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Concanavalin A-stimulated expression of gangliosides with GalNAcβ1-4(NeuAcα2-3)Galβ structure in murine thymocytes

KENTARO HORIKAWA[1], MASAHIKO YAMASAKI[1], MASAO IWAMORI[1]*, HIDEKI NAKAKUMA[2], KIYOSHI TAKATSUKI[2] and YOSHITAKA NAGAI[3]

[1] *Department of Biochemistry, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan*

[2] *Second Department of Internal Medicine, Kumamoto University Medical School, 1-1-1 Honjo, Kumamoto 860, Japan*

[3] *Tokyo Metropolitan Institute of Medical Science, 3-18-22, Honkomagome, Bunkyo-ku, Tokyo 113, Japan*

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We analysed the glycolipids of mouse thymocytes before and after Concanavalin A (Con A) or recombinant interleukin-2 (rIL-2) stimulation by TLC-immunostaining with carbohydrate-specific antiglycolipid antibodies. The thymocytes were cultured in serum-free medium in the presence of 500 ng ml^{-1} Con A, 10 U ml^{-1} rIL-2 or Con A plus rIL-2 for 6, 12, 24, 48, and 72 h, and were found to start proliferating 24 h after cultivation in the presence of Con A or Con A plus rIL-2, the maximum levels being reached at 72 h and 48 h, respectively, in a thymidine uptake experiment. The concentrations of II^{3}Neu-Gg$_4$Cer, Gg$_4$Cer and IV^{3}GalNAcα-Gb$_4$Cer after 48 h Con A stimulation were found to be at almost the original levels. Conversely, II^{3}Neu-Gg$_3$Cer, which was not detected in the thymocytes at the start, began to appear after 48 h stimulation with Con A and Con A plus rIL-2, and IV^{3}Neu-Gg$_5$Cer in the cells 48 h after stimulation with Con A and Con A plus rIL-2 has increased to 41 and 44 times higher than in the original cells, respectively, as judged on TLC-immunostaining with monoclonal antibody YHD-06, which detects the GalNAcβ1-4(NeuAc or NeuGcα2-3)Galβ-structure. These results indicate that the increased synthesis of both gangliosides, in other words, the activation of *N*-acetylgalactosaminyltransferase, is associated with the mitogen-induced proliferation. *N*-Acetylneuraminic acid was the sole sialic acid in II^{3}Neu-Gg$_3$Cer which newly appeared in the cells on stimulation, whereas the sialic acid of IV^{3}Neu-Gg$_5$Cer was a mixture of *N*-acetyl- and *N*-glycoloylneuraminic acids. This result may suggest that the substrates for the two different *N*-acetylgalactosaminyltransferases must be different. This GalNAcβ1-4(NeuAc or NeuGcα2-3)Galβ-structure was also detected on the surface of the Con A or Con A plus rIL-2 stimulated mouse thymocytes on flow cytometric analysis of cells indirectly stained with monoclonal antibody YHD-06.

Keywords: GM2, Con A-stimulation, murine thymocytes, antiglycolipid antibodies

Abbreviations: carbohydrate and glycolipid nomenclature and abbreviations follow the IUPAC-IUB recommendations or the nomenclature system of Svennerholm L. (1963) *J Neurochem* **10**:613–23.

Glycosphingolipids, which are present on the surface of animal cells, are known to be closely associated with cell growth, ontogenesis, differentiation and malignant transformation [1], and provide us with useful markers for characterizing cellular functions. For example, in immunocyte subpopulations, alloantigen-stimulated murine T lymphocyte precursors of both helper and cytolytic T cells express Gb$_4$Cer as a serological marker [2], and cytolytic T cells are differentially distinguished from natural killer cells by Gg$_4$Cer [3, 4]. Also, compensatory expression of Gg$_4$Cer and Thy-1 has been demonstrated in murine thymocytes in the gestation period [5], and II^{3}NeuAc-Gg$_4$Cer, as a cholera toxin receptor, has been found to be distributed widely in murine thymocytes [6]. In addition, cholera toxin has been shown to inhibit mitogenic lectin-mediated T lymphocyte activation, possibly due to its interference with the function of G-protein [7]. These results strongly indicate that glycolipids, particularly ganglioseries glycolipids, are involved in various important immunological functions. Although a change in the glycolipid

* To whom correspondence should be addressed.

