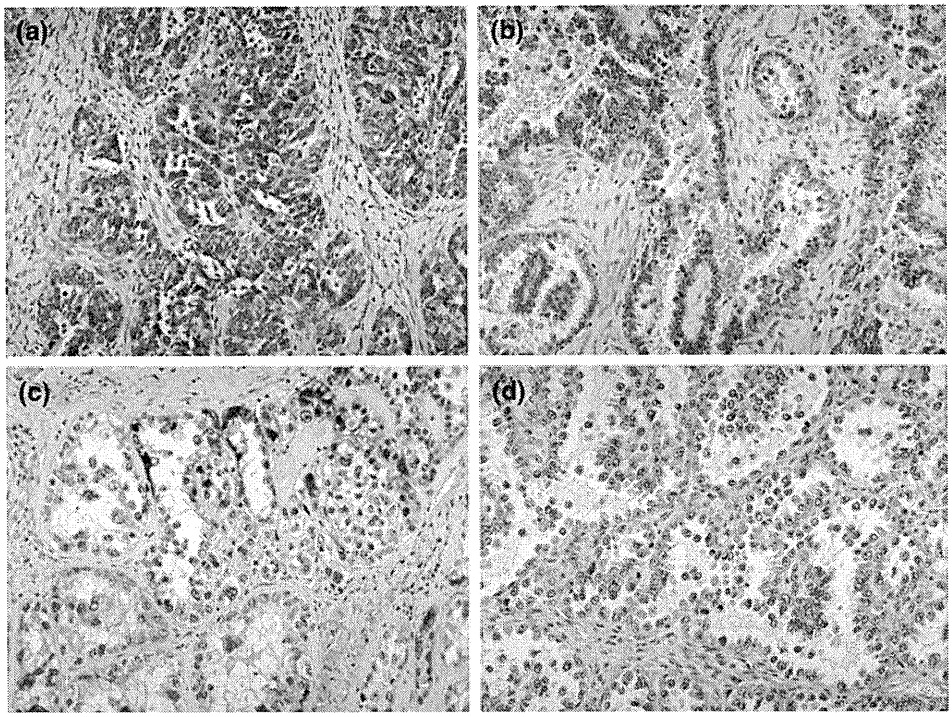

Fig. 1 Examples of immunohistochemical staining of CRABP1 (×400). **a** Maintained CRABP1 expression in a case of serous adenocarcinoma: score 6 (extent: grade 2, intensity: 3). **b** Reduced CRABP1 expression in a case of serous adenocarcinoma: score 1 (extent: grade 1, intensity: 1). **c** Maintained CRABP1 expression in a case of clear cell adenocarcinoma: score 6 (extent: grade 2, intensity: 3). **d** Reduced CRABP1 expression in a case of clear cell adenocarcinoma: score 1 (extent: grade 1, intensity: 1)

CRABP1 expression was reduced and those in which CRABP1 expression was maintained did not exhibit statistically significant differences in either the serous or clear cell adenocarcinoma cases ($P = 0.73$ and $P = 0.52$, respectively, by the χ^2 test). High grade tumors (grade 3) mostly exhibited reduced CRABP1 expression, and CRABP1 expression was frequently maintained in low grade tumors (grade 1 and grade 2) ($P = 0.047$ by Fisher's exact test). Lymph-node metastasis was not associated with CRABP1 expression.

Association of the reduction of CRABP1 expression and overall survival in serous and clear cell adenocarcinoma patients

Overall survival was analyzed in all 40 serous and 26 clear cell carcinoma cases, in which 20 (50%) and 10 (38%) cases, respectively, demonstrated reduction of CRABP1 expression in immunohistochemical analysis. During the median follow-up period of 45.5 months (range 1–133 months), 20 serous adenocarcinoma cases whose CRABP1 expression was reduced exhibited a statistically significant worse prognosis, compared to the other 20 cases whose CRABP1 expression was maintained ($P = 0.0073$ by the Kaplan–Meier method) (Fig. 2). Disease-specific death was documented in only one case (5%) among the 20 cases, which maintained CRABP1 expression. However, disease-linked death occurred in 9 cases (45%) among 20 cases with reduced CRABP1 expression.

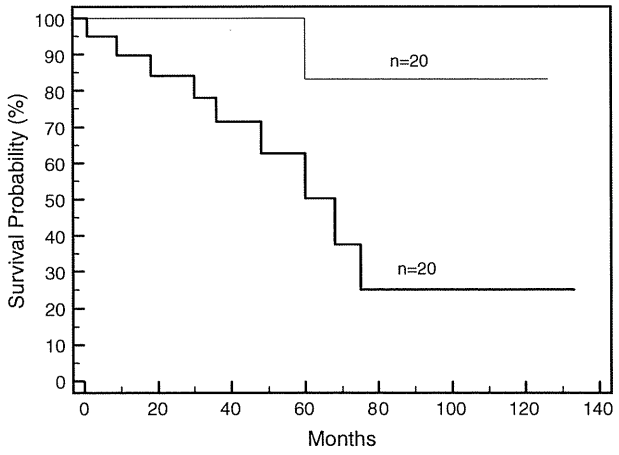

Fig. 2 Overall survival of serous adenocarcinoma cases. The median follow-up period was 45.5 months (range 1–133 months). Overall survival of 20 serous adenocarcinoma cases with reduced CRABP1 expression was significantly worse than that of 20 cases with maintained CRABP1 expression ($P = 0.0073$ by the Kaplan–Meier method). *Broken line* survival probability of maintained-CRABP1-expression cases. *Solid line* survival probability of reduced-CRABP1-expression cases

