Interleukin-3-Associated Expression of Gangliosides in Mouse Myelogenous Leukemia NFS60 Cells Introduced with Interleukin-3 Gene: Expression of Ganglioside GD1a and Key Involvement of CMP-NeuAc:Lactosylceramide α2→3-Sialyltransferase in GD1a Expression†

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Received September 19, 1994; Revised Manuscript Received February 27, 1995⊗

ABSTRACT: Murine interleukin-3 (IL-3)-associated expression of gangliosides has been investigated using a gene transfection technique. A murine IL-3 cDNA was introduced into the parental NFS60-I7 cells that was exclusively dependent on IL-3. We analyzed the glycosphingolipids from the parental cells and the transfected cells by fast atom bombardment mass spectrometry analyses and/or immunostaining techniques using specific antibodies. Two major gangliosides, IV^{3}NeuAc-GgOse$_4$Cer (GM1b) and IV^3-NeuAc,III^{6}NeuAc-GgOse$_4$Cer (GD1α), were expressed, in the parental cells. By contrast, in the IL-3 gene-transfected cells, a ganglioside IV^{3}NeuAc,II^{3}NeuAc-GgOse$_4$Cer (GD1a) was strikingly expressed, in addition to GM1b and GD1α that were already present in the parental cells. In spite of various IL-3-secreting capabilities, all transfectants investigated have exhibited the same ganglioside patterns and expressed GD1a. Furthermore, the appearance of GD1a was a consequence of the up-regulation of a single glycosyltransferase, CMP-NeuAc:lactosylceramide α2→3-sialyltransferase (GM3 synthase). Activities of the other downstream glycosyltransferases that were involved in GD1a synthesis were not significantly different between the parental and the transfected cells. According to these data, the progression of tumor stage by the acquisition of autonomous cell growth ability after IL-3 gene transfection resulted in dramatic changes in cell surface gangliosides and their biosynthetic pathways. GD1a could be considered as an IL-3-associated ganglioside and was expressed in a tight connection with a single glycosyltransferase (GM3 synthase) up-regulation and with IL-3 expression in murine myelogenous leukemia cells.

Glycosphingolipid (GSL)[1] biosynthesis has been demonstrated to shift from one pathway to another during cellular events, including oncogenesis and cell differentiation (van Echten & Sandhoff, 1993). The cell biological significance of the shift and the resulting glycolipid expression are not necessarily clear in all cases. However, regulation of cell differentiation, cell growth, and morphogenesis by the expressed gangliosides has been reported in several cell systems (Saito, 1989, 1993; Nojiri et al., 1986, 1988; Nakamura et al., 1992; Hakomori, 1990). Moreover, GSLs have been demonstrated to bind toxins, bacteria, and viruses (Karlsson, 1989), and to be cell type-specific adhesion ligands for selectins (Phillips et al., 1990; Needham & Schnaar, 1993). In some cases, the shifts of GSL biosynthesis during cell differentiation, oncogenesis, and tissue development have been reported as a consequence of the key regulatory action of the most upstream glycosyltransferases, including the rate-limiting β1→3-*N*-acetylglucosaminyltransferase in neolacto and/or lacto series glycosphingolipids (Nakamura et al., 1992; Holmes et al., 1987; Chou & Jungalwala, 1993). However, the regulation of total metabolic flow in GSL biosynthesis in the other cell systems has not been fully elucidated.

Growth factor receptors are divided into several families according to their structures. They are the tyrosine kinase

† Supported in part by Grant-in-Aid for Scientific Research on Priority Areas No. 05274106 from the Ministry of Education, Science and Culture, Japan.

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1995.

[1] Abbreviations: GSL(s), glycosphingolipid(s); TNF, tumor necrosis factor; IFN, interferon; IL-1, interleukin-1; IL-2, interleukin-2; IL-3, interleukin-3; rIL-3, recombinant interleukin-3; IL-4, interleukin-4; IL-5, interleukin-5; IL-6, interleukin-6; IL-7, interleukin-7; IL-8, interleukin-8; CSF, colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; EPO, erythropoietin; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; MoAb(s), monoclonal antibody (or antibodies); FCS, fetal calf serum; PWM-SCCM, pokeweed mitogen-stimulated spleen cell conditioned medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DEAE, (diethylamino)ethyl; TLC, thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; HPLC, high-performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; glycosphingolipids are designated according to the recommendations of the Nomenclature Committee of the IUPAC (Recommendations of IUPAC-IUB Commission on Biochemical Nomenclature, 1977), and gangliosides are designated as described (Svennerholm, 1964): LacCer, Galβ1→4Glcβ1→Cer; GgOse$_4$Cer or GA1, Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer; II^{3}NeuAc-GgOse$_3$Cer or GM2, GalNAcβ1→4-(NeuAcα2→3)Galβ1→4Glcβ1→Cer; II^{3}NeuAc-GgOse$_4$Cer or GM1a, Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→Cer; IV^{3}NeuAc-GgOse$_4$Cer or GM1b, NeuAcα2→3Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer; IV^{3}NeuAc,II^{3}NeuAc-GgOse$_4$Cer or GD1a, NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→Cer; IV^{3}NeuAc,-III^{6}NeuAc-GgOse$_4$Cer or GD1α, NeuAcα2→3Galβ1→3(NeuAcα2→6)-GalNAcβ1→4Galβ1→ 4Glcβ1→Cer; IV^3(NeuAc)$_2$-GgOse$_4$Cer or GD1c, NeuAcα2→ 8NeuAcα2→3Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer; IV^{3}NeuAc,II^3(NeuAc)$_2$-GgOse$_4$Cer or GT1b, NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→3)Galβ1→4Glcβ1→Cer; GM3 synthase, CMP-NeuAc:LacCer α2→3-sialyltransferase; GM2 synthase, UDP-GalNAc:GM3 β1→4-*N*-acetylgalactosaminyltransferase; GM1a synthase, UDP-Gal:GM2 β1→3-galactosyltransferase; GD1a synthase, CMP-NeuAc: GM1a α2→3-sialyltransferase.

