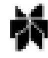

Glycoconjugate Journal 16, 59–65 (1999)
© 1999 Kluwer Academic Publishers. Manufactured in The Netherlands

Activation of human T lymphocytes by ganglioside-containing liposomes

Kazuhiro Yanagihara[1], Eiichi Kato[2], Shigeki Hitomi[1], Junzo Sunamoto[2] and Hiromi Wada[3]*

[1]Department of Thoracic Surgery, Kyoto University, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8397, Japan; [2]Department of Synthetic Chemistry & Biological Chemistry, Graduate School of Engineering, Kyoto University, Yoshida-Hommachi, Sakyo-ku, Kyoto 606-8501, Japan; [3]Department of Medical Systems Control, Field of Medical System Engineering, Institute for Frontier Medical Sciences, Kyoto University, 53 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8397, Japan

We investigated the in vitro stimulatory effect of ganglioside (G_{M3}, G_{D1a}, G_{D1b}, G_{T1b}, or G_{Q1b})-containing liposomes on human immune cells. The effect of ganglioside-containing liposomes on the concentration of cytoplasmic free calcium ions ($[Ca^{2+}]_i$) in human immunocytes was examined using the confocal laser fluorescence microscopic method. The G_{D1a}- and G_{T1b}-containing liposomes significantly increased $[Ca^{2+}]_1$ of human T lymphocytes compared with the G_{M3}-, G_{D1b}- and G_{Q1b}-containing ones. The response of $CD8^+$ and $CD4^+$ cells was significantly higher than that of $CD20^+$ cells. Our results show that the increase in $[Ca^{2+}]_i$ may be caused by not the number of sialic acids contained in the gangliosides but the conformation of the sialic acid moiety to protrude exteriorly from the liposomal membrane surface, and that a sort of receptor recognizing the sialic acid moiety exists on *human* T lymphocytes (both $CD8^+$ and $CD4^+$ cells), which may be involved in the activation of the cells. The present results are almost the same as those obtained for the *rat* T lymphocyte system previously reported. This clearly confirms that a sort of ganglioside surely stimulates T lymphocytes directly, which is not species-specific but conserved in humans and rats among animal species.

Keywords: **ganglioside, liposome, human T lymphocyte, cytoplasmic free calcium ion, sialic acid**

Introduction

The cytoplasmic free calcium ions play a very important role in the initiation of T cell activation as an intracellular signal transducer [1,2]. We have already reported that liposomes containing a ganglioside (G_{T1b} or G_{Q1b}) increased the concentration of cytoplasmic free calcium ions ($[Ca^{2+}]_i$) in *rat T lymphocytes* but that ones containing G_{M3} or G_{D3} did not [3].

Gangliosides are a class of sialic acid-containing glycosphingolipids, which are located ubiquitously in the outer leaflet of the plasma membrane of mammalian cells. Gangliosides act as membrane transducers of both positive and negative signals that regulate cell proliferation and induce differentiation [4,5]. Also, gangliosides are considered to be differentiation markers for T helper lymphocyte subpopulations [6]. In peripheral human T lymphocytes, previous studies demonstrated activated expression of receptors for interleukin-2 (IL-2) and production of IL-2 [7], as well as potentiation of lymphocyte proliferation induced by monoclonal antibodies directed against the disialogangioside, G_{D3} [8].

Exogenous gangliosides, as well as cell surface gangliosides, regulate cell proliferation and induce differentiation. An increase in $[Ca^{2+}]_i$ is observed after direct insertion of exogenous G_{M1} into a human Jurkat cell variant deficient in G_{M1} expression, which results in IL-2 production without cross-linking of CD3/TCR molecules [9].

On the other hand, gangliosides shed from tumor cells have been implicated as suppressors of the host immune response [10]. These suppressive effects of gangliosides are thought to be caused through depression of the B cell function [11] and/or T cell inhibition [12–19].

We paid attention to the position of the sialic acid moiety in both G_{T1b} and G_{Q1b}, which increase $[Ca^{2+}]_i$ in *rat T lymphocytes* as a form of liposomes. The difference in animal species is very important, especially considering the clinical use of ganglioside-containing liposomes as a T cell stimulator in the immunotherapy of human malignancy. Nevertheless, this point has never been investigated at all. In the present study, we investigate the increase in $[Ca^{2+}]_i$ in human lymphocytes, especially *human T lymphocytes,* using the confocal fluorescence microscopic method with ganglioside-containing liposomes.

*To whom correspondence should be addressed. TEL: 81-75-751-3835; FX: 81-75-751-4647; E-mail: wada@frontier.kyoto-u.ac.jp

Materials and methods

Materials

Dipalmitoylphosphatidylcholine (DPPC; Sigma Chemical Co., St. Louis, MO), egg yolk phosphatidylcholine (egg PC; NOF Corp., Tokyo, Japan), cholesterol (Wako Pure Chemicals Co., Tokyo, Japan), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1, 3-benzoxadiazol-4-yl) (DPE-NBD; Avanti Polar Lipids, Inc., Alabaster, AL), phytohemagglutinin P (PHA; DIFCO Laboratories, Detroit, MI), and the gangliosides (G_{M3}, G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b}) (Sigma Chemical Co., St. Louis, MO) were commercially available and used without further purification.

