An Activation-associated Ganglioside in Rat Thymocytes*

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During the activation of rat thymocytes elicited by stimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA) and calcium ionophore A23187, the total amount of gangliosides per cell was demonstrated to increase, reaching a maximum level several times higher than that of resting cells at 48 h after activation when measured at 24-h intervals. The amount of the overwhelmingly predominant ganglioside in resting thymocytes, GD1b(NeuGc,NeuGc) (Nohara, K., Suzuki, M., Inagaki, F., and Kaya, K. (1991) J. Biochem. (Tokyo) 110, 274-278), was found to increase further as a result of activation. Furthermore, another ganglioside, which was barely recognizable in resting thymocytes, was found to increase in activated and proliferating thymocytes to a level similar to that of GD1b. This activation-associated ganglioside was isolated and its structure examined. On the basis of the results of compositional analysis, methylation analysis, sialidase hydrolysis followed by detection with cholera toxin B subunit on TLC, and proton NMR spectroscopy, this ganglioside was clarified to be a rare species of GD1b containing two N-glycolyneuraminic acid residues.

On the other hand, when the thymocytes were activated using concanavalin A (ConA) as a stimulant, the amount of gangliosides per cell was increased more strikingly than that in thymocytes activated with TPA and A23187. In the ConA-activated thymocytes, many other gangliosides, in addition to GD1b (NeuGc,NeuGc), were demonstrated to appear in large amounts. The cause of this difference in gangliosides between thymocytes activated by a combination of TPA and A23187 and those activated with ConA is also discussed.

Resting T lymphocytes or thymocytes become activated when their T cell antigen receptors (TCR)1 recognize antigens presented by accessory cells. The interaction between TCR and antigen triggers numerous biological processes including activation of protein kinase C and elevation of the intracellular calcium level. The latter two events induce interleukin-2 (IL-2) synthesis and IL-2 receptor expression, and eventually cell proliferation (1). These activated and proliferating T lymphocytes play an important role in various immune responses as effector cells. Much evidence has been obtained that gangliosides on the plasma membrane associate with the pathway of T lymphocyte and thymocyte activation, and modulate the activation and proliferation process (2-5). Other evidence suggests that gangliosides inhibit the expression of CD4 molecules on helper T cells (6). It has also been reported that a ceramide analog, which inhibits glycolipid biosynthesis, suppresses mitogen-induced proliferation of T cells and a T cell clone (7). However, in these previous studies, information was not fully obtained on the alterations of gangliosides actually present on the lymphocytes or thymocytes and their alterations during cell activation. To establish definitive functions of gangliosides in the cell activation, such information will be prerequisites.

Recently, we clarified that a unique GM1-derived distosialoganglioside, GD1b(NeuGc,NeuGc), is the overwhelmingly predominant ganglioside of rat thymocytes, most of which is considered to be resting cells (8). In the present study, we found and characterized a ganglioside that appears in activated and proliferating thymocytes obtained by stimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA) and calcium ionophore A23187. Recent studies have revealed that a combination of TPA and a calcium ionophore mimics antigenic stimulation via TCR by activating protein kinase C and augmenting cytosolic calcium, thus inducing proliferation (1). On the other hand, lectin mitogens such as concanavalin A (ConA) have frequently been used to activate T lymphocytes and thymocytes. These lectins are presumed to interact with TCR to activate the cells (9). In this investigation, we also demonstrated that there is a distinctive difference between the gangliosides appearing in activated thymocytes after stimulation with TPA and A23187 and those in cells stimulated with ConA.

EXPERIMENTAL PROCEDURES

Materials—Six- to 9-week-old male Wistar rats supplied by Clea Japan (Tokyo) were used for all the experiments. RPMI 1640 medium and Dulbecco's phosphate-buffered saline (PBS) were obtained from Nissui Pharmaceutical Co. (Tokyo); fetal calf serum was from Life Technologies, Inc.; TPA and A23187 were from Sigma; ConA was from Wako Pure Chemical (Osaka); IL-2 from rat splenocytes was supplied by Collaborative Researc, Becton Dickinson Labware (Bedford, MA). Precast thin-layer plates (HPTLC SiIica Gel 60) for standard TLC analysis and TLC aluminum sheets (silica gel 60) for chola toxin B subunit were purchased from Merck (Darmstadt); Clostridium perfringens sialidase was from Sigma; polysorblyl methacrylate) was from Aldrich; cholera toxin B subunit-biotin conjugate was from List Biological Laboratories (Campbell, CA); Vectastain ABC kit was from Vector Laboratories (Burlingame, CA). As reference gangliosides, GD1b(NeuGc,NeuGc) was isolated from rat thymus (5), GM2(NeuGc) from rat spleen (10), and GM1b(NeuA,NeuAc) from pig brain. Axial-GM3 was prepared from GM3(NeuGc) of rat spleen by mild acid hydrolysis (11).