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metabolism of mitogen-stimulated T and B cells has been demonstrated by metabolic investigations with radioactive precursors, detailed analysis of glycolipids altered in response to lymphocyte proliferation has not been performed until now. Therefore, we characterized the glycolipids in murine thymocytes before and after mitogen stimulation using a sensitive TLC-immunostaining procedure involving carbohydrate-specific monoclonal and polyclonal antibodies.

Materials and methods

The following glycolipids were used as references for TLC and TLC-immunostaining: Gg_3Cer(asialo GM2) from guinea-pig erythrocytes; Gg_4Cer(asialo GM1) from formic acid-treated human brain gangliosides; $IV^3GalNAc\alpha$-Gb_4Cer(Forssman antigen) from horse kidney; II^3NeuGc-Gg_3Cer from murine erythrocytes; and II^3NeuAc-Gg_4Cer, II^3NeuAc-Gg_3Cer, II^3NeuAc-LacCer and $II^3NeuAc\alpha_2$-Gg_3Cer from human brain. IV^3NeuGc-Gg_5Cer was kindly donated by Drs Nakamura and Suzuki of the Tokyo Metropolitan Institute of Medical Science [8]. Two mouse monoclonal antibodies (mAbs): Pyk-2, that reacts with II^3NeuGc-Gg_3Cer, and YHD-06, that reacts with II^3NeuAc-Gg_3Cer, II^3NeuGc-Gg_3Cer and IV^3NeuGc-Gg_5Cer, were purified from the culture supernatants of hybridomas that were established by fusing spleen cells from mice immunized with II^3NeuGc-Gg_3Cer and mouse myeloma cells NS-1. In addition, rabbit polyclonal anti-Gg_4Cer, anti-$IV^3GalNAc\alpha$-Gb_4Cer and cholera toxin B subunit antibodies were prepared through immunization with the purified glycolipids or the glutaraldehyde-treated cholera toxin B subunit with Freund's complete adjuvant. Peroxidase-conjugated affinity-purified goat antimouse immunoglobulin (Ig)G + M antibody and the peroxidase-conjugated IgG fraction of goat antirabbit IgA + G + M antibody were purchased from Jackson Lab. and Cappel Lab., respectively.

Preparation and cultivation of thymocytes

Thymuses were obtained from BALB/c mice (female, 4–5 weeks of age; SLC Japan Co.). A suspension of the thymocytes was prepared by pressing the thymuses through a fine stainless steel mesh and the cell number was determined after staining with erythrosin B in phosphate-buffered saline (PBS). The effects of Con A (Honen Seiyu Co.) and rIL-2 (Genzyme, Boston, USA) on the thymidine uptake by thymocytes were determined by cultivating them at a concentration of 2.5×10^6 ml^{-1} in serum free medium HL-1 (Ventrex Co.) containing insulin, testosterone and transferrin. The cells were cultivated in the presence of various concentrations of Con A, rIL-2 or Con A plus rIL-2 under a humidified atmosphere of 5% CO_2 in air at 37°C for 6, 12, 24, 48 and 72 h. A part of the cells corresponding to 5.0×10^5 of the original thymocytes was used for the thymidine uptake experiment, in which 3.7 kBq of [^{3}H]thymidine (247.9 GBq mmol^{-1}; NEN Research) was added to the cells 6 h before harvesting. Then the cells were collected with a cell harvester (200A, Cambridge Technology Co.) and the radioactivity incorporated into them was counted with a liquid scintillation counter (LS1800, Beckman Co.). Triplicate experiments were performed, cells cultured without Con A or rIL-2 serving as controls. The remaining cells were harvested, washed twice with PBS and then stored at −20°C until use.

Lipid extraction from thymocytes

Thymocytes, 5×10^8, cultured in the presence of 500 ng ml^{-1} Con A, 10 U ml^{-1} rIL-2 or Con-A plus rIL-2 for various times as described above were lyophilized and suspended in 3 ml of chloroform:methanol:water (C:M:W), 20:10:1 by vol, by means of sonication, followed by incubation at 40°C for 60 min. The lipid extracts were collected after centrifugation and the residue was re-extracted with C:M:W, 10:20:1 by vol, and C:M, 1:1 by vol, in the same manner. The lipid extracts were combined in each case and the volume was adjusted with C:M, 1:1 by vol.