Antibodies were also commercially available: anti-CD16b and anti-CD11b from PharMingen (San Diego, CA); and anti-CD20 (B-Lym1), anti-CD14 (Tük4), anti-CD3, anti-CD4, and anti-CD8 from Dako A/S (Glostrup, Denmark).

Ganglioside-containing liposomes

A ganglioside was reconstituted into liposomal membranes by means of the following procedures [3,20,21]. DPPC, which bears a chemically stable saturated fatty acid, was used as the matrix lipid for the observation of $[Ca^{2+}]_i$ with the confocal laser fluorescence microscopic method. Egg PC, which is a naturally occurring lipid, was used for the cell culture. To prepare stable liposomes, a mixture of DPPC (74 mol%), cholesterol (14 mol%), and a ganglioside (Figure 1) (12 mol%) or a mixture of egg PC (62 mol%), cholesterol (26 mol%), and a ganglioside (12 mol%) was dissolved in chloroform / methanol (1:1, v/v) [3,22]. To prepare fluorescent probe-labelled liposomes, DPE-NBD (1 mol%) was added at this stage. On complete removal of the solvent using a rotary evaporator at 30°C, a thin film was formed at the bottom of the flask. After in vacuo overnight, this thin film was swollen in 3.0 ml of calcium and magne-

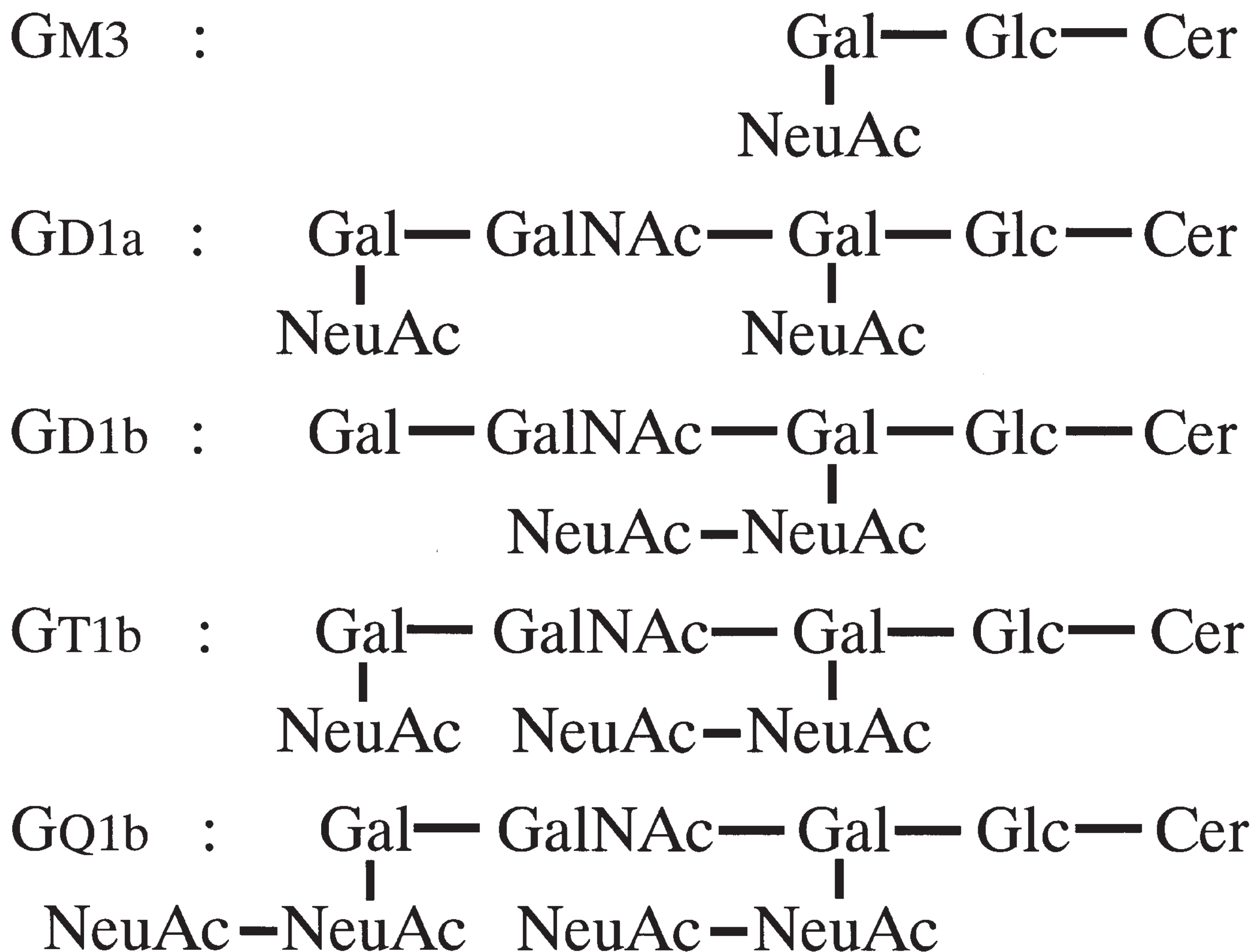

Figure 1. Gangliosides employed in this work (Gal: galactose, Glc: glucose, GalNAc: N-acetylgalactosamine, Cer: ceramide, NeuAc: N-acetylneuraminic acid).