Similarly, during the median follow-up period of 43 months (3–133 months), 10 clear cell adenocarcinoma cases with reduced expression of CRABP1 exhibited worse prognoses, compared to the other 16 cases whose CRABP1 expression was maintained, with statistical significance ($P = 0.049$ by the Kaplan–Meier method) (Fig. 3). Disease-specific death was documented in only two cases (13%) among the 16 cases with maintained CRABP1 expression.

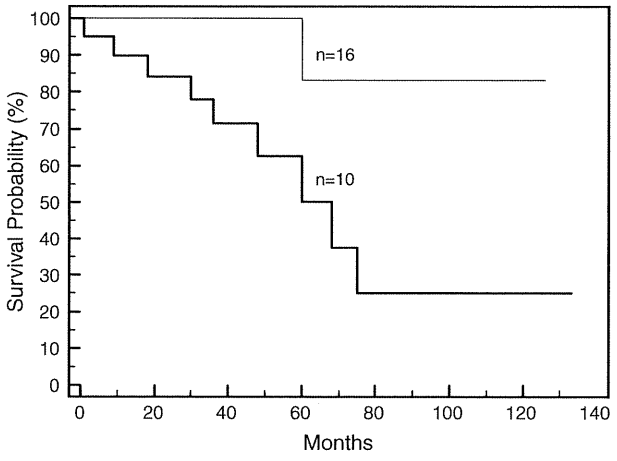

Fig. 3 Overall survival of clear cell adenocarcinoma cases. The median follow-up period was 43 months (range 3–133 months). Overall survival of 16 clear cell adenocarcinoma cases with maintained CRABP1 expression was significantly better than that of 10 cases with reduced CRABP1 expression ($P = 0.049$ by the Kaplan–Meier method). *Broken line* survival probability of maintained-CRABP1-expression cases. *Solid line* survival probability of reduced-CRABP1-expression cases

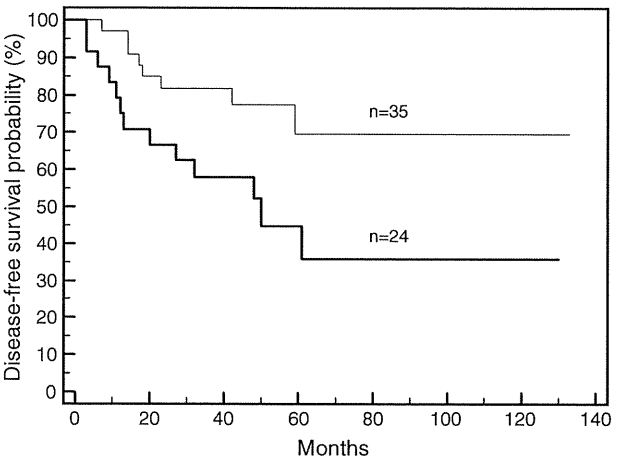

Fig. 4 Disease-free survival of ovarian carcinomas. Complete remission was achieved by surgery, with/without chemotherapy, in a total of 48 ovarian carcinomas, including 35 serous and 24 clear cell carcinoma cases. The median follow-up period was 46 months (range 4–133 months). Disease-free survival of 35 carcinomas with maintained CRABP1 expression was significantly better than that of 24 cases with reduced CRABP1 expression ($P = 0.024$ by the Kaplan–Meier method). *Broken line* survival probability of maintained-CRABP1-expression cases. *Solid line* survival probability of reduced-CRABP1-expression cases

However, it occurred in 5 (50%) among 10 cases with reduced CRABP1 expression.

Association of reduction of CRABP1 expression and disease-free survival in serous and clear cell adenocarcinoma patients

Complete remission was achieved by surgery (with or without chemotherapy) in 36 cases (90%) of 40 serous adenocarcinomas and 23 cases (88%) of 26 clear cell adenocarcinomas. Disease-free survival was next analyzed in these 59 complete-remission cases. During the median follow-up period of 46 months (4–133 months), 20 cases with reduced expression of CRABP1 exhibited worse disease-free survival, compared to the other 35 cases whose CRABP1 expression was maintained, with statistical significance ($P = 0.024$ by the Kaplan–Meier method) (Fig. 4). Recurrence occurred in only 9 cases (26%) among 35 cases with maintained CRABP1 expression; however, it occurred in 11 cases (46%) among 24 cases with reduced CRABP1 expression. Statistically significant differences were not demonstrated in our analysis of each of serous and clear cell adenocarcinomas. The duration from recurrence to death did not demonstrate significant difference between histological sub-types, nor between maintained and reduced CRABP1 expression (data not shown).

