Difference in Glycosphingolipid Compositions of Avian Marek's Disease Lymphoma-Derived Cell Lines and Lymphoid Leukosis Lymphoma-Derived Cell Lines

Yoichi FUJII, Kazuyoshi IKUTA¹⁾, Shiro KATO¹⁾, Takeshi MIKAMI²⁾, and Masaharu NAIKI

Department of Biochemistry and ²⁾Department of Epizootiology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, and ¹⁾Department of Pathology, Research Institute for Microbial Diseases, Osaka University, Osaka 565, Japan

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ABSTRACT. We previously reported that Marek's disease (MD) lymphoma-derived cells, transplantable MD tumor-derived cells and lymphoid leukosis (LL) lymphoma-derived cells, each express different glycosphingolipid (GSL) antigens. The GSL composition of each cell line was compared by thin-layer chromatography and immunological analysis. Six different cell lines derived from MD lymphoma showed a typically identical GSL composition, in which a unique neutral GSL with a carbohydrate chain consisting of more than five sugars was detected as a major component. However, transplantable MD tumor-derived cell lines and LL lymphoma cell lines showed a similar composition in which Forssman GSL, asialo GM₁ ganglioside and other several components were detected. The specific presence of Forssman GSL and asialo GM₁ in these two cell lines was confirmed by immunological reaction of GSLs from each cell line with antisera to each authentic GSL and by the specific antibody elevation in rabbits immunized with each cell line.—**KEY WORDS:** avian lymphoma, ganglioside, glycosphingolipid, lymphoid leukosis, Marek's disease.

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Chicken Marek's disease (MD) has been extensively researched as a lymphoproliferative malignant disease caused by an oncogenic herpesvirus, MD virus. Many lymphoma cell lines [34] have been established from lymphoproliferative lesions of the affected spleens or ovaries by cultivation at 41°C since Akiyama and Kato first succeeded [2]. On the other hand, lymphoblastic leukemia induced by inoculation with the JM strain of MD virus (JMV) had been repeatedly transplanted, and several cell lines from the transplants were established in *in vitro* culture systems in different laboratories [24, 25]. Both cell lines carry T lymphocyte marker antigens [27] and express Marek's disease tumor-associated surface antigens [33]. It has been reported by several researchers, however, that they have different properties in the expression of viral antigens and in reproducibility of the

virus *in vivo* and *in vitro* [1, 30]. The glycosphingolipids (GSLs) are known to be important cell surface components with antigenic activities, such as blood group antigens [22], tumor antigens [5] and marker antigens of some cell populations [16]. We recently reported the different expression of some GSL antigens in the two groups of cell lines. MD lymphoma-derived cell lines express Hanganutziu-Deicher (H-D) antigen [12] but not Forssman antigen [11]; however, JMV transplant-derived cell lines and lymphoid leukosis (LL) lymphoma-derived cell lines, express Forssman antigen but not H-D antigen.

The present paper describes a dramatic difference in GSL composition of MD lymphoma-derived cell lines and JMV transplant-derived cell lines and the similarity between the latter cell lines and LL lymphoma-derived cell lines.

MATERIALS AND METHODS

Glycosingolipids (GLSs): Ceramide monohexoside, Ceramide dihexoside and globoside were purified from human erythrocytes [21]. Ceramide trihexoside was purified from pig spleens [18]. Asialo GM₁ ganglioside was prepared by mild acid hydrolysis of GM₁ ganglioside which was purified from bovine brain [20]. Forssman GSL was purified from goat erythrocytes [19].

Antisera: Rabbit antiserum to cell line RP1 was prepared by subcutaneously immunizing rabbits with a homogenate of RP1 (2×10^8) treated with Freund's complete adjuvant, following the procedure of Sugimoto *et al.* [31]. After the first immunization, 2×10^7 cells were intravenously given every week for three weeks. Ten days after the last injection, the antiserum was harvested. Anti-MSB1/41C and anti-1104B1 were prepared by intravenous immunization with 7×10^6 and 1×10^8 intact cell respective-

ly. One week after booster injections which were similarly given every week for two weeks, the antisera were harvested.

The preparation of anti-Forssman [14], anti-asialo GM₁ [21], and anti-globoside serum [14] were previously prepared.

Cells: The avian lymphoid cell lines used (Table 1) were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum (Flow Lab.) except for adding 2% chicken serum to the above medium for the MSB1/41C in cell line. Each cell line was incubated at 41°C in a humidified atmosphere of 5% CO₂ in air except for BP1 cell line which was adapted at 37°C. HP1, HP2, RP1, BP1, MSB1/41C and two LL lymphoma-derived cell lines were kindly donated by the persons indicated in Table 1.

