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## Sensitive detection of ganglioside GD3 on the cell surface using liposome immune lysis assay.

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## Abstract

We developed a sensitive method for detection of glycosphingolipid (GSL) antigen(s) on the cell surface. As a model of GSL antigen, ganglioside GD3 was used. An IgM monoclonal antibody (DSG-1) specific for ganglioside GD3 was preincubated with standard inhibitor liposomes containing ganglioside GD3. Then carboxyfluorescein-entrapped indicator liposomes containing ganglioside GD3 and complement were added. Release of the marker from the indicator liposomes was specifically inhibited by inhibitor liposomes. The assay system was simple, sensitive, reproducible, and semiquantitative. Pg to ng of ganglioside GD3 could be detected. Furthermore, ganglioside GD3 on the cells was investigated with SK-MEL-28 human melanoma cell line and human red blood cells (HRBC). When SK-MEL-28 melanoma with ganglioside GD3 was used as an inhibitor, specific inhibition was observed. However, HRBC without ganglioside GD3 showed no significant inhibition. The marker release was 50% inhibited by  $1.4 \times 10^6$  SK-MEL-28 melanoma cells/ml. The amount of ganglioside GD3/melanoma cell was estimated to be at least  $1.1 \times 10^{-14}$  g from the standard curve made with the liposomes containing 10% epitope density of ganglioside GD3. This assay system may be useful for detection of GSL antigen on the cell.

**KEYWORDS:** ganglioside GD3, tumor-associated antigen, liposomes, antigen determination, monoclonal antibody

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## Sensitive Detection of Ganglioside G<sub>D3</sub> on the Cell Surface Using Liposome Immune Lysis Assay

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We developed a sensitive method for detection of glycosphingolipid (GSL) antigen(s) on the cell surface. As a model of GSL antigen, ganglioside G<sub>D3</sub> was used. An IgM monoclonal antibody (DSG-1) specific for ganglioside G<sub>D3</sub> was preincubated with standard inhibitor liposomes containing ganglioside G<sub>D3</sub>. Then carboxyfluorescein-entrapped indicator liposomes containing ganglioside G<sub>D3</sub> and complement were added. Release of the marker from the indicator liposomes was specifically inhibited by inhibitor liposomes. The assay system was simple, sensitive, reproducible, and semi-quantitative. Pg to ng of ganglioside G<sub>D3</sub> could be detected. Furthermore, ganglioside G<sub>D3</sub> on the cells was investigated with SK-MEL-28 human melanoma cell line and human red blood cells (HRBC). When SK-MEL-28 melanoma with ganglioside G<sub>D3</sub> was used as an inhibitor, specific inhibition was observed. However, HRBC without ganglioside G<sub>D3</sub> showed no significant inhibition. The marker release was 50 % inhibited by  $1.4 \times 10^6$  SK-MEL-28 melanoma cells/ml. The amount of ganglioside G<sub>D3</sub>/melanoma cell was estimated to be at least  $1.1 \times 10^{-14}$  g from the standard curve made with the liposomes containing 10 % epitope density of ganglioside G<sub>D3</sub>. This assay system may be useful for detection of GSL antigen on the cell.

**Key words :** ganglioside G<sub>D3</sub>, tumor-associated antigen, liposomes, antigen determination, monoclonal antibody

Cell surface glycosphingolipids (GSLs) have been shown to function as receptors for a variety of toxins, lymphokines, and biological mediators (1). GSLs have also been recognized as tumor associated and differentiation markers (2,3). Previously, ganglioside G<sub>D3</sub> was detected on cells from various neoplasms, such as melanoma (4-7), glioma (8), astrocytoma (9), adult T-cell leukemia (10, 11), chronic lymphocytic leukemia

(12,13), acute lymphocytic leukemia, and acute myeloblastic leukemia (14,15). In our previous study, it was also found that ganglioside G<sub>D3</sub> is a prominent constituent of enzootic bovine leukosis (EBL) tumors (16) and is a useful tumor marker of EBL (17). Therefore, a sensitive method for detection of GSLs, including ganglioside G<sub>D3</sub>, on the cell surface may be useful not only for diagnosis of cancers but also a tool in assessing biological functions of GSLs on the cell surface membranes. Although there are reports of sensi-

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tive methods for the detection of GSLs on thin-layer chromatography plates using specific antibodies to GSLs, no sensitive method for the detection of GSLs on living cells has yet been presented.