TLC and TLC-immunostaining

The total lipids were applied on a silica gel TLC plate (Macherey-Nagel Co.) for TLC and TLC-immunostaining. The plates were developed with three solvent systems: I, C:M:5 M NH_4OH:0.5% $CaCl_2$ in water, 60:40:4:5 by vol; II, C:M:0.5% $CaCl_2$ in water, 55:45:10 by vol; or III, C:M:0.5% $CaCl_2$ in water, 65:35:8 by vol; and the glycolipids were visualized by spraying with orcinol−H_2SO_4 and heating at 110°C for 5 min. For TLC-immunostaining, a plate, after separation of glycolipids, was incubated with a blocking buffer (1% ovalbumin and 1% polyvinylpyrrolidone in PBS) at 37°C for 1 h, subsequently with the first antibody diluted with a dilution buffer (3% polyvinylpyrrolidone in PBS) at 37°C for 1 h. After washing the plate five times with 0.5% polyoxyethylene sorbitan monolaurate (Tween 20) in PBS, the antibody remaining on the plate was visualized by incubation with the peroxidase-conjugated second antibody at 37°C for 1 h, washing the plate and reaction with a HRP immunostaining kit (Konica Co.), the sensitivity for the detection of peroxidase of which was about 10 times higher than that with 4-chloro-1-naphthol and H_2O_2 as substrates. For the detection of GM1 by TLC-immunostaining, the cholera toxin B subunit (List Biological Lab. Inc.), at 50 ng ml^{-1} in the dilution buffer, was reacted with the plate at 37°C for 1 h prior to the reaction with the first antibody. The reference glycolipids, of known amounts, were reacted on the same plate and quantitation of the glycolipids was performed with a double wavelength TLC-densitometer (CS9000, Shimadzu Co.) at a sample wavelength of 580 nm and a control wavelength of 700 nm.

Flow cytometric analysis of thymocytes with mAb

Mouse thymocytes (1×10^6) before and after treatment with Con A for 72 h were stained with the monoclonal