GSL analysis: For metabolically labeling GSLs of each cell line, 1 nmole (5 μ Ci) of D-[1-¹⁴C]-galactose (Amersham Int. PLC) was added to 15ml of medium containing approximately 10^6 cells. After culturing for 4 days at 37°C or 41°C, each cell was washed three times with 0.01 M phosphate buffer

Table 1. Nomenclature of avian lymphoma-derived cell lines used

Cell line ^{a)}	Origin	Reference
MD lymphoma-derived cell lines		
MDCC-MSB1 (MSB1)	MD splenic lymphoma	Akiyama & Kato [2]
MDCC-MSB1-41C (MSB1/41C) ^{b)}	MD splenic lymphoma	Matsuda <i>et al.</i> [17]
MDCC-HP1 (HP1)	MD ovarian lymphoma	Powell <i>et al.</i> [27]
MDCC-HP2 (HP2)	MD lymphoma	Powell <i>et al.</i> [27]
MDCC-JP1 (MOGA1)	MD ovarian lymphoma	Kawamura <i>et al.</i> [15]
MDCC-JP2 (MOGA2)	MD ovarian lymphoma	Kawamura <i>et al.</i> [15]
Transplantable MD tumor-derived cell lines ^{c)}		
MDCC-BP1 (BP1)	MD transplant JMV	Sekiya <i>et al.</i> [29]
MDCC-RP1 (RP1)	MD transplant JMV	Nazerian <i>et al.</i> [24]
LL lymphoma-derived cell lines		
LSCC-1104B (1104B)	LL lymphomatous bursa	Hihara <i>et al.</i> [7]
LSCC-1104X5 (1104X5)	LL lymphomatous bursa	Hihara <i>et al.</i> [7]

a) Nomenclatures of cell lines follows Witter [34].

b) In this group of cell lines, only this cell line is transplantable in newborn chickens.

c) The cell lines were established from a transplanted MD tumor which had been maintained for several years by transplantation.

(pH7.0) containing 0.15 M sodium chloride (PBS) and lyophilized. Lipids were extracted with 2ml of chloroform-methanol (2:1, v/v) and again with an equal volume of chloroform-methanol (1:1, v/v). The lipid extracts were evaporated and fully acetylated with 0.5ml of pyridine-acetic anhydride (5:4, v/v) at 60°C overnight. The acetylated GSLs were purified by Florisil (Wako Chem.) column (0.7×7cm) chromatography according to the method of Saito and Hakomori [28]. The GSL fraction was deacetylated by incubating for 4 hr at room temperature with 0.5ml of 0.1 N potassium hydroxide in methanol, and deacetylated GSLs were obtained after neutralization with Dowex 50W X8 (H⁺ form). The radioactivity of an aliquot of each GSL fraction was counted in 3ml of toluene containing 0.01% POPOP and 0.4% PPO by a liquid scintillation counter (LSC-751, Aloka Co., Ltd.). Three thousand cpm of the GSLs from each cell line and standard GSLs were applied on a high-performance thin-layer chromatography (TLC) plate (Merck) and developed with chloroform-methanol-water (60:35:8, v/v/v). After development, the plate was kept in contact with X-Omat S film (Kodak, RP) at 4°C for 2 or 3 weeks. After development of the film, the plate was sprayed with anthrone sulfuric acid reagent [13] and heated at 120°C for 10 min to visualize the standard GSLs. For extraction and analysis of the composition of unlabeled GSL, each cell was cultured and lyophilized after washing. Approximately 100 mg of each cell powder were extracted with 25ml of chloroform-methanol-water (30:60:8, v/v/v) and applied on a DEAE-Sepharose CL-6B (Pharmacia Fine Chem.) column (0.05×5cm). Neutral GSL-containing fractions were eluted with 10ml of the same solvent system. The neutral GSLs were purified by the acetylation procedure described above.