We have previously developed an assay system based on a complement-dependent liposome immune lysis assay (LILA) to measure antibodies to GSLs and protein antigens (18-20). The assay system is simple, fast, and sensitive. Recently, we have also produced mouse monoclonal antibodies specific for ganglioside  $G_{D3}$  (21). In the present study, we applied liposomes for detection of GSL antigen on the cell and developed a sensitive method for detection of cell-surface ganglioside  $G_{D3}$  as a model of GSL antigens on the cell surface.

## Materials and Methods

**Lipids.** Egg yolk phosphatidylcholine (egg PC) (Nippon Fine Chemical, Osaka), cholesterol (Chol) (Sigma Chemical Co., St. Louis, Mo.), L-dimyristoyl-phosphatidylcholine (DMPC) (Nippon Fine Chemical), and ganglioside  $G_{D3}$  (NeuAc-NeuAc) [ $II^3\alpha$  (NeuAc $\alpha$ 2-8NeuAc)-LacCer] (Iatoron, Tokyo) were commercially obtained.

Each lipid was dissolved in chloroform/methanol (2/1, v/v) solution and stored at  $-20^\circ\text{C}$ .

**Monoclonal antibody.** We used an IgM monoclonal antibody (DSG-1) specific for ganglioside  $G_{D3}$  established as described previously (21). The DSG-1 monoclonal antibody was purified by precipitation with 50% ammonium sulfate, followed by Sephadex G-200 (Pharmacia, Uppsala) gel filtration.

**Cells.** The SK-MEL-28 human melanoma cell line (22, 23) which possesses ganglioside  $G_{D3}$  on the cell surface was used as the ganglioside  $G_{D3}$ -positive cell. The culture was maintained in RPMI-1640 medium (Nissui Co., Tokyo) supplemented with 10% fetal calf serum, 2.5  $\mu\text{g}/\text{ml}$  of Fungizon, 100 unit/ml of penicillin, and 100  $\mu\text{g}/\text{ml}$  of streptomycin.

Human red blood cells (HRBC) were obtained from a healthy staff member of the Institute of Cellular and Molecular Biology, Okayama University Medical School and used as the ganglioside  $G_{D3}$ -negative cell.

**Preparation of liposomes.** Indicator liposomes

containing ganglioside  $G_{D3}$  for liposome immune lysis assay were prepared according to the method of Yasuda *et al.* (18): DMPC (0.5  $\mu\text{mol}$ ), Chol (0.5  $\mu\text{mol}$ ), and varied amounts of ganglioside  $G_{D3}$  (NeuAc-NeuAc) (0.005, 0.025, 0.05, and 0.1  $\mu\text{mol}$ : the molar ratios to DMPC are 1, 5, 10, and 20% ganglioside  $G_{D3}$ , respectively) were mixed in a conical flask. After evaporation of the solvent by a rotary evaporator and then by a vacuum pump, 100  $\mu\text{l}$  of 0.05M carboxyfluorescein (CF) (Eastman Kodak Co., Rochester, N.Y.) was added as a release marker. After incubation for 1 min at  $50^\circ\text{C}$ , the lipid film was dispersed by vigorous vortexing. Unencapsulated CF was removed by repeated centrifugation at  $20,000 \times g$  for 20 min in phosphate-buffered saline (PBS) (pH7.2). The final pellet of liposomes was suspended in PBS (1 ml) and stored at  $4^\circ\text{C}$  until use.

Liposomes for standard inhibitors were prepared as follows: Lipid films composed of egg PC (0.5  $\mu\text{mol}$ ), Chol (0.5  $\mu\text{mol}$ ), and varied amounts of ganglioside  $G_{D3}$  (NeuAc-NeuAc) (0.005, 0.0125, 0.025, 0.05 and 0.1  $\mu\text{mol}$ : the molar ratios to egg PC are 1, 2.5, 5, 10, and 20% ganglioside  $G_{D3}$ , respectively) were dispersed in 1 ml of PBS by vortexing, and sonicated with a probe-type sonicator (Sonifier 250, Branson, Danbury, CT) at 20 W for 5 min. Standard noninhibitor liposomes were similarly prepared without  $G_{D3}$ . These inhibitor and noninhibitor liposomes were stored at  $4^\circ\text{C}$  until use.