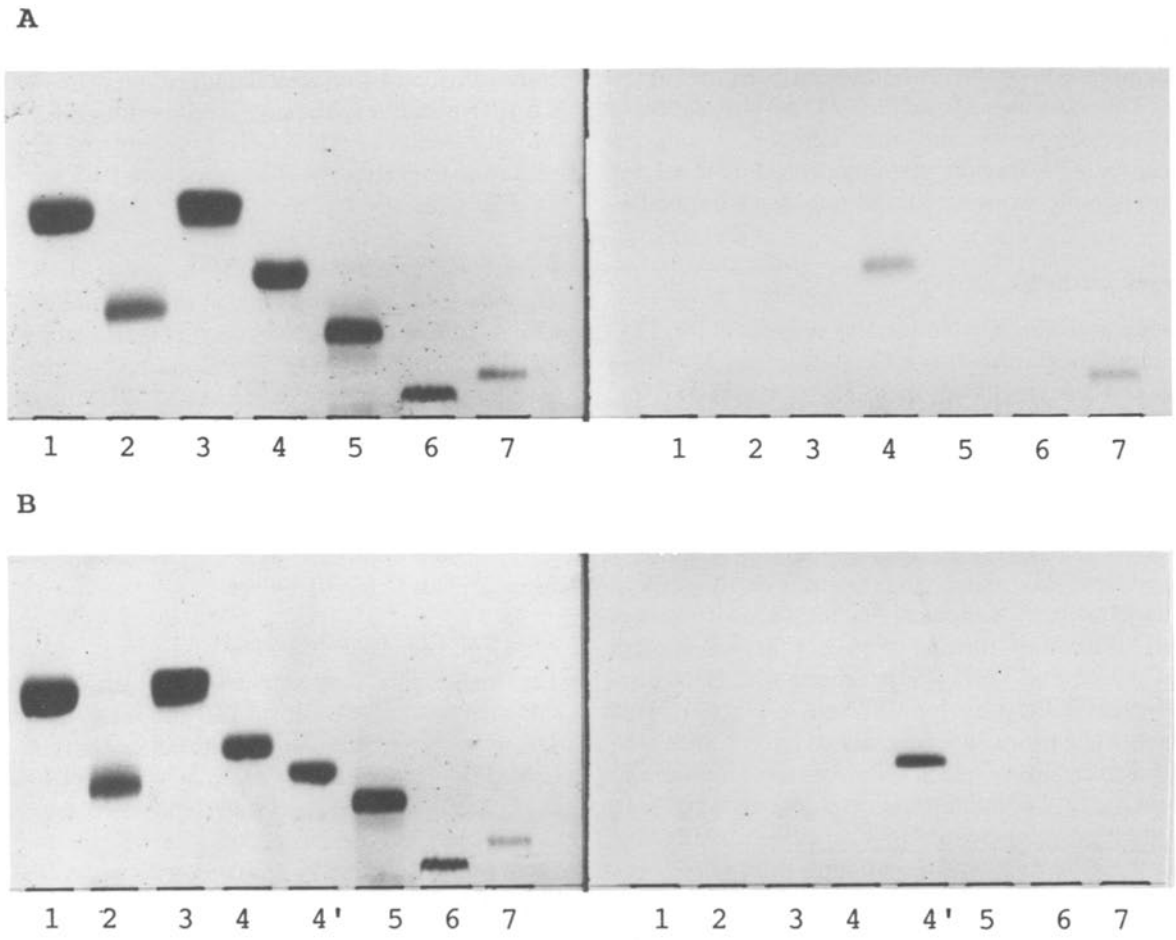

Figure 1. TLC (left) with spraying with orcinol-H_2SO_4 and TLC-immunostaining (right) using YHD-06 (a) and Pyk-2 (b). 1, Gg_3Cer; 2, Gg_4Cer; 3, II^3NeuAc-LacCer; 4, II^3NeuAc-Gg_3Cer; 4′, II^3NeuGc-Gg_3Cer; 5, II^3NeuAc-Gg_4Cer; 6, $II^3NeuAc\alpha_2$-Gg_3Cer; 7, IV^3NeuGc-Gg_5Cer.

anti-II^3Neu-Gg_3Cer antibody at 4°C for 10 min, subsequently with the fluorescein isothiocyanate (FITC)-conjugated antimouse Ig antibody at 4°C for 30 min according to the method described previously [9]. The fluorescence intensity of cells was measured with a flow cytometer (FACScan, Beckton Dickinson). Dead cells were gated out by forward low-angle scattering and using propidium iodide (Sigma Co.), and the data for 10000 cells were collected.

Results

Specificities of mAbs

The epitope structures for mAbs, YHD-06 and Pyk-2, were elucidated by TLC-immunostaining, as shown in Fig. 1. YHD-06 reacted with II^3NeuAc-Gg_3Cer, II^3NeuGc-Gg_3Cer (data not shown) and IV^3NeuGc-Gg_5Cer, but not with Gg_3Cer, Gg_4Cer, II^3NeuAc-LacCer, II^3NeuGc-LacCer, $II^3NeuAc\alpha_2$-Gg_3Cer or II^3NeuAc-Gg_4Cer, indicating that the nonreducing terminal structure of GM2 with *N*-acetyl- or *N*-glycolylneuraminic acid is the epitope (Table 1). Instead, Pyk-2 was only reactive with II^3NeuGc-Gg_3Cer, i.e., not with II^3NeuAc-Gg_3Cer or IV^3NeuGc-Gg_5Cer, as shown in Fig. 1(b). Therefore, both *N*-glycolylneuraminic acid and a part of the GlcCer moiety of GM2 was required for the epitope (Table 1).

Cellular proliferation of murine thymocytes in response to Con A and rIL-2

The proliferation of murine thymocytes in serum free medium, as determined from the [^{3}H]thymidine uptake, gradually decreased, becoming undetectable 48 h after cultivation. The addition of rIL-2 did not have a significant effect on the uptake of [^{3}H]thymidine. Con A, as is well known, was able to induce proliferation of the cells (Table 2), and the coexistence of Con A and rIL-2 was effective in increasing the incorporation rate.

Table 1. Comparison of molecular structures reactive with mAbs YHD-06 and Pyk-2[a].

Structure of ganglioside	*YHD-06*	*Pyk-2*
GalNAcβ1-4Galβ1-4Glcβ1-1Cer 3 | NeuAcα2 (II^3NeuAc-Gg_3Cer)	+	−
GalNAcβ1-4Galβ1-4Glcβ1-1Cer 3 | NeuGcα2 (II^3NeuGc-Gg_3Cer)	+	+
GalNAcβ1-4Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer 3 | NeuGcα2 (IV^3NeuGc-Gg_5Cer)	+	−

[a] +, reactive; −, not reactive.

Table 2. [^{3}H]Thymidine incorporation into thymocytes cultured with rIL-2, Con A and Con A plus rIL-2 for 6, 12, 24, 48 and 72 h[a].