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receptor family, the tumor necrosis factor (TNF) receptor family, the interferon (IFN) receptor family, the hematopoietic receptor family, and others including receptors of interleukin-1 (IL-1), interleukin-2 (IL-2, α-subunit), interleukin-8 (IL-8), and activin. It has been well investigated and reported that gangliosides and their related compounds are associated with the transmembrane signaling of tyrosine kinase receptor family members: platelet-derived growth factor (PDGF), epidermal cell growth factor (EGF), and insulin receptors (Bremer et al., 1984, 1986; Hanai et al., 1988; Nojiri et al., 1991). By contrast, similar association of glycosphingolipids with signal transduction of the other families of cytokine receptors has not been investigated, with the rare exception of ganglioside GM1a with IL-2 receptor (Sharom et al., 1991). Moreover, even hematopoietic factor-associated glycosphingolipids have not been well characterized for the receptor family members, including interleukin-3 (IL-3), interleukin-5 (IL-5), interleukin-6 (IL-6), granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4), interleukin-7 (IL-7), granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), and so on. Among them, IL-3 is known as a multi-CSF (colony-stimulating factor), supports growth and differentiation of hematopoietic stem cells, and activates hematopoietic cell functions at the terminal differentiation stage. Further, receptors of IL-3, GM-CSF, and IL-5 share the same β-subunit, and the signaling through them has also been thought to share common pathways. Thus, it is of great interest to select an IL-3 gene as a model for analyzing hematopoietic factor-associated glycolipids.

In previous studies, murine myeloid leukemia cell line NFS60 was demonstrated to be multifactor-dependent (Holms et al., 1985) and to exhibit the ability to differentiate into neutrophils and macrophages in the presence of IL-3 and GM-CSF and into erythroids in the presence of EPO (Hara et al., 1988). In the present study, we have isolated a subclone NFS60-I7 exclusively responsive to IL-3 and established IL-3-independent clones from the IL-3-dependent NFS60-I7 by introduction of a murine IL-3 gene. We report here the comparison of the gangliosides from the parental NFS60-I7 cells and the transfected cells and analyses of a hematopoietic receptor family member (IL-3)-associated ganglioside GD1a. Furthermore, we report that ganglioside biosynthesis in NFS60 cells shifted from the "asialo" pathway alone to the "asialo" plus so-called "a" pathway after the acquisition of autonomous cell growth ability by IL-3 gene transfection and that this shift was due to key regulatory activation of CMP-NeuAc:lactosylceramide α2→3-sialyltransferase (GM3 synthase).

MATERIALS AND METHODS

Materials. The plasmid pcD-MCGF, a pBR322 derivative containing murine IL-3 cDNA (Yokota et al., 1984), was kindly provided by Dr. K. Arai (Institute of Medical Science, Tokyo University). The eukaryotic expression vector pCR3 containing the neomycin-resistant gene was obtained from Invitrogen (San Diego, CA). Restriction endonucleases were obtained from Nippon Gene (Tokyo) and TAKARA (Kyoto, Japan). A murine recombinant IL-3 (rIL-3) was from Genzyme Corporation (Cambridge, MA). Rabbit anti-mouse IL-3 antiserum was kindly provided by Dr. Sudo (Toray Basic Research Institute, Fujisawa, Japan). Geneticin (G418 sulfate) was from Gibco (Grand Island, NY). Gangliosides GD1a, GT1b, and GM3 (bovine brain) were purchased from BIOSYNTH AG (Staad, Switzerland). Lactosylceramide, GM2, and GM1a were obtained from Sigma (St. Louis, MO). Bovine brain gangliosides GM1b and GD1α were generous gifts from Dr. Y. Hirabayashi (RIKEN, Wako, Japan) (Hirabayashi et al., 1990). Monoclonal and polyclonal antibodies used in this study are listed and summarized in Table 1. A monoclonal antibody (MoAb) GMR17 (mouse IgM), which is most reactive with GD1a and moderately reactive with GM1b and GT1b, was kindly provided by Dr. T. Tai (Tokyo Metropolitan Institute of Medical Science, Tokyo) (Kotani et al., 1992). MoAbs NA-6 (mouse IgM) and KA-17 (mouse IgM), which react specifically with GM1b and GD1α, respectively, were kindly supplied by Dr. Y. Hirabayashi (Hirabayashi et al., 1990). Polyclonal antiasialo-GM1 and anti-GM1a antibodies were purchased from Iatron Laboratories, Inc. (Tokyo). Rabbit anti-mouse IgM antibody was from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). High-performance thin-layer chromatography (HPTLC) glass and aluminum plates were obtained from Merck (Darmstadt, Germany). All other reagents were of the highest grade commercially available.

Table 1: Monoclonal and Polyclonal Antibodies Used in This Study

antibody	specificity	footnote
NA-6	Galβ1→3GalNAcβ1→4Galβ1→ 3 ↑ NeuAcα2	*a*
KA-17	Galβ1→3GalNAcβ1→ 3 6 ↑ ↑ NeuAcα2 NeuAcα2	*b*
GMR-17	Galβ1→3GalNAcβ1→ 3 6 ↑ ↑ NeuAcα2 R[c]	*d*
GA-002	Galβ1→3GalNAcβ1→4Galβ1→	*e*
GA-011	Galβ1→3GalNAcβ1→4Galβ1→ 3 ↑ NeuAcα2	*e*

[a] NA-6 reacts with only GM1b and does not react with GD1a, GD1α, or GT1b (Y. Hirabayashi et al., unpublished data). [b] Hirabayashi et al., 1990. [c] R = OH or NeuAcα2→. GMR-17 was first reported to react only with GD1a, GM1b, and GT1b, and the reactivity with GD1α had not been tested at the time of publication (Kotani et al., 1992). In the present study, however, this MoAb was proved to react with GD1α, as well as with GD1a and GM1b, on an HPTLC plate by the immunostaining method (Magnani et al., 1980). [d] Kotani et al., 1992. [e] Purchased from Iatron Laboratories, Inc. (Tokyo, Japan).

Cells and Cell Culture. A murine myelogenous leukemia cell line NFS60 (Holms et al., 1985) was generously supplied by Dr. J. N. Ihle (Saint Jude Children's Hospital, Memphis, TN). The cells were dependent on several hematopoietic factors, such as IL-3, GM-CSF, G-CSF, and EPO, and maintained as described (Hara et al., 1988). Cells that were exclusively dependent on exogenous IL-3 were used for the transfection of IL-3 cDNA as the parental cells. The cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCCM; final concentration of 4−8%). In the practical

experiments, 100 units/mL murine rIL-3 was used unless otherwise indicated.