sium ion free phosphate-buffered saline (PBS) using a Vortex mixer. The resulting suspension was then extruded using an Extruder (Lipix Biomembrane Inc., Vancouver, Canada) under nitrogen pressure through two stacked polycarbonate membrane filters (Nuclepore®; Coaster Scientific Corp., Cambridge, MA) as follows; five times through 1.0 μm, five times through 0.6 μm, five times through 0.4 μm, ten times through 0.2 μm, and ten times through 0.1 μm [3,23,24]. This procedure was performed at 50°C for DPPC and at room temperature for egg PC. The mean diameter of the uni- or oligolamellar vesicles thus obtained was found to be approximately 100 nm on DLS measurement.

The final concentration of the liposomal suspension was adjusted to the lipid concentration of 3.7×10^{-4} M, which was determined with a U-3400 Spectrophotometer (Hitachi Ltd., Tokyo, Japan) using a Phospholipid C-Test® kit (Wako Chemicals Co., Osaka, Japan) [3,25].

Preparation of lymphocytes

The human peripheral blood utilized in this study was obtained from healthy volunteers. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) density gradient centrifugation at 400 × g for 20 minutes at room temperature. They were then washed twice with and resuspended in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal calf serum (FCS) (Bioproducts, Walkerville, MD).

Cell selection

Non-specific antigens on PBMC were blocked for 20 minutes at 4°C with a 20% volume of human AB type serum (inactivated at 56°C for 30 minutes), Venoglobin I® (5 mg/ml; Green Cross Corp., Osaka, Japan), and RPMI 1640/10% FCS. The elimination of unnecessary cells from PBMC was performed using Dynabeads M-450 goat anti-mouse IgG (Dynal A.S., Oslo, Norway). PBMC were incubated at 4°C for 30 minutes with anti-CD16b, anti-CD11b, anti-CD14, and the following antibodies: anti-CD20 for the collection of T lymphocytes; anti-CD20 and anti-CD8 for the collection of $CD4^+$ lymphocytes; anti-CD20 and anti-CD4 for the collection of $CD8^+$ lymphocytes; and anti-CD3, anti-CD4, and anti-CD8 for the collection of B lymphocytes. After washing three times, the cells were incubated at 4°C for 20 minutes with Dynabeads M-450 goat anti-mouse IgG in an apparatus that allows both gentle tilting and rotating. The cells that formed rosettes with immunomagnetic beads were removed with a Dynal magnetic particle concentrator (Dynal A.S., Oslo, Norway). The negatively selected cells were collected, washed, and resuspended in RPMI 1640/10% FCS. The final concentration of the cell suspension was adjusted to 1×10^7 cells/ml for $[Ca^{2+}]_i$ check systems and 1×10^6 cells/ml for cell proliferation systems. The collected cells were identified with a fluorescence-activated cell sorter (FACS; Becton Dickinson & Co, Mountain View, CA).

Confocal laser fluorescence microscopic method

The observation of $[Ca^{2+}]_i$ using a confocal fluorescence microscope with an argon-ion laser was performed by the following procedure [3,26,27]. Glass observation chambers (ZOG-3; Elekon Science Co., Ltd., Chiba, Japan) were coated with poly-L-lysine hydrobromide (Sigma Chemical Co., St. Louis, MO). Four hundred ml of a human negatively-selected cell suspension (1×10^7 cells/ml) was incubated at 37°C for 15 minutes in this chamber with 2.0 μl of dimethyl sulfoxide containing 1.0 mM 1-(2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)phenoxy)-2-(2-amino-5-methylphenoxy)ethane-N,N,N′,N′-tetraacetic acid, pentaacetoxymethyl ester (Fluo-3-AM; Dojindo, Kumamoto, Japan). The floating cells were removed by washing several times with RPMI 1640 / 10 % FCS. Just after the addition of 20 μl of a liposome suspension (lipid concentration, 3.7×10^4 M), fluorescent images of the fluo-3-loaded cells were taken with a confocal laser fluorescense microscope (MRC-600; Bio-Rad Digilab, Cambridge, MA) and an inverted epifluorescence microscope (Nikon TMD-EFQ; Nikon Co., Tokyo, Japan). The fluo-3-loaded cells were excited at 488 nm using the argon-ion laser and then the fluorescence emission was observed above 515 nm. The number of fluorescent cells was determined at 200 seconds after the addition of the liposome solution (Fig. 2). The % response was calculated as follows:

$$\%\ \text{response} = \frac{(\text{number of fluorescent cells})}{(\text{total number of cells in the same observation field})} \times 100$$

The adhesion of the fluorescent probe-labelled liposomes to the T lymphocyte surface was investigated by the following procedure. After incubation for 15 minutes in the chamber and removal of the floating cells with the medium, 200 μl of the fluorescent probe-labeled liposomal suspension was added. After 200 seconds, the DPE-NBD-labelled liposomes were excited at 488 nm using the argon-ion laser, and then the fluorescence emission was observed above 515 nm.