Complement Fixation Tests: Complement

fixation (CF) was carried out by the semi-quantitative method as described previously [20]. Authentic GSL antigens for the test were prepared by mixing each GSL with lecithin purified from egg yolk [18] and cholesterol (Tokyo Kasei) at ratios of 1:0:10, w/w for Forssman GSL [14], 1:3.5:3.5 for asialo GM₁ ganglioside [20] and 1:1:10 for globoside [14], respectively. These ratios were the most suitable for each GSL antigen to fix the complement. The neutral GSL antigens from each cell line for complement fixation were prepared by mixing each GSL fraction (5.8 μg as hexose content for MSB1, 2.8 μg for BP1, 25 μg for RP1 and 2.3 μg for 1104B) with 1ml of a micellar solution containing 2 μg lecithin and 10 μg cholesterol. Briefly the procedure for the CF test was as follows. For detection of the three GSL antigenic activities in each cell line, two-fold serial dilutions of the GSL antigen preparations (25 μl) were mixed with 4 CF units of the antiserum (25 μl) to Forssman GSL, asialo GM₁ ganglioside or globoside. For detection of the antibody activities against the three authentic GSL antigens in rabbit antisera prepared by immunization with each cell line, two-fold dilutions of each serum (each 25 μl) were mixed with each GSL preparation (25 μl) with a concentration of 2 μg/ml as the GSL content. To each well of the mixture, 2 hemolytic units of guinea pig complement (25 μl) were added and the mixture was kept at 4°C overnight. CF was measured by inhibition of the immunohemolysis of 2% sensitized sheep erythrocytes (25 μl) which were incubated at 37°C for 40 min. Hexose content of each authentic GSL and each cell line-derived GSL fraction was determined with phenol sulfuric acid reagent [3].

RESULTS

First, in our preliminary experiments, we purified cold neutral GSL fraction from

Fig. 1. Autoradiographical analysis of GSLs from avian lymphoid cell lines. MD lymphoma cell lines: MOGA2 (M_1), MOGA1 (M_2), HP2 (M_3), HP1 (M_4) and MSB1 (M_5); a transplantable MD lymphoma cell line: BP1 (MT); and LL lymphoma cell lines: 1104B (L_1) and 1104X5 (L_2) were labeled with [^{14}C]-galactose under the 37°C culture conditions. As authentic samples, asialo GM_1 ganglioside (S_1), Forssman GSL (S_2) and the mixture (S_3) of ceramide monohexoside (A), ceramide dihexoside (B), ceramide trihexoside (C) and globoside (D) were chromatographed on the same thin-layer plate and visualized with anthrone-sulfuric acid reagent.

twelve different cell lines and compared their compositions by TLC (data not shown). The results were quite similar to the autoradiographical patterns shown in Fig. 1. Therefore, each cell line was metabolically labeled with [^{14}C]-galactose, and GSLs from each cell line were purified and analyzed by TLC (Figs 1 and 2). The two autoradiographical patterns showed differences among MD lymphoma-derived cell lines (MSB1, MSB1/41C, HP1, HP2, MOGA1 and MOGA2; the 1st group), JMV transplant-derived cell lines (BP1 and RP1; the 2nd group) and LL lymphoma-derived cell lines (1104B and 1104X5; the 3rd group). The six different cell lines of the 1st group gave an identical GSL pattern, in which a unique

Fig. 2. Autoradiographical analysis of GSLs from several cell lines cultured under different conditions. As the 1st group cell lines, GSLs from three MSB1 cell lines were compared. MSB1 (M_5) has been maintained by multiple passages for more than five years; MSB1 (M_6) maintained for less than one year; and MSB1/41C (M_7) was a subline with a transplantable property. As the 2nd group cell line, GSLs from two RP1 cell lines, which were cultured for several months at 37°C (R_{37}) or 41°C (R_{41}), were compared. Authentic samples (S_1 to S_3) follow Fig. 1.

major GSL migrating more slowly than asialo GM_1 was detected. This major compound was determined to be a neutral GSL because of negative binding to a DEAE-Sepharose CL-6B column and negative reaction with resorcinol-HCl reagent [32]. Two cell lines of the 2nd group gave a similar pattern to those of two cell lines of the 3rd group. In the 2nd and 3rd groups of cell lines, several spots were detected between the globoside position and the main spot which was detected in the 1st group.

By using the same metabolic labeling method, we tried to compare the GSL compositions of some of the identical cell

lines which were cultured under different conditions or which had different properties. The RP1 cell line, belonging to the 2nd group, was cultured at 37°C or 41°C for long time. The GSL composition did not change under these two different conditions (Fig. 2) and gave a pattern similar to that of BP1 cell line cultured at 41°C (Fig. 1). The GSL compositions in three different MSB1 cell lines were compared. One cell line was continuously cultured for approximately five years and another was cultured for only one year. MSB1/41C was recently cloned and

distinguished from the MSB1 cell line by its unique transplantable property [17]. All the cell lines gave an identical pattern, as shown in Fig. 2. A major component was detected at the globoside-migrating position and another at a lower position than asialo GM₁ ganglioside.