Transfection of IL-3 Gene pcD-MCGF. The pcD-MCGF was linearized with restriction endonuclease *Cla*I. The plasmid DNA was then transfected into the IL-3-dependent parental cells according to an electroporation technique using a Gene Pulser (Nippon BioRad, Tokyo) as described previously (Suda et al., 1988). After transfection, the cells were cultured in RPMI-1640 containing 10% FCS and 100 units/mL rIL-3 for 48 h. IL-3-independent cells were then isolated as colonies by culturing the cells in methylcellulose semisolid medium in the absence of rIL-3 for 14−21 days. Several IL-3-independent sublines were subcloned and established. To examine the effect of exogenous IL-3, both parental and transfected cells were cultured at various initial concentrations in the presence or absence of 100 units/mL rIL-3.

Construction and Transfection of pCR3-MCGF. Because pcD-MCGF had no suitable restriction sites, full-length murine IL-3 cDNA was amplified by polymerase chain reaction (PCR) from the plasmid using primers 5′-ATT-CTG-CAG-GAT-CCC-TTG-GAG-GAC-CAG-AA-3′ (sense) and 5′-ACC-GAG-CTC-GAA-TTC-TGG-TAT-CTT-TAT-AGT-C-3′ (antisense). The amplified fragment was subcloned into pCR3 vector, and a clone was isolated and designated as pCR3-MCGF-#4. The sequence of the pCR3-MCGF-#4 insert was analyzed by the dideoxynucleotide chain termination method (Sanger et al., 1977), and the deduced amino acid sequence was confirmed to be identical to that of pcD-MCGF. The plasmid was linearized with *Sca*I and transfected into NFS60-I7 cells as described earlier. The cells were selected in the presence of G418 (200 μg/mL) and 100 units/mL rIL-3 in the liquid growth medium. After 3−4 weeks, the polyclonal transfected cell lines NFS60-#4W1 and NFS60-#4W2 were established to be independent on exogenous IL-3.

Southern Blot and RNA Blot Analyses. High molecular weight DNA was prepared from parental cells and IL-3 gene transfectants and digested with restriction endonucleases *Pst*I and *Hin*dIII (Nippon Gene, Tokyo). Southern blot hybridization was performed with a ^{32}P-labeled 0.6 kb *Pst*I/*Nco*I fragment of the murine IL-3-specific cDNA according to standard methods (Mason & Williams, 1985). Total cellular RNA was extracted from NFS60-I7 and NFS60-H7 cells using the guanidium/CsCl method; RNA blot analysis was carried out as described in Sambrook et al. (1989) using the ^{32}P-labeled 0.6 kb IL-3-specific cDNA fragment.

Biological Assay for IL-3 Activity. IL-3 activity in conditioned media was measured according to the method of colorimetric MTT assay using another IL-3-dependent FDC-P2 cell proliferation (Ihle et al., 1985). The conditioned media were collected at a cell density of 2×10^6 cells/mL. FDC-P2 cells were incubated for 24 h at 2×10^5 cells/mL in 100 μL of RPMI-1640 medium containing 10% FCS in the presence of 10% conditioned medium.

Analyses of Gangliosides. Cells for glycosphingolipid analyses were harvested at the mid-logarithmic or late-logarithmic growth stage and stored at −80 °C until use. We compared ganglioside patterns from the parental and IL-3-transfected cells when the harvest times were identical to each other. For glycolipid analysis, the parental NFS60-I7 cells were cultured in the presence of 8% PWM-SCCM unless otherwise stated. Glycosphingolipids were prepared by the combination of chloroform/methanol extraction and DEAE-Sephadex A-25 column chromatography (Yu & Ledeen, 1972). After mild alkaline treatment, dialysis, and lyophilization, ganglioside fractions were separated on HPTLC plates with a solvent system of chloroform/methanol/0.2% $CaCl_2$ (50:50:10, v/v/v). To purify each component, the fractions were further separated by high-performance liquid chromatography (HPLC) using a DEAE-Iatrobeads column (6RSP-8010, Iatron Laboratories, Inc., Tokyo) and then an Aquasil column (Senshu Kagaku, Tsukuba, Japan). The patterns of ganglioside fractions from the parental and transfected cells were also compared by using cells metabolically labeled with [^{14}C]Gal, followed by extraction, purification, separation by HPTLC, and autoradiography (Sonderfeld et al., 1985). Preparative HPTLC was conducted as described previously (Ebel et al., 1992). Fast atom bombardment mass spectrometry (FAB-MS) was conducted on a JEOL SX-102A mass spectrometer equipped with a DA-7000 datalizer. Gangliosides were analyzed by negative ion FAB-MS in triethanolamine as a matrix.

TLC Immunostaining. Immunostaining was performed by a modification of the method described by Magnani et al. (1980). Briefly, the gangliosides were chromatographed on HPTLC aluminum sheets, reacted successively with one MoAb, a second rabbit anti-mouse IgM antibody, and an ^{125}I-labeled protein A solution (Amersham), and then autoradiographed. The information on sialidase-sensitive structures of the gangliosides was sought through a similar TLC immunostaining method combined with sialidase pretreatment on the plate. Namely, the gangliosides separated on HPTLC plates were treated with sialidase (*Clostridium perfringens*) on the plate and then detected with antiasialo-GM1 or anti-GM1a antibodies.

Activities of Glycosyltransferases in NFS60-I7 and IL-3-Transfected Cells. For glycosyltransferase assay, the parental NFS60-I7 cells were cultured in the presence of 8% PWM-SCCM. The total membranous fractions from the IL-3-dependent parental cells and the IL-3-transfected cells were prepared and stored until use, and glycosyltransferase assays were essentially carried out by the method described in our earlier work (Nakamura et al., 1992). The conditions for GM3 synthase in the parental and transfected cells were as described previously (Nakamura et al., 1991). The activities of the other glycosyltransferases, GM2 synthase, GM1a synthase, and GD1a synthase, were assayed according to conditions on the basis of earlier works with slight modifications (Yanagisawa et al., 1987; Basu et al., 1965; Busam & Decker, 1986). The conditions used for all glycosyltransferase assays in this study are summarized in Table 2.