Cell proliferation assay (WST-1 assay)

For the quantitative colorimetric assaying of cell proliferation, we used the sulfonated tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate (WST-1) [28,29]. WST-1 (16.3 mg) and 0.2 mM 1-methoxy-5-methyl-phenazinium methylsulfate (Cell Counting kit; Dojindo Lab., Kumamoto, Japan) (5.0 ml) were dissolved in 20 mM HEPES buffer (pH 7.4). One hundred μl of a human negatively-selected cell suspension (1×10^6 cells/ml) was incubated at 37°C under 5% CO^2 for

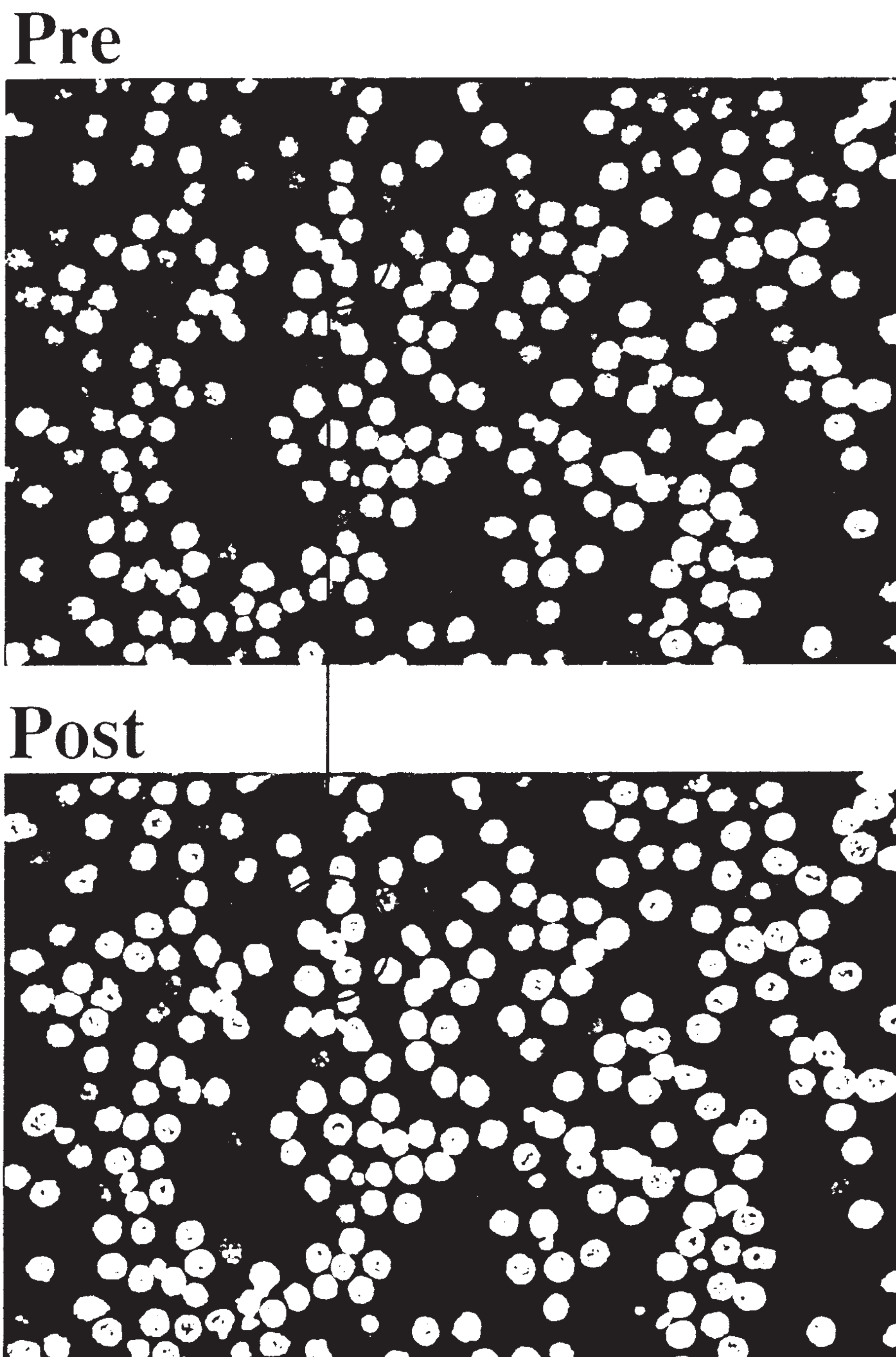

Figure 2. Confocal images of cells stimulated by G_{D1a}-containing liposomes. In the red circles, the % response was 83.3% (five fluorescent cells among a total six cells) (Pre: before the addition of liposomes, Post: 200 seconds after the addition of liposomes).