**Liposome immune lysis assay (LILA).** LILA was performed according to the microplate method developed by us (18). PBS containing 3% bovine serum albumin was used for dilution of DSG-1 antibody. All the other dilutions were performed with gelatin veronal-buffered saline (0.1% gelatin, 10mM veronal buffer (pH7.4), 145 mM NaCl) supplemented with 0.5 mM  $\text{MgCl}_2$  and 0.15 mM  $\text{CaCl}_2$  ( $\text{GVB}^{2+}$ ). Twenty-five microliters of DSG-1 antibody solution and 5  $\mu\text{l}$  of 50-fold-diluted indicator liposome suspension from stock liposomes were added to each well of microtiter plates (Sumitomo Bakelite Co., Tokyo). After addition of 25  $\mu\text{l}$  of 200-fold-diluted fresh guinea pig serum as complement, the mixture was incubated for 1 h at  $37^\circ\text{C}$  in a moist chamber. To stop the reaction, 100  $\mu\text{l}$  of veronal-buffered saline containing 10 mM sodium ethylenediaminetetraacetic acid was added to each well.

The fluorescence ( $F_t$ ) of each well was automatically measured with a spectrophotometer (MTP-32, Corona Electric Co., Katsuta) at an excitation wavelength of 490 nm and an emission wavelength of 530 nm. The percentage of specific marker release ( $R_t$ ) was estimated as

$$(F_t - A)/(F_1 - A) \times 100,$$

where  $F_T$  is fluorescence obtained by lysing with 25  $\mu$ l of a 0.5 % Triton X-100 solution instead of complement, and A is that obtained by adding 25  $\mu$ l of GVB<sup>2+</sup> instead of DSG-1 antibody.

**Inhibition assay.** Twenty-five microliters of appropriately diluted DSG-1 antibody solution and 25  $\mu$ l of varied concentrations of inhibitor liposomes or cell suspensions were added to each well of microtiter plates (Sumitomo), mixed well and incubated overnight at 4 °C. Then indicator liposomes and complement were added as described in LILA. The mixture was further incubated for 1 h at 37 °C. The percentage of inhibition was calculated as

$$100 - (R_t/R_0) \times 100,$$

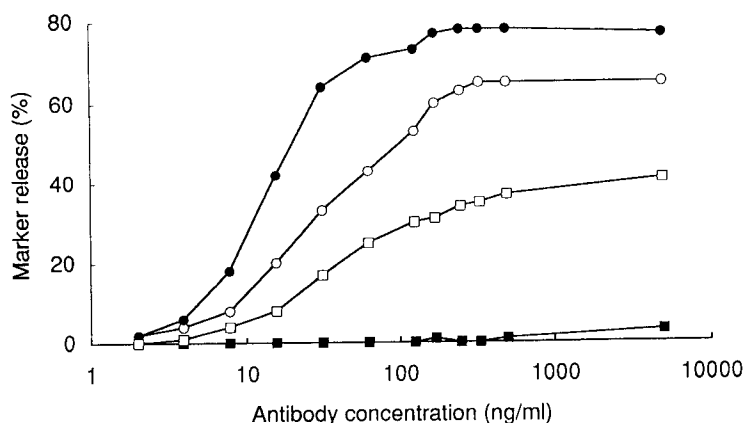
where  $R_0$  is marker release (%) obtained by adding 25  $\mu$ l of standard noninhibitor liposomes which bear no ganglioside G<sub>D3</sub>.

## Results and Discussion

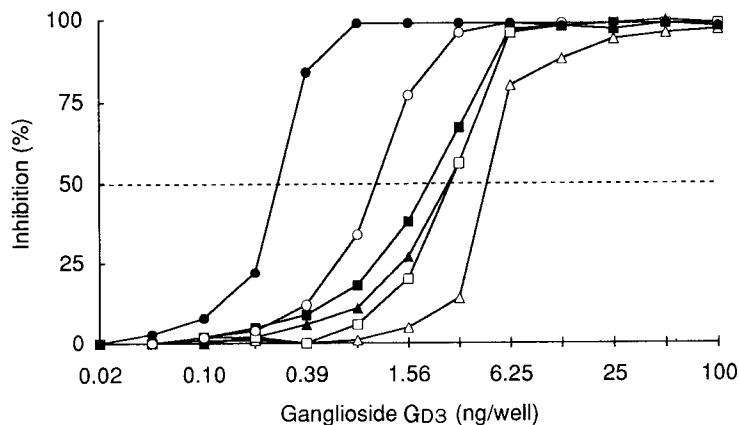
The inhibition assay using liposomes was investigated to develop a simple and sensitive assay system for quantitative analysis of ganglioside G<sub>D3</sub>. The principle of the assay is inactivation of antibody by preincubation with liposomes

containing reacting glycolipid antigen.