RESULTS

Acquisition of Autonomous Growth by IL-3 Gene Transfection. A subline that was exclusively dependent on exogenous IL-3 was established from the original NFS60 cells and designated as NFS60-I7. Following IL-3 gene transfection into the NFS60-I7 cells using the electroporation method, 10 sublines showing autonomous growth without exogenous IL-3 were established from the parental cells. The transfected cell lines extracellularly secreted various amounts of IL-3 into conditioned media (Table 3). In the subsequent experiments, we mainly studied a clone designated NFS60-H7 that extracellularly produced the highest IL-3 activity

Table 2: Assay Conditions for Ganglioside Glycosyltransferases in NFS60 Cells

synthase	GM3	GD1a	GM2	GM1a
acceptor μM	LacCer (200)	GM1a (300)	GM3 (50)	GM2 (200)
donor (nmol)	CMP-[^{14}C]NeuAc (5.0)	CMP-[^{14}C]NeuAc (5.0)	UDP-[^{14}C]GalNAc (1.0)	UDP-[^{14}C]Gal (10.0)
metal ion (μM)	$MnCl_2$ (20)	$MnCl_2$ (20)	$NiCl_2$(10)	$MnCl_2$ (10)
detergent (μg)	Triton CF54 (75)	Triton CF54 (75)	Triton X-100 (125)	sodium deoxycholate (2.5)
cacodylate buffer (μmol)	3.75	3.75	12.5	3.75
pH	6.2	7.3	6.8	7.3
CDP-cholin (nmol)			62.5	125
protein (μg)	200	200	84	170
reaction volume (μL)	25	25	25	25
reaction time (h)	2−3	2	2	2

Table 3: IL-3 Activity of the IL-3 Gene-Transfected Cell Lines

clone	IL-3 Activity[a] extracellular (units/mL)	IL-3 Activity[a] intracellular (U/107 cells)
high producing type		
NFS60-H7	280	55
NFS60-H4	180	ND[b]
intermediate type		
NFS60-M3	90	ND[b]
low producing type		
NFS60-L5	<10	12
NFS60-L8	<10	ND[b]

[a] IL-3 activity was measured according to the colorimetric MTT assay using FDC-P2 cells as described in the text. FDC-P2 cells (2×10^4) were incubated for 24 h in 100 μL of RPMI-1640 medium containing 10% FCS in the presence of 10% conditioned medium (extracellular IL-3) or cellular extract (intracellular IL-3) of each IL-3 gene transfectant. [b] ND, not determined.

and clone NFS60-L5, which was one of the lowest IL-3 secretors into the conditioned media among the established sublines. As a first step, we characterized the transfected NFS60-H7 and NFS60-L5 cells by Southern and RNA blot analyses. Southern blot analysis showed that a band in addition to those found in the parental cells was exhibited in NFS60-H7 and NFS60-L5 cells (data not shown). RNA blot analysis revealed that the parental NFS60-I7 cells never expressed the IL-3 message (Figure 1, lane a). In contrast, IL-3 gene-transfected NFS60-L5 cells expressed low but significant amounts of the message (lane b). Moreover, NFS60-H7 cells expressed significant and high amounts of the transcripts (lane c). Consequently, it could be thought that exogenous IL-3 gene was successfully integrated into chromosomes and expressed constitutively. Furthermore, the extracellular IL-3 producibility was dependent upon the amount of message of the integrated IL-3 gene.

Autonomous Cell Growth Potential and IL-3 Producibility of the IL-3 Gene Transfectants. The 10 transfected cell lines were divided into three groups according to the IL-3-producing capabilities to the conditioned media. As summarized in Table 3 (left column), they were high-producing cells secreting IL-3 at a range from 100 to 280 units/mL, an intermediate type of cells ranging from 10 to 100 units/mL, and low producers with less than 10 units/mL. In spite of their IL-3-producing capabilities, they showed no difference in growth rates in the culture medium with or without exogenous IL-3, as shown in Figure 2A.

Figure 3 showed the effect of anti-IL-3 antiserum on the growth of NFS60 cells. In the presence of 5 units/mL exogenous rIL-3, the parental NFS60-I7 cells exhibited good survival and proliferation for at least 24 h. When the antiserum was added to this NFS60-I7 cell culture at

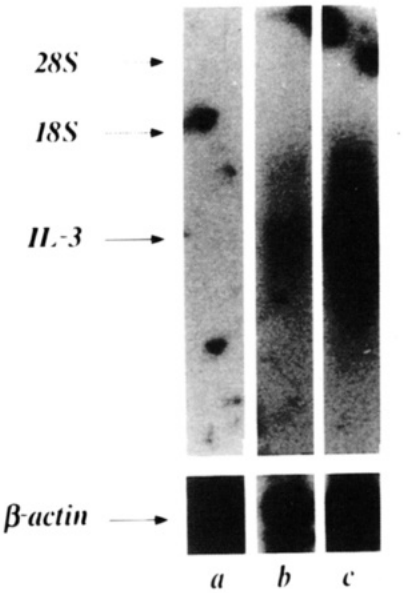

FIGURE 1: RNA blot analysis of the IL-3-dependent parental cells and the IL-3 gene-transfected cells. Total cellular RNA was extracted using the guanidium/CsCl method. Each 20 μg of total RNA from NFS60-I7 (lane a), NFS60-L5 (lane b), and NFS60-H7 (lane c) cells was electrophoresed in 0.75% agarose gel, blotted to nylon membrane Biodyne B (Nippon Genetics, Tokyo, Japan), hybridized with ^{32}P-labeled murine IL-3-specific cDNA *Nco*I/*Pst*I fragment, and detected with autoradiography.

concentrations of 1.0%, 2.0%, and 10%, the growth of the parental cells was remarkably suppressed to 65%, 33%, and 10% of the control, respectively. In contrast, this antiserum never inhibited the proliferation of NFS60-H7 and NFS60-L5 cells, even when it was added to these cultures at a concentration of 10%.