68 hours in 10 µl of PBS with or without ganglioside-containing liposomes on a 96-well plate. After incubation in 10 µl of the WST-1 solution for an additional 4 hours (total 3 days incubation), the number of cells were determined using a microplate reader at the test wavelength of 415 nm, with a reference wavelength of 630 nm.

Statistic analysis

Values are expressed as means ± standard deviation. Statistical significance was evaluated by means of analysis of variance and Scheffe's multiple comparison test using the SPSS system for Windows. A p-value of less than 0.05 was considered significant.

Results

Changes of $[Ca^{2+}]_i$ in PBMC after the addition of ganglioside-containing liposomes

The differences in the changes of $[Ca^{2+}]_i$ in human PBMC after the addition of various ganglioside-containing liposomes were observed (Table 1). The change produced on the addition of G_{D1a}-containing liposomes was significantly higher than that on the addition of conventional liposomes without a ganglioside (NON), G_{M3}- or G_{D1b}-containing liposomes. The change produced on the addition of G_{T1b}- and G_{Q1b}-containing liposomes was higher, but not significantly so, than that on the addition of NON, G_{M3}- or G_{D1b}-containing liposomes.

Changes of $[Ca^{2+}]_i$ in various types of cells after the addition of G_{T1b}-containing liposomes

The changes of $[Ca^{2+}]_i$ after the addition of G_{T1b}-containing liposomes with various types of cells were observed. The % responses were as follows: 3.65 ± 1.96% for CD20+, 45.7 ± 15.3% for CD4+, and 71.2 ± 10.3% for CD8+ (Figure 3). The response of CD8+ cells was significantly higher than that of CD4+ or CD20+ cells, while the response of CD4+ cells was significantly higher than that of CD20+ cells.

Changes of $[Ca^{2+}]_i$ in human T lymphocytes after the addition of ganglioside-containing liposomes

The % response in the changes in $[Ca^{2+}]_i$ of negatively-selected human T lymphocytes after the addition of various ganglioside-containing liposomes was observed (Table 1). The response after the addition of G_{D1a}- or G_{T1b}-containing liposomes was significantly higher than that after the addition of NON, G_{M3}-, G_{D1b}- or G_{Q1b}-containing liposomes. This was confirmed by an adhesion test involving fluorescent probe-labelled liposomes on confocal laser fluorescence microscope observation. Although G_{D1a}- or G_{T1b}-containing liposomes definitely adhered to T lymphocytes, NON, G_{M3}-, G_{D1b}- or G_{Q1b}-containing liposomes hardly adhered to them (Figure 4).

Table 1. Response of $[Ca^{2+}]_i$ elevation.

Liposomes	*vs. PBMC (%)*	*vs. pan T cells (%)*
NON	0.99 ± 0.20	2.00 ± 0.79
GM3	0.27 ± 0.47	1.44 ± 1.08
GD1a	9.70 ± 4.19*	37.7 ± 18.9
GD1b	0.45 ± 0.42	1.10 ± 1.21
GT1b	6.71 ± 2.15	35.6 ± 5.53
GQ1b	4.52 ± 3.73	4.21 ± 1.79

The responses in the change of $[Ca^{2+}]_i$ in human PBMC and human T lymphocytes after the addition of various ganglioside-containing liposomes are shown (*, **: $P < 0.05$, Scheffe's F test, *: vs. NON, GM3, and GD1b, **: vs. NON, GM3, GD1b, and GQ1b, PBMC: peripheral blood mononuclear cells, NON: conventional liposomes without a ganglioside, GM3, GD1a, GD1b, GT1b, GQ1b: G_{M3}-, G_{D1a}-, G_{D1b}-, G_{T1b}-, G_{Q1b}-containing liposomes).

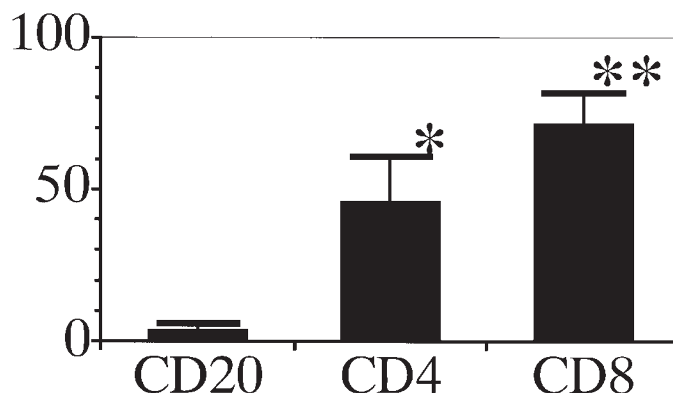

Figure 3. $[Ca^{2+}]_i$ in various cells with G_{T1b}-containing liposomes. The vertical axis shows the % response. The horizontal axis shows the cell types (*, **: $P < 0.05$, Scheffe's F test, *: vs. CD20, **: vs. CD20 and CD4; CD20: CD20+ cells; CD4: CD4+ cells; and CD8: CD8+ cells).