Cultivation time	*Control*	*rIL-2*	*Con A*	*Con A+rIL-2*
6 h	2467 ± 136	2436 ± 392	2811 ± 248	2839 ± 218
12 h	804 ± 91	695 ± 102	912 ± 67	873 ± 93
24 h	204 ± 9	274 ± 18	2474 ± 315	2781 ± 468
48 h	36 ± 10	104 ± 20	12998 ± 978	21331 ± 1718
72 h	36 ± 6	48 ± 8	18051 ± 890	19815 ± 3003

[a] Thymocytes corresponding to 5.0×10^5 of the original thymocytes were used for this experiment, in which 3.7 kBq of [^{3}H]thymidine was added to the cells 6 h before harvesting. The radioactivity incorporated into the cells was expressed as counts min^{-1}, and triplicate experiments were performed. Control: thymocytes cultured without rIL-2 or Con A; rIL-2, those with 10 U ml^{-1} rIL-2; Con A: those with 500 ng ml^{-1} Con A; Con A+rIL-2: those with 500 ng ml^{-1} Con A and 10 U ml^{-1} rIL-2. 6 h, 12 h, 24 h, 48 h, and 72 h denote the thymocytes cultured for 6, 12, 24, 48, and 72 h, respectively.

Change in glycolipids of murine thymocytes before and after Con A stimulation

Mouse thymocytes, 5×10^8, were cultured in a plastic dish in serum free medium with Con A, rIL-2 or Con A plus rIL-2 for 48 h, and then the glycolipids were compared by TLC-immunostaining with mAbs, YHD-06 and Pyk-2, cholera toxin, and polyclonal rabbit anti-Gg_4Cer and anti-IV^3GalNAcα-Gb_4Cer antibodies (Figs 2, 3). The total lipid extracts were directly applied on TLC plates to minimize the loss of glycolipids.

As shown in Fig. 2, IV^3Neu-Gg_5Cer was detected on normal thymocytes and the concentration corresponding to the original thymocytes was reduced after cultivation with rIL-2 for 48 h because the number of viable cells was significantly lowered due to the death of cells. In contrast, thymocytes cultured in the presence of Con A or Con A plus rIL-2 for 48 h expressed IV^3Neu-Gg_5Cer and II^3Neu-Gg_3Cer at concentrations, higher than those in the original thymocytes. Three bands of IV^3Neu-Gg_5Cer in the total lipid extracts with Con A and Con A plus rIL-2 were estimated to differ in the *N*-substitution of neuraminic acid and the length of fatty acids. Whereas, II^3Neu-Gg_3Cer newly synthesized in the cells in response to Con A-stimulation was identified as II^3NeuAc-Gg_3Cer, because of the negative reaction with mAb Pyk-2 (Fig. 3A).

The concentrations corresponding to the original thymocytes of both gangliosides were determined by TLC-immunostaining with mAb YHD-06 (Table 3). In the original thymocytes, IV^3Neu-Gg_5Cer was present at the concentration of 10.1 nmol in 1×10^{10} cells, but no II^3Neu-Gg_3Cer was detected. Although IV^3Neu-Gg_5Cer disappeared in thymocytes cultured in serum free medium with rIL-2 and without rIL-2 or Con A, mainly due to cellular death, it began to increase 24 h after cultivation in medium containing Con A or Con A plus rIL-2, the highest concentrations being reached at 72 h and 48 h after cultivation, respectively. The rate of the increase in IV^3Neu-Gg_5Cer in cells stimulated by rIL-2 plus Con A was more enhanced than that in cells stimulated by Con A alone. The effect of Con A was essential for the increase in IV^3Neu-Gg_5Cer, and the coexistence of rIL-2 was effective in enhancing the increase. A 44-fold increase was observed 72 h after stimulation with Con A plus rIL-2. Also, II^3NeuAc-Gg_3Cer was