These data suggest that the autonomous growth capability of the IL-3 gene transfectants was not dependent upon extracellular IL-3 producibility. To further confirm this, we performed two additional experiments as follows. Firstly, the growth of the low IL-3 producing NFS60-L5 cells was examined at various cell concentrations ranging from 1×10^3 to 1×10^5 cells/mL in the presence or absence of exogenous IL-3. As shown in Figure 2B, the cells in any initial cell concentration proliferated at almost the same growth rate in spite of the presence or absence of exogenous IL-3. Secondly, the intracellular IL-3 activities were measured using NFS60-H7 and NFS60-L5 cells, as shown in Table 3 (right column). In contrast to the extracellular IL-3 activity, the intracellular activity did not show as much difference between the high and low IL-3 producing sublines. That is, NFS60-H7 cells exhibited intracellular IL-3 activity only about 5 times that in NFS60-L5 cells. These data suggest that, not the secreted IL-3, but the intracellular IL-3

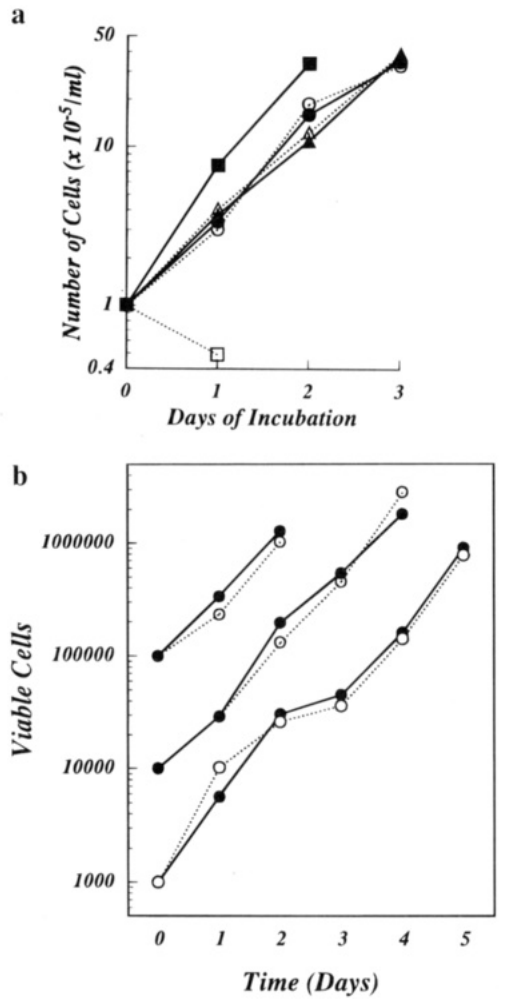

FIGURE 2: (A) Cell growth of the IL-3-dependent parental cells and the IL-3 gene-transfected cells. The parental NFS60-I7 cells (■, □), the transfectant NFS60-L5 cells (▲, △), and NFS60-H7 cells (●, ○) were cultured at an initial cell concentration of 1.0×10^5 viable cells/mL in RPMI-1640 medium containing 10% FCS in the presence (closed symbols with solid lines) or absence (open symbols with dotted lines) of 100 units/mL rIL-3. Cell growth was measured by determining viable cell concentrations at various given times. Values represent means of duplicate experiments. (B) Effect of exogenous IL-3 on cell proliferation of the IL-3 gene transfectant NFS60-L5, low IL-3 producing, cells. Cell growth was started at initial cell concentrations of 1×10^3, 1×10^4, or 1×10^5 viable cells/mL in RPMI-1640 medium containing 10% FCS in the presence (broken line) or absence (solid line) of 100 units/mL rIL-3, and viable cells were counted at various given times. Values represent means of duplicate experiments.

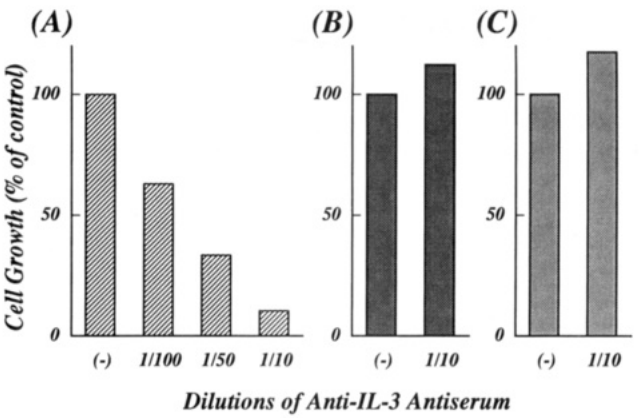

FIGURE 3: Effect of anti-IL-3 antiserum on cell growth of the parental NFS60-I7 cells and the IL-3 transfectants NFS60-H7 and NFS60-L5. The NFS60-I7 cells were cultured for 24 h in RPMI-1640 medium containing 10% FCS in the presence of 5 units/mL rIL-3 and varying dilutions (0−10%) of rabbit anti-IL-3 antiserum (A). The transfectant NFS60-H7 (B) and NFS60-L5 (C) cells were cultured for 24 h in the same culture medium containing 10% FCS in the presence or absence of 10% anti-IL-3 antiserum. Cell proliferation was determined according to the colorimetric MTT assay, as described in the text.

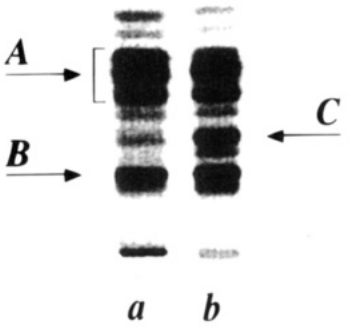

FIGURE 4: Autoradiography of metabolically labeled gangliosides from NFS60-I7 and NFS60-H7 harvested at the mid-logarithmic growth phase. Gangliosides from NFS60-I7 (lane a) and NFS60-H7 (lane b) cells labeled with [^{14}C]Gal were chromatographed in chloroform/methanol/0.2% aqueous $CaCl_2$ (50:40:10, v/v/v) and detected by autoradiography. Arrows A, B, and C indicate the positions of Gx1, Gx2, and GxN, respectively.

produced in the cells would be enough to show the autonomous cell growth ability of the IL-3 gene transfectants.

Parental and Transfected Cells Show Different Ganglioside Patterns. By using metabolically labeled or nonlabeled cells, gangliosides were isolated from the parental NFS60-I7 and the IL-3 gene-transfected NFS60-H7 cell lines. They were separated by HPTLC and detected by autoradiography or resorcinol hydrochloride staining, respectively. Analysis of metabolically labeled cells harvested at the mid-logarithmic growth phase showed that gangliosides from the parental cell line were composed of two major compounds (Figure 4, lane a). The two major components were designated as Gx1 and Gx2, the bands indicated by arrows A and B, respectively. In contrast, gangliosides from the transfected NFS60-H7 cells, which were harvested at the same growth stage, showed striking differences from those of the parental cells (Figure 4, lane b). Namely, NFS60-H7 cells exhibited another major ganglioside, designated GxN and indicated by arrow C in Figure 4, in addition to gangliosides Gx1 and Gx2 that were already expressed in the parental NFS60-I7 cells. The intensities of Gx1, Gx2, and the other minor components from the transfected cells were almost the same as those from the parental cells. However, only GxN intensity was quite different from that of NFS60-I7 cells. This suggested that the synthesis of GxN was highly activated in NFS60-H7 cells and closely associated with IL-3 gene transfection.