Cell proliferation with ganglioside-containing liposomes

The cell proliferation of T lymphocytes was investigated using the WST-1 assay method to explain the relation between the elevation of $[Ca^{2-}]_i$ after the addition of ganglioside-containing liposomes and cell proliferation. With negatively-selected T lymphocytes, the absorbance after incubation with various ganglioside-containing liposomes was as follows: 0.1012±0.0296 for PBS, 0.0963±0.019 for EPC (liposomes composed of egg PC and cholesterol with no ganglioside), 0.1017 ± 0.0165 for G_{M3}, 0.1063 ± 0.0173 for G_{D1a}, 0.1100 ± 0.012 for G_{T1b}, and 0.4651 ± 0.0368 for PHA (Figure 5). With all the ganglioside-containing liposome systems, no significant proliferation of T lymphocytes was observed under the conditions employed.

Discussion

Gangliosides play roles in both the stimulation and inhibition of cell proliferation [4,5]. We have already reported that G_{T1b}- or G_{Q1b}-containing liposomes increase $[Ca^{2+}]_i$ in *rat T lymphocytes,* while G_{M3}- or G_{D3}-containing ones did not [3]. In this study G_{D1a}- and G_{T1b}-containing liposomes increased $[Ca^{2+}]_i$ also in *human T lymphocytes,* while G_{M3}-, G_{D1b}- or G_{Q1b}-containing ones did not. This clearly reveals that the direct stimulation of T lymphocytes by a sort of ganglioside is not species-specific. We thought the reason why G_{Q1b}-containing liposomes did not increase $[Ca^{2+}]_i$ in the present study but did so in the previous rat study was the high purity of the G_{Q1b} used in the present study compared with that in the previous study, which was suspected