Preparation of Activated Thymocytes—Thymocytes were prepared from rat thymuses by teasing out through a stainless steel mesh into RPMI 1640 medium supplemented with 12 mM HEPES, 5 x 10-5 M 2-mercaptoethanol, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (complete medium) and washed once. The cells were cultured at a density of 3 x 106/ml in complete medium containing TPA (10 ng/ml), A23187 (60 ng/ml), and supplementary rat IL-2 (2

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1 The abbreviations used are: TCR, T cell antigen receptor; TPA, 12-O-tetradecanoylphorbol-13-acetate; ConA, concanavalin A; IL-2, interleukin-2; PBS, phosphate-buffered saline; NeuGc, N-glycolyneuraminic acid. The abbreviations for gangliosides are: GD1b, sialylol-2-5Galβ1-3GalNAcpβ1-4Galβ1-4Glcβ1-1ceramide; GD1b, sialylol-2-8sialylol-2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1ceramide; GD1b, sialylol-2-3Galβ1-3-sialylol-2-6GalNAcβ1-4Galβ1-4Glcβ1-1ceramide. Other gangliosides are designated according to Svennerholm (26). Species of the sialic acids were indicated in parentheses behind the abbreviations.
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units/ml or with ConA (8 µg/ml) at 37°C in humidified 5% CO₂. After incubation for the indicated period, the activated and proliferating cells were harvested, washed three times with PBS, and subjected to investigation of their gangliosides.

Preparation of Gangliosides—Total glycolipids were extracted from cells and partitioned by the method of Svennerholm and Fredman (12). The crude glycolipids obtained in the upper phase were treated with 0.5 N NaOH in methanol at 40°C for 1 h, dialyzed, and evaporated. An aliquot was used for two-dimensional TLC. The other portion was fractionated by DEAE-Sephadex column chromatography (13). After neutral lipids had been eluted with 10 volumes of chloroform/methanol/water (3:7:1), gangliosides were separated into mono-α, di-, tri-, and tetrasialogangliosides by stepwise elution with 10 volumes each of chloroform/methanol (3:7) containing a 0.1 volume of 0.2, 0.5, or 2.0 mM aqueous ammonium acetate.

Isolation of the Activation-associated Ganglioside (GX)—The activation-associated ganglioside, tentatively named GX, was isolated from the disialogangliosides of activated thymocytes stimulated with TPA and A23187 by preparative TLC using a solvent system of 1-propanol, 28% NH₄OH, water (75:5:25). GX recovered from the plates was purified with a Sep-Pak C₁₈ cartridge (14).

Compositional Analysis and Methylation Analysis—Ganglioside-bound sialic acids were quantified by the thiobarbituric acid method using NeuGc as a standard (15). Carbohydrate and fatty acid compositions were analyzed as described previously (10, 16). Methylation analyses were performed according to the previous method (8).

Sialidase Hydrolysis Followed by Detection with Cholera Toxin B Subunit on TLC—Gangliosides were hydrolyzed with C. perfringens sialidase as described previously (10). After the products had been chromatographed on a TLC aluminum sheet, the sheet was divided into two pieces, and one of the pieces was detected with 20% sulfuric acid. The other piece of the sheet was immersed in 0.5% polyvinyl sulfate. After washing three times, the peroxidase activity was detected with 4-chloro-1-naphthol/H₂O₂ solution (17).

NMR Spectroscopy—Proton NMR spectra were obtained with a JEOL JNM-GX400 NMR spectrometer as described previously (10, 16).

RESULTS

Alteration in the Amount of Gangliosides—When the thymocytes were cultured with TPA and A23187 as stimulants, and with supplementary IL-2 to ensure growth, the cells proliferated efficiently as shown in Fig. 1. Blast transformation was observed in all cultures at 24 h onward after activation when measured at 24-h intervals (data not shown). In the activated thymocytes, the amount of gangliosides per cell was demonstrated to be a disialoganglioside, as mentioned below, the presence of GX in the ConA-activated thymocytes was verified by TLC of the disialogangliosides obtained by DEAE-Sephadex column chromatography (data not shown).