Univariate and multivariate cox proportional hazards analysis for effect of alteration of CRABP1 expression on overall survival

Univariate analysis demonstrated that advanced stage (stage II, III, and IV) and reduced expression of CRABP1 effected overall survival of the diseases. In order to further support our belief that reduced expression of CRABP1 independently relates to the prognosis of ovarian serous and clear cell adenocarcinomas, multivariate Cox proportional hazards analysis was performed (Table 1). Clear cell type, advanced stage (stage II, III, and IV), and reduced expression of CRABP1 were shown to be independent factors for overall survival of the diseases. Especially, the adjusted hazard ratio (HR) of reduced expression of CRABP1 was 8.189 (95% CI, 2.186–30.672, $P = 0.0019$).

Discussion

Although the ovarian cancer incidence rate has been slowly falling over the past 20 years, it still accounts for 3–5% of all cancers in women. In addition to its human toll, it exacts a huge financial burden; in the US about $2.2 billion is spent annually on ovarian cancer treatments (in 2004 dollars) (www.cancer.org/docroot/cri/content/cri_2_4_1x_what_are_the_key_statistics_for_ovarian_cancer_33.asp, 2009).

Among the histological sub-types of ovarian cancers, the serous adenocarcinoma is the most frequent type. Although rarer, most serous adenocarcinoma cases have already disseminated to other pelvic tissues and the peritoneum, or have metastasized to regional lymph nodes, at the time of initial diagnosis and are thus extremely more difficult to cure (DiSaia and Creasman 2002). Cytoreductive surgery

Table 1 Multivariate cox proportional hazards analysis for effect of alteration of CRABP1 expression on overall survival

Variable	Univariate analysis	Multivariate analysis		
	P value	Adjusted HR	95% CI	*P* value
Age (years)	0.83			0.67
<60		1		
≥60		1.259	0.436–3.631	
Histology	0.89			0.019
Serous		1		
Clear cell		4.598	1.299–16.275	
Initial stage	0.049			0.0055
I		1		
II/III/IV		7.806	1.844–33.044	
CRABP1 expression	0.0041			0.0019
Maintained		1		
Reduced		8.189	2.186–30.672	

The adjusted HR of reduced expression of CRABP1 was 8.189 (95% CI, 2.186–30.672), compared to maintained expression CRABP1, showing statistical significance (*P* value was 0.0019)

HR hazard ratio

followed by combination chemotherapy using taxane and platinum improves the prognosis of some ovarian cancer patients; however, there remain serious problems in the management of this disease where additional prognostic markers would be extremely helpful.

Clear cell adenocarcinoma, which represents only 5–10% of all the ovarian carcinomas in the Caucasian-dominated United States cases (DiSaia and Creasman 2002; Kurman 1994; Berek 2002) accounts for a much larger percentage (23%) of ovarian carcinoma cases in Japan (Our unpublished data). Clear cell carcinomas are unusually resistant to standard platinum-based chemotherapy, and their prognosis is extremely poor (Sugiyama et al. 2000). Thus, clinical and basic research targeting understanding serous and clear cell adenocarcinomas has been a high priority.

In our present study, expression of CRABP1 protein as a potential prognostic marker was investigated in 100 ovarian carcinomas of various histological sub-types, including serous and clear cell adenocarcinomas. CRABP1 was demonstrated to be a useful factor for predicting the prognosis of serous and clear cell adenocarcinomas. Overall survival was significantly poorer in the cases with reduced CRABP1 expression, in both serous and clear cell adenocarcinomas, compared to the cases whose CRABP1 expression was maintained ($P = 0.0073$ and $P = 0.049$, respectively). Also, the disease-free survival of the serous and clear cell carcinoma cases with reduced CRABP1 expression was significantly poorer, compared to the cases whose CRABP1 expression was maintained ($P = 0.024$). The fact that distribution of the tumor stages was not different between the cases with reduced CRABP1 expression and those with maintained CRABP1 expression implies that the reduction of CRABP1 expression predicted the prognosis irrespective of the stage of the disease. Multivariate analysis showed that reduced expression of CRABP1 was a significantly important prognostic factor.

Reduced expression of CRABP1 was observed by immunohistochemical analysis in 31% of 86 ovarian cancer cases. Especially in serous and clear cell adenocarcinomas, reduced expression of CRABP1 was detected in 50 and 38% of cases, respectively. However, reduced expression was found in endometrioid and mucinous adenocarcinomas in only 2 (8%) of 24 and 1 (10%) of 10 cases, respectively.

So, how it is that loss of CRABP1 expression would have an effect on ovarian tumor phenotype? Evidence that hypermethylation of the *crabp1* gene was more than just a reflection of the hypermethylation CIMP phenotype was recently shown when restoration of CRABP1 expression in esophageal carcinoma cells (ESCC) lacking the protein reduced cell growth by inducing arrest at G_0–G_1, whereas knockdown of the gene in cells expressing CRABP1 promoted cell growth (Tanaka et al. 2007). Among 113 primary ESCC tumors, the absence of immuno-reactive CRABP1 was significantly associated with de-differentiation of cancer cells and with distant lymph-node metastases in the patients (Tanaka et al. 2007). These results indicate that CRABP1 appears to have an active tumor-suppressor function in esophageal epithelium, and its epigenetic silencing may play a pivotal role during esophageal carcinogenesis. In our ovarian cancer cases, CRABP1 expression was associated with histological grade of the tumor, but not with lymph-node metastasis.