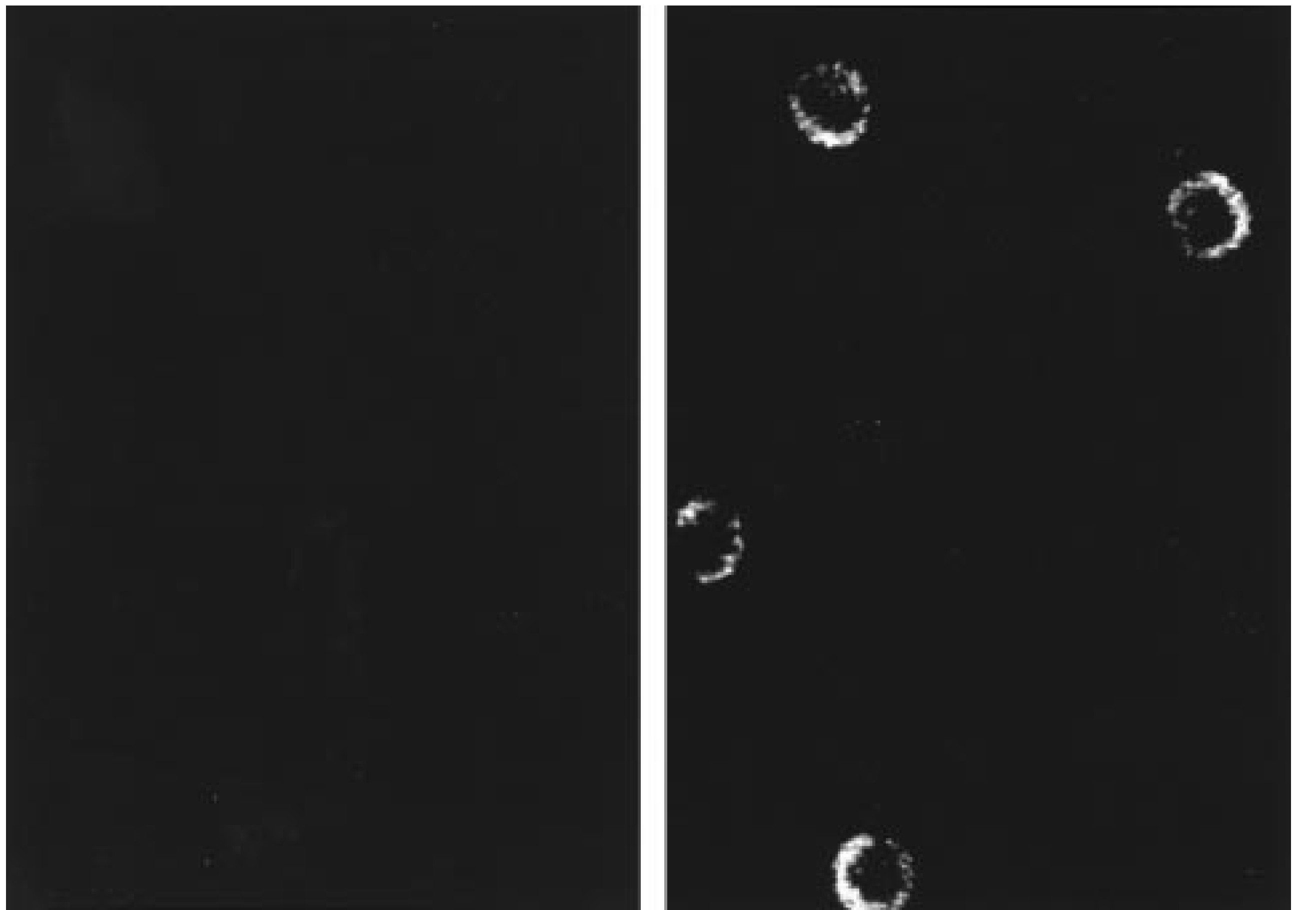

Figure 4. Confocal images of T lymphocytes with DPE-NBD-labelled liposomes. Left panel, T lymphocytes treated with G_{M3}-containing liposomes; right panel, T lymphocytes treated with G_{D1a}-containing liposomes (DPE-NBD, 1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)).

to be contaminated by G_{T1b}. The properties of gangliosides reconstituted in liposomes were similar to those of intact cell membranes [30], and differed from those of free gangliosides shed from the tumor cell surface.

The elevation of $[Ca^{2+}]_i$ did not occur after the addition of conventional liposomes without any gangliosides. Cell proliferation was also not induced on incubation with conventional liposomes without gangliosides. These facts show that conventional liposomes themselves have no stimulatory effect on T lymphocytes.

Confocal images of DPE-NBD-labelled liposomes revealed that the liposomes containing G_{D1a} or G_{T1b} adhered to T lymphocytes but those containing G_{M3}, G_{D1b} or G_{Q1b} did not. This suggests that the receptor for gangliosides or the sialic acid moiety on the T lymphocyte membrane can recognize and bind to G_{D1a} and G_{T1b} among the gangliosides studied.

The large difference in the change of $[Ca^{2+}]_i$ between G_{D1a}-containing and G_{D1b}-containing liposomes suggests that this stimulatory effect is not related to the number of sialic acid groups of the gangliosides, but that the conformation of the sialic acid moiety on the membrane surface might be important. In other words, considering the conformation of gangliosides reconstituted into the liposomal membrane, the sialic acid moieties of G_{D1a} and G_{T1b} protrude exteriorly from the membrane surface of the liposomes, but that of G_{M3} or G_{D1b} lays on the membrane surface of the liposomes. Moreover, the large difference in the change of $[Ca^{2+}]_i$ in T lymphocytes between G_{T1b}-containing and G_{Q1b}-containing liposomes suggests that this stimulatory effect is related to the number of the sialic acid moiety to protrude exteriorly from the membrane surface of the liposomes. The significant difference in the change of $[Ca^{2+}]_i$ may be caused by the configuration of the sialic acid moiety on the liposomal membrane surface that is recognized by a specific receptor on T lymphocytes.

The difference in the response between B lymphocytes ($CD20^+$ cells) and T lymphocytes ($CD4^+$ cells and $CD8^+$ cells) may depend on whether the specific receptor(s) for gangliosides exists or not on B or T lymphocytes. A sort of receptor for the sialic acid moiety should exist on human T lymphocytes to stimulate them. While there is no clear

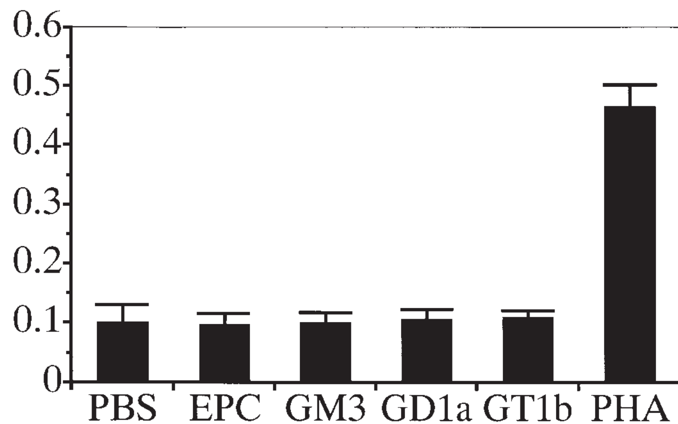

Figure 5. Cell proliferation with ganglioside-containing liposomes. With all the ganglioside-containing liposome systems, no significant proliferation was observed. The vertical axis shows the absorbance (415 nm – 630 nm). The horizontal axis shows the sort of ganglioside reconstituted into the liposomes (PBS: only PBS was added; EPC: conventional liposomes without a ganglioside; GM3, GD1a, GT1b: G_{M3}-, G_{D1a}-, G_{T1b}-containing liposomes; and PHA: phytohemagglutinin P).

explanation for the difference between $CD4^+$ cells (helper T lymphocytes) and $CD8^+$ cells (cytotoxic T lymphocytes) in the changes of $[Ca^{2+}]_i$.

The cytoplasmic free calcium ions play a very important role in the initiation of T cell activation as an intracellular signal transducer [1,2]. But the reason for the discrepancy between the change of $[Ca^{2+}]_i$ and cell proliferation after the addition of G_{D1a}- or G_{T1b}-containing liposomes was not clarified in this study. This will need more careful investigation.