In order to know the appropriate concentration of the DSG-1 monoclonal antibody and optimal epitope density of ganglioside G<sub>D3</sub> in indicator liposomes for the inhibition assay, LILA was performed using indicator liposomes containing various molar ratios of ganglioside G<sub>D3</sub>. As shown in Fig. 1, no significant marker release was observed with the indicator liposomes containing 1 % ganglioside G<sub>D3</sub>. As for the liposomes containing 5 % ganglioside G<sub>D3</sub>, a gradual decrease in release of the marker was observed from an antibody concentration of 250 ng/ml and no significant marker release was observed less than 4 ng/ml of antibody. However, when the liposomes containing 10 % ganglioside G<sub>D3</sub> were used, the release of the marker retained maximal up to 250 ng/ml of antibody and began to decrease at an antibody concentration of 170 ng/ml. On the other hand, the liposomes containing 20 % ganglioside G<sub>D3</sub> showed lower extent of release of the marker than the liposomes containing 5 % ganglioside G<sub>D3</sub>. Therefore, the liposomes containing 10 % ganglioside G<sub>D3</sub> and the DSG-1 antibody concentration of 170 ng/ml,



**Fig. 1** Reactivity of DSG-1 monoclonal antibody with liposomes containing various molar ratios of ganglioside G<sub>D3</sub>. CF-entrapped indicator liposomes composed of DMPC (0.5  $\mu$ mol), Chol (0.5  $\mu$ mol), and various amounts of ganglioside G<sub>D3</sub> (0.005, 0.025, 0.05, and 0.1  $\mu$ mol: the molar ratios to DMPC are 1 % (■), 5 % (○), 10 % (●), and 20 % (□), respectively) were prepared and used for liposome immune lysis assay (LILA). Specific release of the marker was measured and calculated as described in Materials and Methods.



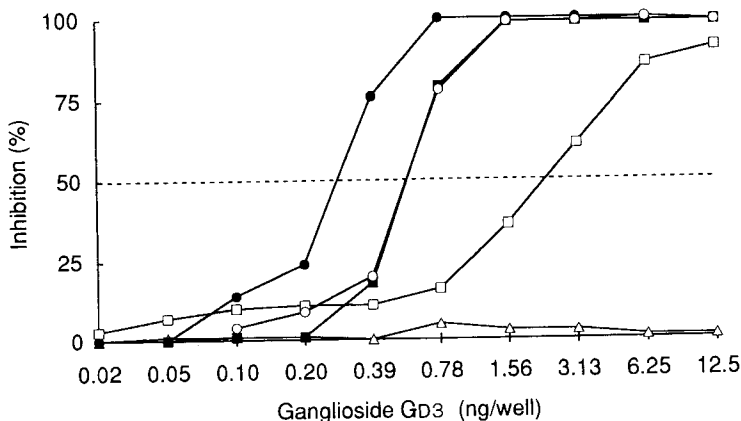
**Fig. 2** Comparison of preincubation conditions in inhibition assay. Inhibitor liposomes composed of egg PC ( $0.5\ \mu\text{mol}$ ), Chol ( $0.5\ \mu\text{mol}$ ), and ganglioside  $G_{D3}$  ( $0.05\ \mu\text{mol}$ ) were prepared as described in Materials and Methods. Twenty-five microliters of inhibitor liposomes and  $25\ \mu\text{l}$  of DSG-1 antibody ( $170\ \text{ng/ml}$ ) were mixed and incubated for various hours at  $37^\circ\text{C}$  or  $4^\circ\text{C}$ . Then LILA was performed. The axis of abscissa indicates the amount of ganglioside  $G_{D3}$  in inhibitor liposomes per well. The preincubation conditions are as follows: for 1h ( $\triangle$ ), 2h ( $\blacktriangle$ ), 3h ( $\blacksquare$ ) at  $37^\circ\text{C}$ , for 1h ( $\square$ ), 3h ( $\circ$ ), and overnight ( $\bullet$ ) at  $4^\circ\text{C}$ .

the highest concentration which gave the gradual decrease in marker release, were selected as most suitable.