The results of Wu et al. implied that *crabp1* expression was impaired specifically in clear cell adenocarcinoma of the ovary by promoter CpG island hypermethylation. This hinted at a phenotypic preference in ovarian tumors (Wu et al. 2007; Barton et al. 2008). This also suggested that other mechanisms, including loss of heterozygosity, mutations, and post-transcriptional and post-translational alterations

that have not yet been reported, might be involved in the reduction of *crabp1* expression in other sub-types of ovarian tumors, such as serous adenocarcinomas.

CRABP1 was shown to be expressed selectively in the mesenchymal tissues at the junction of the epithelium and the mesenchyme, functioning in mesenchymal/epithelial interaction (Bhasin et al. 2003). The prognostic significance of epithelial-mesenchymal transition (EMT) was recently demonstrated in various carcinomas, including ovarian tumors (Bagnato and Rosanò 2007; Smit et al. 2009; Vasko et al. 2007; Soltermann et al. 2008; Al-Saad et al. 2008; Shim et al. 2009). In our study, CRABP1 expression was observed in the cytoplasm of the normal epithelial cells of the ovarian surface, the fallopian tube, and the adenocarcinomas.

It is possible that de-differentiation of ovarian carcinoma cells is triggered by reduction of CRABP1 and may similarly represent an epithelial-mesenchymal transition, which results in the poor prognosis. Based on these findings, it is implied that CRABP1 may normally act to induce or maintain differentiation of ovarian cells and that reduction of CRABP1 expression may lead to a failure to differentiate or a de-differentiation of tumor cells of serous and clear cell adenocarcinoma of the ovary, resulting in an early recurrence and poor overall survival.

Altered expression of CRABP1 may present as a potential target for molecular therapy in serous adenocarcinoma, which is the most frequent histological ovarian tumor type, and also for clear cell carcinoma, which often exhibits chemo-resistance. It should be noted with caution that retinoids have been proposed to have such beneficial cancer-preventive functions that they were recently used in large scale human clinical trials to reduce lung cancer incidence in high-risk individuals. However, the obtained antagonistic clinical results of RA prophylactic treatments were in direct contradiction with the previous promising in vivo and in vitro studies (Poulain et al. 2009).

Recent studies have shown that the hypermethylation of specific marker genes, including *Igfbp-3*, 18S and 28S rDNA, can act as potential prognostic markers in ovarian carcinoma (Wiley et al. 2006; Chan et al. 2005). While CIMP-related hypermethylation can be rather indiscriminant, it is thought to contribute to tumor progression by silencing important tumor-suppressor genes. The relatively frequent loss of *crabp1* gene expression in the serous and clear cell sub-types of ovarian adenocarcinomas suggests it may be such a tumor-suppressor gene, and it is reasonable to assume that loss of expression is in part due to promoter hypermethylation, but awaits experiments to demonstrate this hypothesis.

In conclusion, we have demonstrated that reduction of CRABP1 expression was observed most specifically in serous and clear cell adenocarcinomas of the ovary. The present study is the first to demonstrate that the reduced expression of CRABP1 has a potential as a prognostic marker for ovarian cancers. Further study is necessary to clarify how CRABP1 protein expression was altered and how CRABP1 affects ovarian carcinoma cells.

Acknowledgments We would like to thank Dr. G.S. Buzard, CDCP, for his constructive critique of our manuscript. We are also deeply grateful to Ms. S. Sugiyama and Ms. K. Nakano for their excellent support in collecting patient medical records.

Conflict of interest statement None.

References

Al-Saad S, Al-Shibli K, Donnem T, Persson M, Bremnes RM, Busund LT (2008) The prognostic impact of NF-kappaB p105, vimentin, E-cadherin and Par6 expression in epithelial and stromal compartment in non-small-cell lung cancer. Br J Cancer 99:1476–1483

Bagnato A, Rosanò L (2007) Epithelial-mesenchymal transition in ovarian cancer progression: a crucial role for the endothelin axis. Cells Tissues Organs 185:85–94

Barton CA, Hacker NF, Clark SJ, O'Brien PM (2008) DNA methylation changes in ovarian cancer: implications for early diagnosis, prognosis and treatment. Gynecol Oncol 109:129–139

Berek JS (2002) Novak's gynecology, 13th edn. William and Wilkins, Baltimore

Bhasin N, Maynard TM, Gallagher PA, LaMantia AS (2003) Mesenchymal/epithelial regulation of retinoic acid signaling in the olfactory placode. Dev Biol 261:82–98

Boylan JF, Gudas LJ (1992) The level of CRABP-I expression influences the amounts and types of all-trans-retinoic acid metabolites in F9 teratocarcinoma stem cells. J Biol Chem 267:21486–21491

Chan MW, Wei SH, Wen P, Wang Z, Matei DE, Liu JC, Liyanarachchi S, Brown R, Nephew KP, Yan PS, Huang TH (2005) Hypermethylation of 18S and 28S ribosomal DNAs predicts progression-free survival in patients with ovarian cancer. Clin Cancer Res 11:7376–7383

Chang B, Liu G, Xue F, Rosen DG, Xiao L, Wang X, Liu J (2009) ALDH1 expression correlates with favorable prognosis in ovarian cancers. Mod Pathol 22:817–823