To obtain preliminary information about the structures of Gx1, Gx2, and GxN, gangliosides were subjected to HPLC, GLC, and sialidase treatment analysis on HPTLC, followed by immunostaining. By HPLC analysis using a DEAE-Iatrobeads column, Gx2 and GxN were fractionated into the disialo fraction, while Gx1 was eluted in the monosialo region (data not shown). Subsequently, sialic acid species of the ganglioside mixture from NFS60-I7 and NFS60-H7 were analyzed as trimethylsilylated derivatives by GLC. According to the chromatograms, no NeuGc was found in the fraction, whereas a significant peak was detected at the same retention time of NeuAc (data not shown). This

Table 4: Structures of Selected Gangliosides

GM1a	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer 3 ↑ NeuAcα2
GM1b	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer 3 ↑ NeuAcα2
GM1α	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer 6 ↑ NeuAcα2
GD1a	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer 3 3 ↑ ↑ NeuAcα2 NeuAcα2
GD1α	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer 3 6 ↑ ↑ NeuAcα2 NeuAcα2
GD1c	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer 3 ↑ NeuAcα2 8 ↑ NeuAcα2

suggested that all sialic acid species of the ganglioside mixtures were those of NeuAc types. Then, information on the sialic acid positions was obtained by neuraminidase (*C. perfringens*) treatment on the HPTLC plates, followed by staining with antibodies against asialogangliosides and GM1a. Neuraminidase from *C. perfringens* cleaves off sialic acid at positions III and IV of the $GgOse_4$ backbone (Table 4). Among three major components, only Gx1 and Gx2 were degraded to $GgOse_4Cer$ by *C. perfringens* neuraminidase treatment and then detected by a specific antibody against asialo-GM1 (data not shown). As Gx1 was fractionated into a monosialo fraction and proved to have an asialo-GM1 backbone, Gx1 could be IVNeuAc-$GgOse_4Cer$ (GM1b), a ganglioside first found in rat ascites hepatoma AH7974F (Matsumoto et al., 1981; Taki et al., 1986), or IIINeuAc-GgOse4Cer (GM1α)[2] (Table 4). Since Gx2 was fractionated into a disialo fraction and proved to have an asialo-GM1 backbone, Gx2 could be either IVNeuAc,IIINeuAc-$GgOse_4$-Cer (GD1α), a ganglioside also found in rat ascites hepatoma cells and murine lymphoma cells (Taki et al., 1986; Murayama et al., 1986), or IV$(NeuAc)_2$-$GgOse_4Cer$ (GD1c), a ganglioside reported by Bartoszewicz et al. (1986) (Table 4). In contrast, GxN treated with *C. perfringens* neuraminidase was detected not by an antiasialo-GM1 antibody but by a specific antibody against GM1a (data not shown). This means that GxN has a II^{3}NeuAc-$GgOse_4Cer$ (GM1a) backbone. As GxN was fractionated into a disialo fraction, GxN could be IVNeuAc,IINeuAc-$GgOse_4Cer$ (GD1a) (Table 4).

[2] Unpublished data: GM1α has been detected and characterized in another cell line (Y. Hirabayashi *et al.*).

Isolation and Characterization of Gx1, Gx2, and GxN. To further characterize Gx1, Gx2, and GxN, we purified them by HPLC using an Aquasil column or by preparative HPTLC, conducted FAB-MS analysis, and stained them with specific antibodies on HPTLC plates.

After sequential chromatographies, Gx1 was further separated into two components designated Gx1a and Gx1b. Subsequently, we analyzed Gx1a by negative FAB-MS to confirm the proposed structures. The $GgOse_4Cer$−specific sequence was represented by the fragment ions at *m/z* 648/646 (Cer^- 24:0/18 and 24:1/18), 810/808 ($HexCer^-$), 972/970 (Hex_2Cer^-), and 1175/1173 ($Hex_2HexNAcCer^-$) (Figure 5A). However, neither the fragment ion at *m/z* 1263 ($NeuAcHex_2Cer^-$) nor that at *m/z* 1466 ($NeuAcHex_2HexNAcCer^-$) was detected. The fragment ions were all accompanied by a series of 28 amu lower mass ions, representing another GM1b homologue with Cer 22:0/18. The molecular ions appeared as $[M - H]^-$ at *m/z* 1628, 1626, and 1600 for the GM1b homologues ($NeuAcHex_3HexNAcCer^-$) with Cer 24:0/18, 24:1/18, and 22:0/18, respectively (Figure 5A). In addition, Gx1a comigrated with the authentic GM1b on HPTLC (chloroform/methanol/0.2% $CaCl_2$, 50:50:10, v/v/v) (see Figure 6A). Furthermore, the terminal sugar sequence of Gx1 was analyzed by TLC immunostaining with a specific MoAb NA-6 against GM1b. The bands corresponding to Gx1a and Gx1b were strongly stained with NA-6 (Figure 6A). This indicates that both compounds have the same terminal NeuAcα2→3Galβ1→3GalNAc sequence without NeuAc substitution at the 6 position of GalNAc and at the 3 position of the second hexose from the reducing end. Therefore, Gx1 was determined to be GM1b.

Although Gx2 was purified by sequential chromatographies and preparative thin-layer chromatography, the negative and positive ion FAB-MS results were of poor quality due to the presence of impurities (data not shown). However, the molecular ions appeared as $[M - H]^-$ at *m/z* 1919 and 1917 for the $NeuAc_2Hex_3HexNAcCer^-$ sequence with Cer 24:0/18 and 24:1/18, respectively. In addition, the fragment ions appeared at *m/z* 808/810 ($HexCer^-$), 970/972 (Hex_2Cer^-), and 1626/1628 ($NeuAcHex_3HexNAcCer^-$). Although the ions at *m/z* 1261/1263 ($HeuAcHex_2Cer^-$) were not detected, the fragment ions at *m/z* 1464/1466 ($NeuAcHex_2HexNAcCer^-$) were found. Further, the nonreducing terminal sequence of Gx2 was revealed to be NeuAcα2→3Gal instead of NeuAcα2→8NeuAcα2→3Gal by TLC immunostaining with a MoAb GMR17 (see Figure 6B, lane d, band C). This suggested that Gx2 could be GD1α but not GD1c. Subsequently, the HPTLC mobility of Gx2 was analyzed using GD1α as an authentic standard. Although Gx2 showed almost the same mobility as GD1α, the Gx2 bands migrated just a little bit slower than those of the standard (see Figure 6C). However, Gx2 was strongly reactive with a specific MoAb KA-17 against GD1α, indicating that the terminal sugar sequence of Gx2 was NeuAcα2→3Galβ1→3(NeuAcα2→6)GalNAc (Figure 6C, lane f). According to these data, we concluded that Gx2 was GD1α.