The preincubation condition of the DSG-1 antibody with inhibitor liposomes was investigated. Serially diluted inhibitor liposomes containing 10% ganglioside  $G_{D3}$  were mixed with  $170\ \text{ng/ml}$  of DSG-1 antibody, and incubated for various hours at  $37^\circ\text{C}$  or  $4^\circ\text{C}$ . After incubation, LILA was done with indicator liposomes containing 10% ganglioside  $G_{D3}$ . Fig. 2 shows the results of the experiments. The preincubation of DSG-1 antibody with inhibitor liposomes resulted in the inhibition of marker release from indicator liposomes. Preincubation at  $4^\circ\text{C}$  showed stronger inhibition than at  $37^\circ\text{C}$ , and prolonged preincubation caused the stronger inhibition. This is due to the reaction kinetics of antibodies binding to antigens (25), that is, dissociation constants may have increased greatly at  $37^\circ\text{C}$  compared with  $4^\circ\text{C}$ . The strongest inhibition of marker release from indicator liposomes was observed in overnight preincubation at  $4^\circ\text{C}$ . Therefore, the

overnight reaction at  $4^\circ\text{C}$  was adopted as standard condition for the preincubation of the inhibition assay.

In order to clarify the sensitivity of the inhibition assay, inhibitor liposomes containing various molar ratios of ganglioside  $G_{D3}$  to egg PC were prepared and inhibition assay was studied as described in Materials and Methods. The standard inhibition curve is shown in Fig. 3. In reaction with inhibitor liposomes containing 1% epitope density of ganglioside  $G_{D3}$ , no inhibition was detected. On the other hand, inhibition curves were obtained by inhibitor liposomes containing 2.5%, 5%, 10%, and 20% epitope density of ganglioside  $G_{D3}$ , and the amount of ganglioside  $G_{D3}$  in inhibitor liposomes at 50% inhibition was calculated to be 2.3 ng, 560 pg, 280 pg, and 560 pg, respectively. The results indicated that pg to ng detection of ganglioside  $G_{D3}$  was possible using this inhibition assay system, and suggested that inhibition assay was useful in semiquantitative determination of ganglioside  $G_{D3}$  in the cells containing 2.5% or more epitope



**Fig. 3** Results of inhibition assay using inhibitor liposomes composed of various epitope densities of ganglioside G<sub>D3</sub>. Inhibitor liposomes were prepared from lipid mixture solution containing egg PC (0.5 μmol), Chol (0.5 μmol), and ganglioside G<sub>D3</sub> (0.005, 0.0125, 0.025, 0.05, and 0.1 μmol: the molar ratios to egg PC are 1% (△), 2.5% (□), 5% (■), 10% (●), and 20% (○), respectively) as described in Materials and Methods.

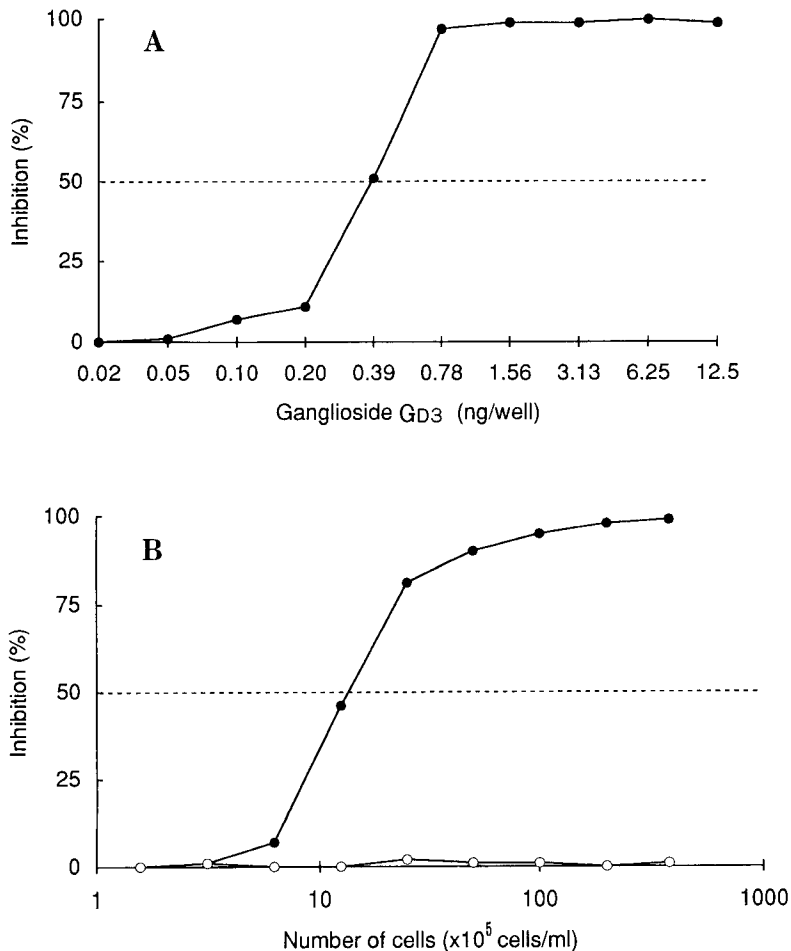
density of ganglioside G<sub>D3</sub>.