GSL fractions from each cell line were purified in amounts too small to be chemically characterized. Therefore, we tried to identify the existence of globoside, asialo GM₁ and Forssman GSL in GSL fractions by the reactivities with antiserum to each

Table 2. Structures of several GSLs

Compound	Chemical structure ^{a)}
Ceramide monohexoside	Gal-Cer
Ceramide dihexoside	Gal α 1-4Glc-Cer
Ceramide trihexoside	Gal α 1-4Gal β 1-4Glc-Cer
Globoside	GalNAc β 1-3Gal α 1-4Gal β 1-4Glc-Cer
Forssman GSL	GalNAc α 1-3GalNAc β 1-3Gal α 1-4Gal β 1-4Glc-Cer
Asialo GM ₁ ganglioside	Gal β 1-3GalNAc β 1-4Gal β 1-4Glc-Cer

a) Gal, D-galactose; Glc, D-glucose; GalNAc, N-acetyl-D-galactosamine; GlcNAc, N-acetyl-D-glucosamine; NeuGc, N-glycolyl-neuraminic acid; NeuAc, N-acetyl-neuraminic acid; Cer, Ceramide (N-acylsphingosine)

Table 3. Complement fixation of neutral GSL fractions isolated from different cell lines with antisera against each authentic GSL

GSL	Rabbit antiserum ^{a)}		
	Forssman	asialo GM ₁	Globoside
Forssman	2.7 ^{b)}	— ^{c)}	—
Asialo GM ₁	—	1.7	—
Globoside	—	—	6.3
BP1	58	— ^{d)}	—
RP1	16	260	65
1104B	55	110	220
MSB1	—	—	120

a) Rabbit anti-Forssman GSL, anti-asialo GM₁ and anti-globoside were used at dilutions of 1: 320, 1: 160 and 1: 320, respectively, according to the titer of each antiserum shown in Table 4.

b) The lowest concentration (ng/ml) as hexose content giving complete inhibition of immunohemolysis.

c) Negative at concentration of less than 175 ng/ml and ^{d)}2.5 μ g/ml, respectively.

Table 4. Complement fixation of rabbit antisera against different cell lines with several authentic GSLs

Rabbit antiserum	GSL antigen ^{a)}		
	Forssman	Asialo GM ₁	Globoside
Anti-Forssman	1280 ^{b)}	— ^{c)}	—
Anti-asialo GM ₁	—	640	—
Anti-globoside	—	—	1280
Anti-MSB1/41C	—	—	320
Anti-RP1	1280	1280	2560
Anti-1104X5	640	—	—

a) Authentic GSLs were used at a concentration of 2 µg/ml.

b) The highest dilution of antisera giving complete inhibition of immunohemolysis.

c) Negative at dilutions of less than 10-fold.

authentic GSL. Their chemical structures are shown in Table 2. The antigenic activity of Forssman GSL was clearly demonstrated in the neutral GSL fractions from the BP1, RP1 and 1104B cell lines, but not in the MSB1 cell line (Table 3). Asialo GM₁ was detected in those from RP1 and 1104B but not from MSB1 and BP1. Globoside antigen was detected in those from MSB1, RP1 and 1104B1 but not in that from BP1. On the other hand, antibodies to Forssman GSL were stimulated in rabbits immunized with cell lines RP1 and 1104X5 but not with cell line MSB1/41C (Table 4). Anti-asialo GM₁ was elevated only in the antiserum to RP1 but not in the antiserum to 1104X5, while the GSL fractions from both RP1 and 1104B5 had the antigenic activity. Antibodies to globoside was detected in the antisera to MSB1/41C and RP1 but not in the antiserum to 1104X5, while the GSL fractions from these three groups of cell lines had antigenic activity.

DISCUSSION

The six different cell lines which were derived from MD lymphoma classified as the 1st group have very similar GSL com-

positions (Figs 1 and 2). They all have a major neutral GSL component which moves slower than asialo GM₁ ganglioside, although MOGA1 and MOGA2 showed another band at a lower position than that of the major GSL. The chemical structure of the GSL and its antigenicity are still under study using the TLC-immunostaining method. All six cell lines showed minor bands at the positions of globoside and ceramide trihexoside. The existence of globoside in MSB1 and MSB1/41C was immunologically confirmed (Tables 3 and 4). Both TLC and immunological analysis showed that they lack asialo GM₁ and Forssman GSL. On the other hand, the two cell lines derived from transplants of MD lymphoma (the 2nd group) showed a similar GSL composition but different from cell lines of the 1st group. They both showed six bands below the globoside-migrating position. Both cell lines expressed Forssman GSL antigen, and the identical GSL band was visualized by TLC in each preparation. In both cell preparations, GSLs which moved at the same position as globoside or asialo GM₁ was detected by TLC, but only in RP1 homogenate were they immunologically detected. These results suggest that not

only concerning their oncogenic genome but also the synthesis of GSLs, there is a large distinction between the 1st group and 2nd group cell lines.