DiSaia PJ, Creasman WT (2002) Clinical gynecologic oncology, 6th edn. Mosby, Inc, St. Louis

Greenspan DL, Connolly DC, Wu R, Lei RY, Vogelstein JT, Kim YT, Mok JE, Muñoz N, Bosch FX, Shah K, Cho KR (1997) Loss of FHIT expression in cervical carcinoma cell lines and primary tumors. Cancer Res 57:4692–4698

Huang Y, de la Chapelle A, Pellegata NS (2003) Hypermethylation, but not LOH, is associated with the low expression of MT1G and CRABP1 in papillary thyroid carcinoma. Int J Cancer 104:735–744

Kurman RJ (1994) Blaunstein's pathology of the female genital tract, 4th edn. Springer, New York

Lee HS, Kim BH, Cho NY, Yoo EJ, Choi M, Shin SH, Jang JJ, Suh KS, Kim YS, Kang GH (2009) Prognostic implications of and relationship between CpG island hypermethylation and repetitive DNA hypomethylation in hepatocellular carcinoma. Clin Cancer Res 15:812–820

Liu RZ, Sharma MK, Sun Q, Thisse C, Thisse B, Denovan-Wright EM, Wright JM (2005) Retention of the duplicated cellular retinoic acid-binding protein 1 genes (*crabp1a* and *crabp1b*) in the zebrafish genome by subfunctionalization of tissue-specific expression. FEBS J 272:3561–3571

Means AL, Thompson JR, Gudas LJ (2000) Transcriptional regulation of the cellular retinoic acid binding protein I gene in F9 teratocarcinoma cells. Cell Growth Differ 11:71–82

Poulain S, Evenou F, Carré MC, Corbel S, Vignaud JM, Martinet N (2009) Vitamin A/retinoids signalling in the human lung. Lung Cancer 66:1–7

Shim HS, Yoon BS, Cho NH (2009) Prognostic significance of paired epithelial cell adhesion molecule and E-cadherin in ovarian serous carcinoma. Hum Pathol 40:693–698

Smit MA, Geiger TR, Song JY, Gitelman I, Peeper DS (2009) A Twist-Snail axis critical for TrkB-induced EMT-like transformation, anoikis resistance and metastasis. Mol Cell Biol 29:3722–3737

Soltermann A, Tischler V, Arbogast S, Braun J, Probst-Hensch N, Weder W, Moch H, Kristiansen G (2008) Prognostic significance of epithelial-mesenchymal and mesenchymal-epithelial transition protein expression in non-small cell lung cancer. Clin Cancer Res 14:7430–7437

Sugiyama T, Kamura T, Kigawa J, Terakawa N, Kikuchi Y, Kita T, Suzuki M, Sato I, Taguchi K (2000) Clinical characteristics of clear cell carcinoma of the ovary: a distinct histological type with poor prognosis and resistance to platinum-based chemotherapy. Cancer 88:2584–2589

Tanaka K, Imoto I, Inoue J, Kozaki K, Tsuda H, Shimada Y, Aiko S, Yoshizumi Y, Iwai T, Kawano T, Inazawa J (2007) Frequent methylation-associated silencing of a candidate tumor-suppressor, CRABP1, in esophageal squamous-cell carcinoma. Oncogene 26:6456–6468

Ueno Y, Enomoto T, Otsuki Y, Sugita N, Nakashima R, Yoshino K, Kuragaki C, Ueda Y, Aki T, Ikegami H, Yamazaki M, Ito K, Nagamatsu M, Nishizaki T, Asada M, Kameda T, Wakimoto A, Mizutani T, Yamada T, Murata Y (2006) Prognostic significance of *p53* mutation in suboptimally resected advanced ovarian carcinoma treated with the combination chemotherapy of paclitaxel and carboplatin. Cancer Lett 241:289–300

Vasko V, Espinosa AV, Scouten W, He H, Auer H, Liyanarachchi S, Larin A, Savchenko V, Francis GL, de la Chapelle A, Saji M, Ringel MD (2007) Gene expression and functional evidence of epithelial-to-mesenchymal transition in papillary thyroid carcinoma invasion. Proc Natl Acad Sci USA 104:2803–2808

Wiley A, Katsaros D, Fracchioli S, Yu H (2006) Methylation of the insulin-like growth factor binding protein-3 gene and prognosis of epithelial ovarian cancer. Int J Gynecol Cancer 16:210–218

Williams SJ, Cvetkovic D, Hamilton TC (2009) Vitamin A metabolism is impaired in human ovarian cancer. Gynecol Oncol 112:637–645

Wu Q, Lothe RA, Ahlquist T, Silins I, Tropé CG, Micci F, Nesland JM, Suo Z, Lind GE (2007) DNA methylation profiling of ovarian carcinomas and their in vitro models identifies HOXA9, HOXB5, SCGB3A1, and CRABP1 as novel targets. Mol Cancer 6:45

International Journal of Clinical Medicine, 2011, 2, 588-592
doi:10.4236/ijcm.2011.25097 Published Online November 2011 (http://www.SciRP.org/journal/ijcm)

Integrating Complimentary and Alternative Medicine in Form of Active Hexose Co-Related Compound (AHCC) in the Management of Head & Neck Cancer Patients

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Received September 4th, 2011; revised October 17th, 2011; accepted October 26th, 2011.