Characterization of the Activation-associated Ganglioside GX—On DEAE-Sephadex column chromatography, ganglioside GX, which increased markedly in the thymocytes activated with TPA and A23187, was obtained in the disialoganglioside fraction. GX was isolated from the other disialogangliosides by preparative TLC. The TLC profile of the isolated GX is shown in Fig. 3. GX showed a similar mobility to that of G₁₀₂₆₄(NeuAc,NeuAcΔc) in a neutral solvent system (Fig. 3A) and migrated more slowly than G₁₀₂₆₄(NeuGc,NeuGc) in a solvent system containing ammonium hydroxide (Fig. 3B).

Compositional analyses showed that GX contained Gal, Glc, GalNAc, and N-glycolyneuraminic acid in a molar ratio of 2:0.1:1:0.1:1.8. The fatty acid constituents were C₁₆:0 (29%), C₁₈:0 (30%), C₂₀:0 (18%), C₂₂:0 (10%), and C₂₄:0 (8%)

As shown in Fig. 4, sialidase hydrolysis of GX yielded a ganglioside with the same RF value as G₃₅₆. The hydrolysis product was identified as G₃₅₆ by detection with cholera toxin B subunit (Fig. 4B).

Methylation analysis of GX on hexose and hexosamine gave the same derivatives as those from G₃₅₆, that is 2,3,4,6-tetra-O-Me-Gal, 2,3,6-tri-O-Me-Glc, 2,6-di-O-Me-Gal, and 4,6-di-O-Me-GalNAc.

Fig. 2. Two-dimensional TLC profiles of gangliosides from thymocytes before and after cell activation. The gangliosides from resting thymocytes (A, 1.2 x 10⁶ cells), thymocytes activated with TPA and A23187 for 48 h (B, 1.6 x 10⁶ cells), and thymocytes activated with ConA for 72 h (C, 1.3 x 10⁶ cells) were applied to each plate and developed with chloroform, methanol, 0.2% CaCl₂ (80:40:9) in the first dimension (to the left) and with 1-propanol, 28% NH₄OH, water (75:5:25) in the second dimension (upward). The gangliosides were visualized with resorcinol reagent (26). N, neutral glycolipid.

Fig. 1. Proliferation of thymocytes stimulated with a combination of TPA and A23187, or with ConA, and changes in the amount of gangliosides per cell.
From the results of compositional analysis, sialidase hydrolysis, and methylation analysis, GX was suggested to be Glb(Neu&Neu&), were found to appear in large amounts. The signal at 4.85 ppm (J = 7.0 Hz) in Glb(Neu&Neu&) was assigned to the PGalNAcH1 and assumed to show an upfield shift compared with that of PGalH1 in GDlb(NeuAc,NeuAc) (4.94 ppm, J = 9.2 Hz) due to the difference in sialic acid species. Though the signal of PGlcH1 in GX overlapped other signals, further information could not be obtained because of an insufficient amount of the sample. Signals of protons on the C4 and C5 of sphingosine were also detected around 5.35 and 5.55 ppm.

On the basis of the above results, GX was concluded to be Glb(Neu&Neu&), were found to appear in large amounts. The signal at 4.85 ppm (J = 7.0 Hz) in Glb(Neu&Neu&), were assigned to the PGalNAcH1 and assumed to show an upfield shift compared with that of PGalH1 in GDlb(NeuAc,NeuAc) (4.94 ppm, J = 9.2 Hz) due to the difference in sialic acid species. Though the signal of PGlcH1 in GX overlapped other signals, further information could not be obtained because of an insufficient amount of the sample. Signals of protons on the C4 and C5 of sphingosine were also detected around 5.35 and 5.55 ppm.

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asialo-G_{M1} and then G_{M1B}, which is a common pathway for the synthesis of G_{D1c}. More recently, Nakamura et al. (24) reported the occurrence of G_{D1c} as the major disialoganglioside in WHT/Ht mouse thymocytes. Moreover, they tentatively identified the other major disialoganglioside in the thymocytes as the rare species of G_{D1b}, G_{D1b}(NeuGc,NeuGc), by sialidase hydrolysis followed by TLC immunostaining (24). These reports, taken together with our present study and a previous report on G_{D1c} in rat thymocytes (8), suggest that rats and mice have similar pathways of ganglioside synthesis in T lineage lymphoid cells, at least partly. Data on the structures of gangliosides in T lineage lymphocytes obtained from rats and mice may be mutually suggestive, although still fragmentary with regard to the changes occurring during cell maturation and cell activation with various mitogens.

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