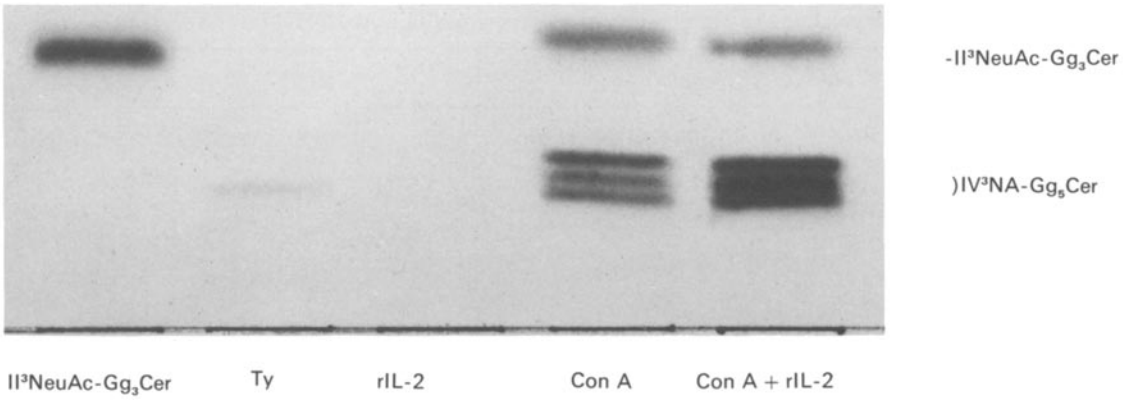

Figure 2. TLC-immunostaining, using mAb YHD-06, of the total lipid extracts from murine thymocytes and thymocytes cultured for 48 h. Lipid extracts from 1.5×10^7 cells were chromatographed with solvent system I. II^3NeuAc-Gg_3Cer, standard; Ty, thymocytes; rIL-2, the thymocytes cultured with rIL-2; Con A, those with Con A; Con A + rIL-2, those with Con A plus rIL-2.

detected in the thymocytes 48 h after cultivation with Con A or Con A plus rIL-2.

We determined the concentrations of II^3Neu-Gg_4Cer with cholera toxin, Gg_4Cer with anti-Gg_4Cer antibody and IV^3GalNAcα-Gb_4Cer with anti-IV^3GalNAcα-Gb_4Cer antibody in the lipid extracts of thymocytes, and thymocytes cultured with rIL-2, Con A and Con A plus rIL-2 for 48 h (Fig. 3(b–d)), but the changes in the concentrations corresponding to the original thymocytes of these glycosphingolipids were not as significant as those of II^3Neu-Gg_3Cer and IV^3Neu-Gg_5Cer.

Flow cytometric analysis of murine thymocytes before and after stimulation with Con A

Mouse thymocytes before and after stimulation with Con A for 48 h were stained by the indirect membrane immunofluorescence technique with mAb YHD-06, subsequently with the FITC-conjugated antimouse Ig antibody, and then analysed with a FACScan, as shown in Fig. 4. The fluorescence intensity of normal thymocytes was identical with that of control cells stained without the first antibody. However, after stimulation the cells became positive as to the antibody, indicating that the GalNAcβ1-4(NeuAc or NeuGcα2-3)Galβ-structure of the epitope is newly expressed in mouse thymocytes in response to Con A-stimulation to give a new phenotypic structure.

Discussion

The present work was conducted to clarify the glycosphingolipids that can be used as phenotypic markers for the characterization of murine immunocytes, since several

Table 3. Concentrations of II^3Neu-Gg_3Cer and II^3Neu-Gg_5Cer in mouse thymocytes cultured without Con A or rIL-2, and with Con A, rIL-2 and Con A plus rIL-2.

	Recovery[b] *of cells*	*Concentrations*[a] *of*							
		II^3Neu-Gg_3Cer (nmol)				*IV^3Neu-Gg_5Cer* (nmol)			
		Control[c]	*rIL-2*[d]	*Con A*[e]	*Con A+rIL-2*[f]	*Control*	*rIL-2*	*Con A*	*Con A+rIL-2*
Ty[g]	5.0×10^8	N.D.	N.D.	N.D.	N.D.	10.1	10.1	10.1	10.1
6 h	4.2×10^8	N.D.	N.D.	N.D.	N.D.	6.3	6.4	17.2	8.3
12 h	2.4×10^8	N.D.	N.D.	N.D.	N.D.	N.D.	4.7	6.3	27.5
24 h	3.3×10^8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	31.3	57.3
48 h	3.7×10^8	N.D.	N.D.	12.4	67.7	N.D.	N.D.	114.2	453.5
72 h	3.4×10^8	N.D.	N.D.	45.8	61.7	N.D.	N.D.	419.3	447.6

[a] Concentrations of gangliosides were expressed in 1×10^{10} of the original thymocytes used for the experiment.
[b] Recovery of cells means the number of thymocytes recovered after cultivation. The original number of thymocytes was 5.0×10^8 in this experiment.
[c] Control: thymocytes cultured without rIL-2 or Con A;
[d] rIL-2: those thymocytes with 10 U ml^{-1} rIL-2;
[e] Con A: those thymocytes with 500 ng ml^{-1} Con A;
[f] Con A+rIL-2: those thymocytes with 500 ng ml^{-1} Con A and 10 U ml^{-1} rIL-2.
[g] Ty: thymocytes. 6 h, 12 h, 24 h, 48 h, and 72 h denote the thymocytes cultured for 6, 12, 24, 48, and 72 h, respectively.
[h] N.D.: not detected.