ABSTRACT

Objectives: *The Active Hexose Correlated Compound (AHCC), is produced from mushroom mycerium and rich in alpha glucans was administered to the cancer patients along with chemotherapy to see if it is having any beneficial effects on the final outcome in terms of reducing side effects of chemotherapy, maintaining the general condition and having effect on tumor control. Methods*: *Twenty five patients were administered AHCC along with conventional palliative chemotherapy regimen out of which sixteen patients received paclitaxel, and cisplatinum/carboplatin, nine patients received combination of cisplatim and 5-Flurouracil. All the patients were having advance stage (T3/T4) head and neck cancers. Thirteen patients were cancer of cheek, followed by cancer of tongue* (4), *oro-pharyngeal cancer* (6) *and cancer of naso-pharynx* (2). *Results*: *All the patients tolerated AHCC well with no added symptoms. Twenty patients reported that they are feeling stronger than before at the time of initiation of chemotherapy cycles. Almost all the patients reported to have better appetite after they started taking AHCC. Twelve patients who required blood transfusion before chemotherapy cycles, decrease in the rate of fall in hemoglobin was observed in these patients and only three patients required blood transfusion before subsequent chemotherapy cycles. In* 22 *patients definite reduction of chemotherapy side effects like nausea, vomiting, drop in total leucocytes count, loose motion/constipation etc. were observed, which reduced the hospital stay of these patients. Tumor regressed in* 11 *patients,* 8 *patients had stable disease and in rest of the patients, the disease progressed. Conclusions*: *AHCC up to* 3 *g is safe to administer and definitely helps cancer patients in reducing side effects of chemotherapeutic drugs, getting a sense of wellbeing and improved intake maintains general condition as well as prepare them to continue and tolerate further cycles in a better way.*

Keywords: *Head and Neck Cancer, Quality of Life, AHCC*

1. Introduction

India contributes to maximum number of Tobacco Related Cancer Deaths in the world [1]. The use of tobacco also attributes towards tuberculosis, heart diseases and other lung conditions in addition to neoplastic diseases [1,2]. According to the national cancer registry (http://www.ncrpindia.org/) data, the incidence of cancer of head and neck region is highest in male, where as cancer of esophagus is highest in female in the state of Meghalaya, one of the north-eastern states of India [1]. North eastern region (NER) of India consists of eight small states namely Assam, Arunachal Pradesh, Nagaland, Meghalaya, Manipur, Mizoram, Tripura and Sikkim. These states are mainly home to various tribal populations, belonging to various ethnic origins. As their domicile changes, their living condition, food pattern, disease spectrum and life span changes. Evidences show that majority of the population inhabiting NER use tobacco in some or other forms in addition to alcohol and supari (*arica-nut*) and combined use of all these factors are responsible for initiation of Oropharyngeal, Lungs and upper aerodigestive tract cancers. Amongst all these NER states the incidence of tobacco related cancers (TRC) are very high. According to the national cancer registry programme (NCRP) data, the difference of inci-

dence of TRC in NER is too high in comparison to rest of the country [1].

Majority of these patients present with an advance stage disease, where the nutrition status of these patients remain low. Other poor prognostic features like old age, stage of the disease at presentation, neck node status, presence/absence of other metastatic sites are also very important, while considering these patients for any kind of treatment. With above mentioned features, many times it becomes difficult for the patients to tolerate various forms of anticancer treatment. Consequently it becomes very important to maintain the general condition of the patients at an optimum level so that the patients can tolerate further treatment. Therefore this trial was undertaken to see if complimentary and alternative medicine (CAM) in form of AHCC can maintain the nutrition/ immunity at optimum level, so the cancer patients having advance stage disease shall be able to tolerate further treatment like chemotherapy and radiotherapy for better tumor control. AHCC has never been tried in the management of head and neck cancer in the clinical study.

2. Materials and Methods

2.1. Cases

Twenty five patients of advance stage (T3: 13 & T4: 12) Head & Neck Cancer Patients were enrolled in this project. Thirteen patients were cancer of Cheek, followed by cancer of tongue (4), oro-pharyngeal cancer (6) and cancer of naso-pharynx (2) (**Table 1**). All the patients were either having residual or recurrent tumors subsequent to their primary treatment. Hence, these patients were managed with palliative chemotherapy treatment.

2.2. Active Hexose Correlated Compound (AHCC)

AHCC is an enzyme fermented extract of the mycelia of Basidiomycetes mushroom obtained through the mushroom (Lentinus edodes), containing a mixture of polysaccharides, amino acids, lipids and minerals. The final product is obtained by hot water extraction after culturing media and then treating them with enzymes. The predominant components of AHCC are oligosaccharides of which major portions are alpha-glucans having an effect on the immune system. It has been proven as a biological response modifier in experimental animals as well as human being. AHCC samples were provided by Amino Up Chemical Co. Ltd., Sapporo, Japan for conducting this trial.