Further studies on lymphocyte proliferation *in vivo* with ganglioside-containing liposomes and the anti-tumor effects of these lymphocytes are ongoing in our laboratory. On the basis of the conformation of the sialic acid moiety of the gangliosides (G_{D1a} and G_{T1b}), the preparation of artificial sialolipids as human T lymphocyte stimulators is also in progress in our laboratory (unpublished data). We are pursuing the possibility of the clinical use of sialolipid-containing liposomes as a T lymphocyte (especially cytotoxic T lymphocyte-) stimulator in the immunotherapy of human malignancy.

Acknowledgements

Two of the authors (K.Y. and E.K.) are Research Fellows of the Japan Society for the Promotion of Science. This work was supported in part by the Japan Society for the Promotion of Science (to H.W.) (RFTF 96100204), and the Terumo Foundation of Science and Technology (to J.S.).

The authors thank Dr. Shin Kawamata for the assistance with the FACS analysis.

References

1 Lew PD, Wollheim CB, Waldvogel FA, Pozzan T (1984) *J Cell Biol* **99**: 1212–20.
2 Weiss A, Imboden JB (1987) *Adv Immunol* **41**: 1–38.
3 Kato E, Akiyoshi K, Furuno T, Nakanishi M, Kikuchi A, Kataoka K, Sunamoto J (1994) *Biochem Biophys Res Comm* **203**: 1750–55.
4 Spiegel S, Fishman PH, Weber RJ (1985) *Science* **230**: 1285–87.
5 Hakomori S (1990) *J Biol Chem* **265**: 18713–16.
6 Ebel F, Schmitt E, Peter-Katalinic J, Kniep B, Mülradt PF (1992) *Biochemistry* **31**: 12190–97.
7 Welte K, Miller G, Champman PB, Yuasa H, Natoli E, Kunicka JE, Cordon-Cardo C, Buhrer C, Old LJ, Houghton AN (1987) *J Immunol* **139**: 1763–71.
8 Hersey P, Schibeci SD, Townsend P, Burns C, Cheresh DA (1986) *Cancer Res* **46**: 6083–90.
9 Gouy H, Deterre P, Debré P, Bismuth G (1994) *J Immunol* **152**: 3271–81.
10 Ladisch S, Kitada S, Hays EF (1987) *J Clin Invest* **79**: 1879–82.
11 Miller HC, Esselman WJ (1975) *J Immunol* **115**: 839–43.
12 Offner H, Thieme T, Vandenbark AA (1987) *J Immunol* **139**: 3295–305.
13 Pique C, Mahé Y, Scamps C, Tétaud C, Tursz T, Wiels J (1991) *Mol Immunol* **28**: 1163–70.
14 Offner H, Vandenbark AA (1985) *Cell Immunol* **95**: 84–94.
15 Chu JWK, Sharom FJ (1993) *Immunology* **79**: 10–17.
16 Lengle EE, Krishnaraj R, Kemp RG (1979) *Cancer Res* **39**: 817–22.
17 Merritt WD, Bailey JM, Pluznik DM (1984) *Cell Immunol* **89**: 1–10.
18 Whisler RL, Yates AJ (1980) *J Immunol* **125**: 2106–11.
19 Marcus DM, Dustira A, Diego I, Osovitz S, Lewis DE (1987) *Cell Immunol* **104**: 71–78
20 Hope MJ, Bally MB, Webb G, Cullis PR (1985) *Biochim Biophys Acta* **812**: 55–65.
21 Mayer LD, Hope MJ, Cullis PR (1986) *Biochim Biophys Acta* **858**: 161–68.
22 Deleers M, Servais JP, Laveleye F (1984) *Biochem Biophys Res Comm* **123**: 178–85.
23 Sunamoto J, Mori Y, Sato T (1992) *Proc Japan Acad* **68,** Ser B: 69–74.
24 Ariyasu T, Ike O, Hitomi S, Wada H, Okumira Y, Sunamoto J (1996) *J Bioactive Compatible Polymers* **11**: 191–202.
25 Takayama M, Itoh S, Nagasaki T, Tanimizu I (1977) *Clin Chim Acta* **79**: 93–98.
26 Nagao Y, Yamada H, Nakanishi M (1992) *Immunol Lett* **34**: 151–4
27 Nakato K, Furuno T, Inagaki K, Teshima R, Terao T, Nakanishi M (1992) *Eur J Biochem* **209**: 745–49.
28 Ishiyama M, Shiga M, Sasamoto K, Mizoguchi M, He PG (1993) *Chem Pharm Bull* **41**: 1118–22.
29 Liu SQ, Saijo K, Todoroki T, Ohno T (1995) *Nature Med* **1**: 267–71.
30 Sharom FJ, Chiu ALH, Chu JWK (1991) *Biochim Biophys Acta* **1094**: 35–42.

Received 29 October 1998, revised 24 November 1998, accepted 4 December 1999