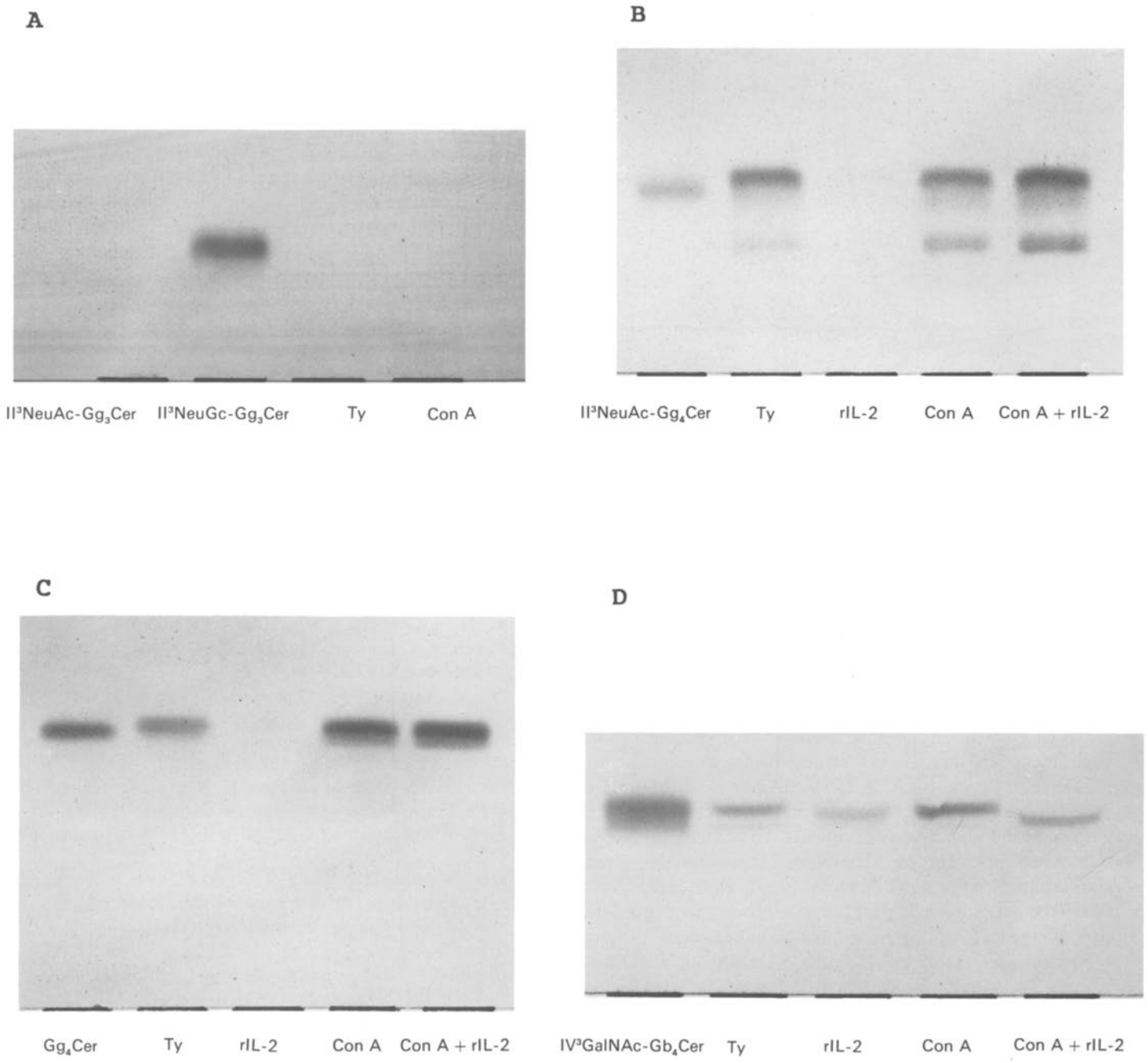

Figure 3. TLC-immunostaining, using mAb Pyk-2 (A), cholera toxin (B), anti-Gg_4Cer antibody (C) and anti-$IV^3GalNAc\alpha$-Gb_4Cer antibody (D), of the total lipid extracts from thymocytes and thymocytes cultured for 48 h. Lipid extracts from 1.5×10^7 (A, D), 1.5×10^5 (B) and 1.5×10^6 (C) cells were chromatographed with solvent system I (A), II (B, C) and III (D). II^3NeuAc-Gg_3Cer, II^3NeuGc-Gg_3Cer, II^3NeuAc-Gg_4Cer, Gg_4Cer and $IV^3GalNAc\alpha$-Gb_4Cer, standards. Ty, thymocytes; rIL-2, the thymocytes cultured with rIL-2; Con A, those with Con A; Con A + rIL-2, those with Con A plus rIL-2.