Majority of the patient received Taxane based chemotherapy along with Platinum (Cispliatin/Carboplatin) (16). The dose of chemotherapy was customized depending on the general condition of each of the patients. Rest patients received platinum with 5 Fluorouracil combinations. Twelve patients also received targeted Monoclonal Antibody treatment in the form of epidermal growth factor receptor (EGFR) inhibitor. Some of the patients were heavily pre-treated with very low general condition. As such patients with head and neck cancers present with low general conditions because of less oral intake. Previous treatment history of the patients includes 6 patients had undergone surgery, 12 patients had history of radiotherapy and 16 patients had history of chemotherapy, prior to recruitment under this AHCC trial (**Table 2**). These patients acted as their own control.

All the patients were administered AHCC 3 g of dried extract every day morning 3 days prior to the chemotherapy with water scheduled date and followed up to one week post chemotherapy either with water or milk. The reasons being maximum toxicity of the chemotherapy drugs are observed within first one week following administration.

Table 1. Division of patients according to primary tumor site.

Tumor sub-site	Stage	No. of patients
Cheek	T3N2bM0	7
	T4N2bM0	6
Tongue	T3N2aM0	3
	T4N2cM0	1
Oro-pharynx	T3N2cM0	6
Naso-pharynx	T3N2cM0	2

Table 2. Treatment history.

Treatment Modality	*No. of Cycles/radiation dose*	*No. of patients*
Chemotherapy		
Paclitaxel/Docetaxel + Cisplatin/Carboplatin	6 - 18	16
5-Flurouracil + Cisplatin/Carboplatin	12 - 24	09
Concurrent Chemo-radiation		08
EGFR Inhibitor	Cetuximab/Nimotuzumab	12
Radiotherapy		
Radical radiotherapy	60 – 66 Gy	06
Post-op radiotherapy	50 Gy	06
Surgery		06

2.3. Assessment

The hematological parameters like hemogram, liver and kidney function tests were performed before each cycle of chemotherapy and followed 3 days after completion of chemotherapy. CT scan was done after completion of three cycles and two weeks after sixth cycle of chemotherapy to evaluate the tumor response. The patients were given a questionnaire on the quality of life issues and asked specific questions regarding their general feeling, sleep pattern, social interaction etc.

3. Results

All the patients tolerated AHCC well with no added symptoms. Twenty patients reported that they are feeling better and stronger than before at the time of initiation of chemotherapy cycles (**Figure 1**). In most of the patients, the sleep pattern became regular than before and the patients started interacting with visitors normally than before (**Table 3**). Almost all the patients reported to have better appetite after they started taking AHCC. No patients with AHCC required appetizer.

Sixteen patients who required blood transfusion before chemotherapy cycles, decrease in the rate of fall in hemoglobin was observed in these patients and only three patients required blood transfusion before subsequent chemotherapy cycles. Only 7 patients required growth factor with AHCC compared to 12 patients without it. Also no patients required platelet concentrate transfusion in AHCC group compared to 3 in without AHCC. The comparative charts are given in **Figure 1**. In 22 patients definite reduction of chemotherapy side effects like nausea, vomiting, loose motion/constipation etc. were observed, which reduced the hospital stay of these patients. While the requirement of antiemetic dropped from 7 - 14 days before AHCC group to 3 - 5 days in AHCC group, only 2 patients required hospitalization because of loose motion in AHCC group compared to 6 patients without AHCC group (**Figure 2** and **Table 3**). Patiaents were evaluated both clinically as well as radiologically to determine tumor response. CT scan/MRI of the primary tumor as well as neck nodes were performed after two weeks of completion of at least six cycles of chemotherapy. Tumor regressed in 11 patients, 8 patients had stable disease and in rest of the patients, disease progressed (**Table 4**).

4. Discussion

AHCC is an alpha-glucan rich nutritional supplement extracted from the *mycelia* of shiitake (*Lentinula edodes*) of the basidiomycetes family of mushrooms. The final product is obtained by hybridization of several types of mushrooms and is very effective biological modifier.

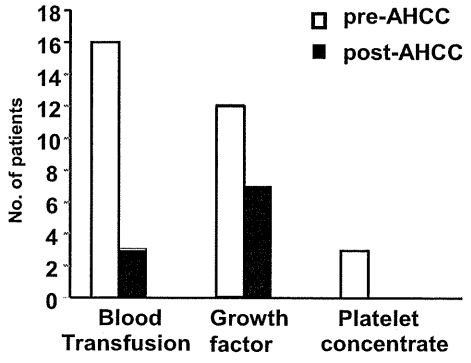

Figure 1. Comparison of hematological parameters in patients with and without AHCC, bringing down the blood transfusion rate (16 vs 3). Total leukocyte and platelet count also showed a slight fall in the patients with AHCC requiring no platelet concentrate transfusion and only 7 patients required growth factor supplement with AHCC.

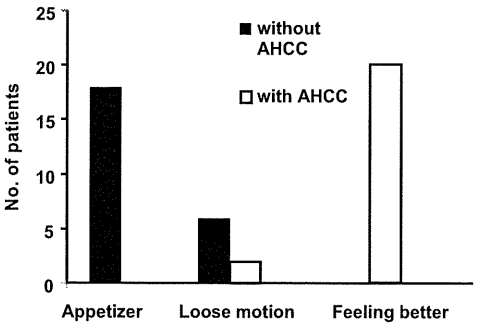

Figure 2. Comparison of quality of life concepts in patients requiring appetizer, having loose motion and better general condition with and without AHCC. While no patients with AHCC required any appetizer and only 2 patients were hospitalized for loose motion.