Several reports have been published concerning the differences in biological properties between the 1st group and the 2nd group cell lines. The 1st group cell lines contain multiple copies of the MDV genomes [23] and can produce MDV by cocultivation with susceptible chick fibroblasts or inoculation into chicks [2]. A small population of producer cells express MDV-specific intracellular and membrane antigens [2]. On the other hand, the 2nd group cell lines contain smaller numbers of copies of MDV genomes [8] and can not become virus-producers *in vitro* or *in vivo* [9]. Both groups of cell lines do indeed express MD tumor-associated surface antigens; however, their antigenic specificities are different. Rabbit anti-MSB1 and anti-RP1 reacted with the corresponding cells [31]. Heterophilic H-D antibody was found in chicken anti-MSB1 sera as one of the MD tumor-specific antibodies because absorption with H-D antigen-positive sheep erythrocytes dramatically reduced the antibody activity [10]. The H-D antigen was expressed on the 1st group cell lines but not on the 2nd group cell lines [12]. The antigenic molecules were detected in MSB1 cell lines as three different GSLs with N-glycolyl-neuraminic acid at the carbohydrate terminal [6]. The above biological differences between the two group cell lines seem to explain the drastic difference in the GSL compositions of these cells.

Two different LL cell lines of the 3rd group showed a very similar GSL composition (Fig. 1). The TLC pattern was quite different from that of the 1st group cell lines, though it rather resembled that of the 2nd group cell lines. Even in some antigenic expressions, the 3rd group cell lines were different from the 1st group but similar to

the 2nd group. They expressed Forssman antigen and asialo GM₁ antigen but not H-D antigen, like the 2nd group cell lines. It is not understandable how these similarities between the 2nd and 3rd group cell lines come about, but if avian leukosis virus (ALV) can affect the induction of Forssman and asialo GM₁ antigen synthesis in the chicken cell lines, it may be speculated that the original MD transplants from which the 2nd group cell lines were derived could have obtained ALV genomes by natural infection during multiple passages through transplantation from chick to chick [9, 25].

Changes of GSL composition between a parent cell line and sublines or same cell lines cultured under different culture conditions were examined (Fig. 2). One subline MSB1/41C could grow attached to the vessels, forming a monolayer sheet at 41°C and it became transplantable in newborn chick while the parent cell MSB1 grew floating in the medium and was not transplantable. Some workers showed that the carbohydrate chain has an important role in the adhesion of cells [4, 26]. The GSL compositions of these cells were, however, quite similar in spite of the difference of the transplantability in our experiments. From these results, we suspect that the ability to attach to the culture dish and the transplantability may be not related with the carbohydrate chains of GSLs in the membranes of MSB1 cell lines.

MSB1 cells which had been adapted to culture at 37°C contained 5 times more MDV genomes per cell than the cells cultured at 41°C [7]. However, RP1 cell lines which were adapted to grow at 37°C showed no change in GSL composition in comparison with the parent cells cultured at 41°C. These results indicate that, even if such a temperature dependent MDV genome increase occurs, it cannot control the synthesis of carbohydrate chains of GSLs.

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要 約

鶏マレック病および白血病リンパ腫由来細胞株の糖脂質組成における差違：藤井陽一・生田和良¹⁾・加藤四郎¹⁾・見上 彪・内貴正治(北海道大学獣医学部家畜生化学教室，²⁾家畜伝染病学教室，¹⁾大阪大学微生物病研究所感染病理学研究部)——各々異なる糖脂質性抗原が発現されるマレック病(MD)腫瘍由来，可移植性同腫瘍株由来および白血球(LL)腫瘍由来各細胞株の糖脂質組成の差違を薄層クロマトグラフィーで比較し，免疫学的に確認した。MD腫瘍由来6細胞株は各々類似の糖脂質組成を示し，5糖以上の糖鎖を持つ中性糖脂質が1主成分として検出された。移植MD腫瘍由来細胞2株とLL腫瘍由来細胞2株は比較的似た糖脂質組成を示し，MD腫瘍株には認められないForssman糖脂質やasialo GM₁などが検出された。