GxN was also purified by sequential chromatographies and by preparative thin-layer chromatography and subjected to negative ion FAB-MS analysis. Not GD1α nor GD1c, but a GD1a-related sequence was represented by the fragment ions at *m/z* 808 ($HexCer^-$), 970/972 (Hex_2Cer^-), 1173/1175 ($Hex_2HexNAcCer^-$), 1261/1263 ($NeuAcHex_2Cer^-$), 1464/

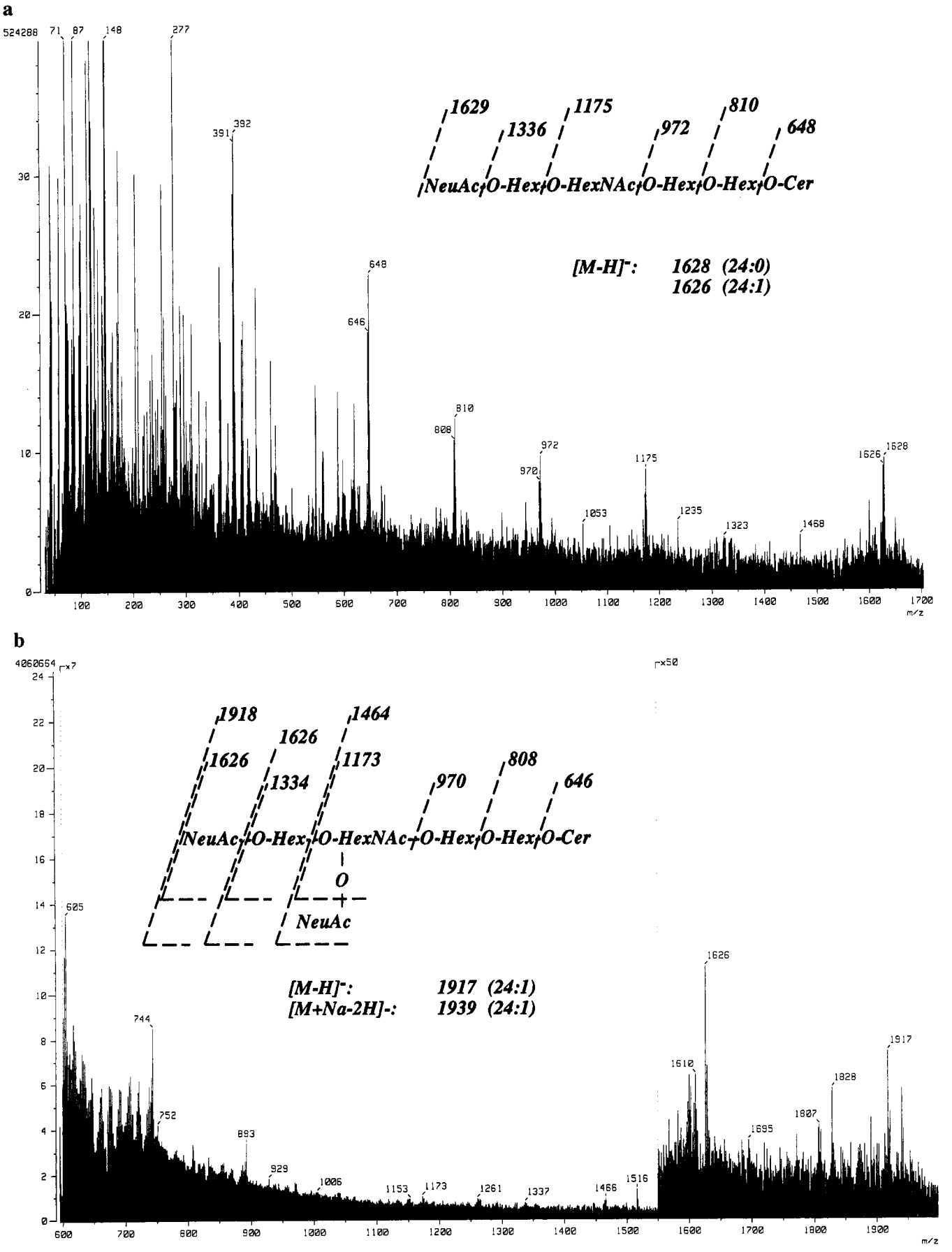

FIGURE 5: Negative fast atom bombardment mass spectra and fragmentation patterns of gangliosides from NFS60-H7 cells: (A) monosialoganglioside Gx1a; (B) disialoganglioside GxN.

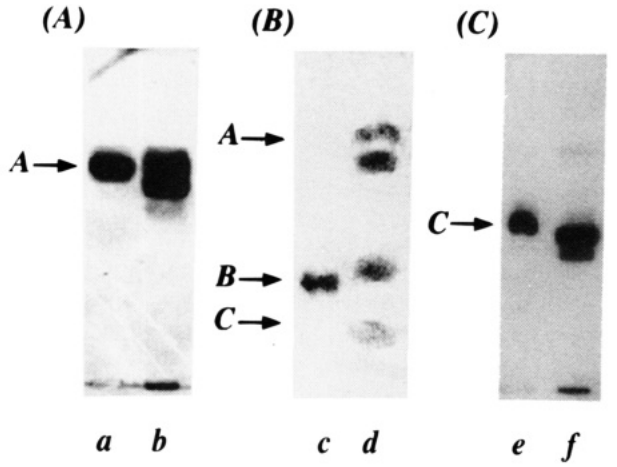

FIGURE 6: TLC immunostaining analyses of gangliosides from NFS60-H7 cells by specific monoclonal antibodies. After development, plates were coated with poly(isobutyl methacrylate) in *n*-hexane, and gangliosides were stained with MoAb NA-6 (A), MoAb GMR-17 (B), and MoAb KA-17 (C). Gangliosides were chromatographed in chloroform/methanol/0.2% aqueous $CaCl_2$ [50:45:10 in (A), 50:50:10 in (B), and 40:55:10 in (C), v/v/v]. Lanes a, c, and e: authentic ganglioside GM1b, GD1a, and GD1α, respectively. Lanes b, d, and f: 1 μg of gangliosides from NFS60-H7 cells. The positions of authentic gangliosides are indicated by arrows: A, GM1b; B, GD1a; C, GD1α. In panel B, only the authentic GD1a was developed in lane c and visualized by the immunostaining method.