Table 3. Quality of life concept.

	Without AHCC	With AHCC
Confinement to bed	14 - 16 hrs/day	8 - 10 h/d
Talking to people	not	yes
Sleep pattern	irregular	regular
Required antiemetic for		
Chemo related Nausea/vomiting	7 - 14 Days	3 - 5 Days

Table 4. Tumor response of the patients.

	No. of patients
Complete Response	00
Partial Response	11
Stable Disease	08
Progressive Disease	06

These intercellular chemical messengers trigger white blood cell production and activity [3,4]. The therapeutic effect is predominantly seen in higher basidiomycete family [5]. Studies show that AHCC also enhances production of cytokines, including interferon γ, tumor necrosis factor-α and interleukins (IL-2,4,6,10) [4]. In this present study majority of the patients, who received AHCC along with chemotherapy showed less fall in their hemoglobin level and total leukocyte count. However, researchers have found the influence of AHCC upon the innate immune system in animal studies and published the results in 1992. AHCC significantly increased natural killer (NK) cell activity in cancer patients, and also enhanced the effects of killer T-cells, and cytokines (interferon, IL-12, TNF-alpha) [3]. AHCC stimulates cell-mediated immunity by activating the white blood cells, particularly natural killer cells and macrophages, which directly attack abnormal cells, virus-infected cells or external vital and bacterial pathogens that enter the body. The fundamental mechanism of activating immunity is by means of stimulating the number of dendritic cells as these cells control the activities of B & T lymphocytic cells who are the ultimate mediators of immunity and hence, affected by AHCC. It also exhibits immunomodulating effects partially by regulating thymic apoptosis. Nomura T *et al.* has published that besides immunogenic, AHCC is also having anti-teratogenic effects in animal models [7]. Effective immunity has got direct impact on tumor control and better tolerability of chemotherapeutic agents. In our series, it was significant that almost all the patients who received AHCC, tolerated chemotherapy better compared to their previous cycles of chemotherapy they had received without AHCC. At least eighteen out of the twenty-five patients acted as control of their own.

One major retrospective study suggests that AHCC intake has a preventive effect in postoperative hepatocellular carcinoma patients [8,9]. The study has compared the outcomes of 113 post-operative liver cancer patients taking AHCC with 156 patients in the control group. The results showed the rate of recurrence of malignant tumors was significantly lower (34.5% versus 66.1%) and patient survival was significantly higher in the AHCC group (79.6% vs. 53.2%). We tried AHCC for the first time in the patients suffering from cancers of head & neck region and obtained good results.

AHCC has been studied extensively for safety in human trials as well as safety with conventional chemotherapy [10,11]. There are also few studies regarding the interaction of AHCC with various kinds of chemotherapeutic agents [12-15]. There is a great deal of scientific evidence that AHCC not only helps to prevent the side effects of chemotherapy, but enhances its primary effectiveness as well. Several animal studies have shown that AHCC was able to relieve the side effects of several standard chemotherapy drugs like 5-FU, cisplatin, cyclophosphamide, mercaptopurine, methotraxate etc. "Severe" (50% to 100%) hair loss or alopecia caused by cytosine arabinoside (Ara-C) was reduced to slight, when AHCC was taken simultaneously. The ability of AHCC to enhance the effectiveness of chemotherapy was demonstrated in a study where rats were implanted with a cell line of spontaneous mammary adenocarcinoma. In the present study, the patients were administered AHCC along with chemotherapeutic agents like paclitaxel, docetaxel, cisplatin, 5-FU and certain monoclonal antibodies like cetuximab and nimotuzumab (**Table 2**). All the patients tolerated AHCC well. Routine side effects like nausea, vomiting, loose motions were less in these patients.

5. Conclusions

It can be concluded that AHCC is safe to administer and definitely helps cancer patients in reducing side effects of chemotherapeutic drugs, getting a sense of wellbeing and improved intake maintains general condition as well as prepare them to continue and tolerate further cycles in a better way. In advance stage disease achieving partial response/stable disease is also of significance, particularly when all the patients were having either recurrent or residual disease. Whether AHCC is responsible for the regression of the tumors, further trials are required to see the effects of AHCC on tumor control. Also dose enhancement trial has to be undertaken.

6. Acknowledgements

This work was carried out as a collaborative project between the North Eastern Indian Gandhi Regional Institute of Health and Medical Sciences and National Institute of Biomedical Innovation. This project was supported by the grant from the MEXT and MHLW, Japan and Amino up chemical Co. Ltd, Sapporo, Japan. We thank Haruko Ryo, National Institute of Biomedical Innovation for her critical observations and proof reading.

REFERENCES

[1] National Cancer Registry P Publication 2007. http://www.ncrpindia.org

[2] P. Jha, B. Jacob, V. Gajalakshmi, P. C. Gupta, N. Dhingra, R. Kumar, D. N. Sinha, R. P. Dikshit, D. K. Parida, R. Kamadod, J. Boreham, R. Peto and RGI-CGHR Investigators, "A Nationally Representative Case-Control Study of Smoking and Death in India," *New England Journal of Medicine*, Vol. 358, No. 11, 2008, pp. 1137-1147.