The availability of inhibition assay system using liposomes for detection of ganglioside G<sub>D3</sub> on the cell surface was studied. SK-MEL-28 melanoma cell line, as a positive control, and HRBC, as a negative control, were used for this inhibition assay system. Previously, cell surface ganglioside G<sub>D3</sub> of SK-MEL-28 melanoma cell line was purified and its structure was established by enzymatic degradation and methylation analysis by mass spectrometry (23). Cell surface glycolipids of HRBC were already investigated and no ganglioside G<sub>D3</sub> existed on HRBC (24). Results are shown in Fig. 4B. HRBC showed no significant inhibition of marker release at all. However, the inhibition of marker release was caused by SK-MEL-28 melanoma cells. The cell number of SK-MEL-28 at 50% inhibition was calculated to be  $1.4 \times 10^6$  cells/ml. The amount of ganglioside G<sub>D3</sub>/melanoma cell was estimated to be at least  $1.1 \times 10^{-14}$  g from the standard curve made with liposomes containing 10% epitope density of ganglioside G<sub>D3</sub> (Fig. 4A).

In this report, the reactivity of DSG-1 anti-

body depended on the density of ganglioside G<sub>D3</sub> inserted in liposomes. The reactivity was the strongest with both indicator and inhibitor liposomes containing 10% ganglioside G<sub>D3</sub>. The result shows that DSG-1 monoclonal antibody effectively recognizes the 10% epitope density of ganglioside G<sub>D3</sub>. Similar density dependent recognition of ganglioside G<sub>M3</sub> by a monoclonal antibody was previously reported by Nores *et al.* (26). They reported the threshold density detectable by the antibody in all-or-none fashion at about 8 mol % of G<sub>M3</sub> concentration.

The reactivity of the monoclonal antibody DSG-1 with various gangliosides and neutral GSLs was examined previously (21). DSG-1 antibody reacted only with ganglioside G<sub>D3</sub> and did not react with any structurally related gangliosides which contain the same terminal NeuAca2-8NeuAca2-3Gal sequence as ganglioside G<sub>D3</sub>, such as G<sub>T1a</sub> and G<sub>Q1b</sub>, or other GSLs. In the present study, release of the marker from indicator liposomes was inhibited by SK-MEL-28 melanoma cells, possessing ganglioside G<sub>D3</sub> on the cell surface, but not by HRBC



**Fig. 4** Detection of ganglioside  $G_{D3}$  on SK-MEL-28 human melanoma cells and human red blood cells (HRBC) by inhibition assay. A: Standard curve of inhibition by inhibitor liposomes containing 10% molar ratio of ganglioside  $G_{D3}$  to egg PC. B: Inhibition curve obtained with SK-MEL-28 human melanoma cells (●) and HRBC (○).

without ganglioside  $G_{D3}$ . In addition, ganglioside  $G_{D3}$  on SK-MEL-28 cells was detected by FACS analysis using the DSG-1 monoclonal antibody (data not shown). These results demonstrate that ganglioside  $G_{D3}$  on the cell surface was specifically detected by the inhibition assay system. The amount of ganglioside  $G_{D3}$  on the surface of the sample cells was estimated to be at least  $1.1 \times 10^{-14}$  g/cell. Although this assay system has a problem as to the quantification, the

application of this assay system may be useful for semi-quantitative detection of ganglioside  $G_{D3}$  on the cell.

This assay system is simple, fast, and a homogeneous one where no separation step is employed. Only a small quantity of living cells is needed. Furthermore, the assay system may be a useful procedure for the detection of glycolipid antigens not only on the cell but also in the serum.



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