glycosphingolipids were known to be associated with murine immunocyte subpopulations. The ganglio-series glycolipids are particularly important for murine immunocytes. Gg_4Cer is not only a cell surface marker of natural killer cells [10], but is also highly expressed in fetal thymocytes [11]. II^3NeuAc-Gg_4Cer in murine lymphocytes serves as a signal mediator in cholera toxin-dependent inhibition of lymphocyte proliferation by a mitogen or an antigen [12]. Therefore, we first attempted to analyse the change in glycosphingolipids on mitogen-induced proliferation of murine thymocytes by utilizing monoclonal and polyclonal antibodies directed to the ganglio-series glycosphingolipids. As

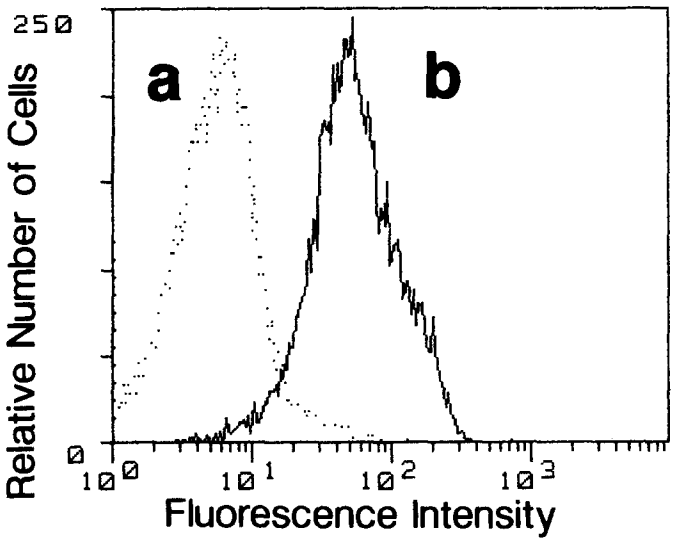

Figure 4. Fluorescence histogram of mouse thymocytes before and after 48 h cultivation with 500 ng ml^{-1} of Con A. The cells were stained with mAb YHD-06, followed by the FITC-conjugated antimouse Ig antibody. Dead cells were gated out by forward low-angle scattering and using propidium iodide. (a) Thymocytes; (b) thymocytes cultured for 48 h with Con A.

described in this paper, among the antibodies tested, YHD-06, that reacts with the GalNAcβ1-4(NeuAc or NeuGcα2-3)Galβ-structure, was able to differentiate proliferating thymocytes from resting cells. The responsible antigens were identified as II^3NeuAc-Gg_3Cer and IV^3(NeuAc or NeuGc)-Gg_5Cer, which were also proved to increase in association with cell growth, as demonstrated by the thymidine uptake experiment. The increase in the gangliosides was significantly higher than that in Gg_4Cer, II^3(NeuAc or NeuGc)-Gg_4Cer and IV^3GalNAcα-Gb_4Cer, and was characteristic of the change in the phenotype of murine thymocytes during Con A-induced proliferation. Therefore, expression of glycosphingolipids having the epitope structure for mAb YHD-06 will be a useful marker for the mitogen-induced proliferation of murine thymocytes. In fact, the fluorescence intensity of thymocytes stained with mAb YHD-06 after mitogen stimulation was stronger than that before the stimulation, indicating that the glycolipids are expressed on the surface of proliferating cells.

Probably, *N*-acetylgalactosaminyltransferase(s), a key enzyme for elongation of the carbohydrate chain of gangliosides, to II^3Neu-LacCer and IV^3Neu-Gg_4Cer might be activated in response to mitogen stimulation. Although a change in the metabolism of glycosphingolipids on mitogen stimulation was demonstrated in splenic T cells by metabolic investigations with radioactive precursors, [^{14}C]galactose and *N*-acetyl[^{14}C]glucosamine, characterization of the glycosphingolipids altered on the stimulation was insufficient [13, 14]. On the basis of our findings, analysis of the metabolism of ganglio-series gangliosides is now in progress in our laboratory to clarify the metabolic basis of the phenotypic alteration on mitogen stimulation.

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