1466 ($NeuAcHex_2HexNAcCer^-$), and 1626/1628 ($NeuAcHex_3HexNAcCer^-$) (Figure 5B). The molecular ions appeared as $[M - H]^-$ at *m/z* 1917 and as $[M + Na^+ - 2H]^-$ at *m/z* 1939 for the GD1a homologue with Cer 24:1/18 (Figure 5B). In addition, GxN almost comigrated with authentic GD1a on HPTLC (chloroform/methanol/0.2% $CaCl_2$, 50:50:10, v/v/v) (see Figure 6B), although the band migrated a little bit faster than that of the standard. Furthermore, the terminal sugar sequence of GxN was analyzed by TLC immunostaining with a MoAb GMR17. GxN was reactive with GMR17, indicating the compound has the terminal NeuAcα2→3Galβ1→3GalNAc sequence (Figure 6B, lane d). All of these data indicated that GxN was GD1a.

Expression of GD1a in NFS60 Cells Transfected with the Plasmid Containing IL-3 cDNA and the Neomycin-Resistant Gene. There is a possibility that the inquisition of the independence of growth on exogenous IL-3 and the expression of GD1a in the IL-3 gene transfectants were just coincidental findings somehow related to the cloning method. To exclude this possibility, we constructed plasmid pCR3-MCGF-#4, containing full-length IL-3 cDNA and the neomycin-resistant gene, transfected it into NFS60-I7 cells, and selected the gene-transfected cells in the presence of G418 and rIL-3. For a control, we transfected the same plasmid without IL-3 cDNA, designated pCR3-#19, into NFS60-I7 cells and selected the control cell line NFS60-#19C5 under the same conditions. While the control cell line NFS60-#19C5 was still dependent on exogenous IL-3, the polyclonal neomycin-resistant NFS60-#4W1 and NFS60-#4W2 cells were proved to acquire the autonomous cell growth potential without exogenous IL-3 (data not shown). As demonstrated in Figure 7A, NFS60-#4W1 and NFS60-#4W2 exhibited GD1a, in addition to GM1b and GD1α (lanes a and b, respectively). Since the NFS60-#19C5 cells did not express GD1a (data not shown), GD1a expression, as well as the acquisition of autonomous cell growth, was not just a coincidental finding related to the cloning method, but a consequence of IL-3 gene transfection to NFS60 parental cells.

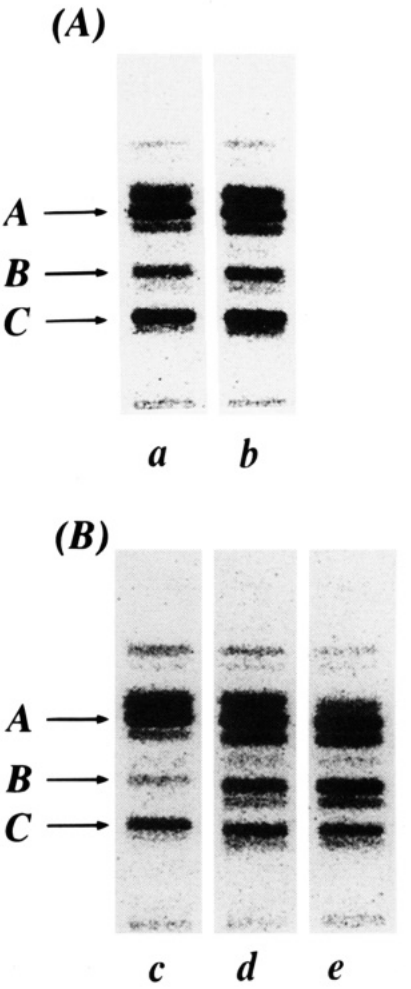

FIGURE 7: (A) Ganglioside analysis of NFS60-I7 cells transfected with the plasmid containing both IL-3 cDNA and neomycin-resistant gene. The ganglioside fractions were extracted and purified from the pCR3-MCGF-transfected NFS60-#4W1 and NFS60-#4W2 cell lines that were metabolically labeled with [^{14}C]Gal for 24 h. Lane a, NFS60-#4W1; lane b, NFS60-#4W2 cells. (B) Effect of a large amount of exogenous rIL-3 on GD1a synthesis in the parental NFS60-I7 cells. The cells were cultured for 72 h in the presence of PWM-SCCM or 300 units/mL rIL-3 and labeled with [^{14}C]Gal for 24 h in the presence of the same growth factor. The gangliosides were extracted, purified according to the method described in Sonderfeld et al. (1985), separated on HPTLC plates, and autoradiographed. Lane c, NFS60-I7 cells cultured with PWM-SCCM; lane d, NFS60-I7 with 300 units/mL rIL-3; lane e, NFS60-H7 cells without IL-3. The positions of GM1b, GD1a, and GD1α were indicated by arrows A, B, and C, respectively.

Expression of GD1a in the Other Transfectants with Various IL-3-Producing Capabilities and the Effect of Exogenous IL-3 on Ganglioside Expression in the Parental NFS60-I7 Cells. Although it was confirmed that GD1a was expressed in clone NFS60-H7, it should be verified whether GD1a was expressed in the other IL-3-transfected clones. In addition, it was of interest to elucidate the connection between the amount of GD1a expressed and the IL-3-secreting capability. To further test whether GD1a was expressed in the other clones, and whether IL-3-producing capabilities have any connection with GD1a expression, we compared the ganglioside pattern of the low IL-3 producing NFS60-L5 with that of the high producing NFS60-H7. As

Table 5: Quantitative Data of Gangliosides from NFS60-H7 and NFS60-L5

	NFS60-H7	NFS60-L5
total NeuAc[a]	2.62	2.43
gangliosides[b]		
Gx1	1.86	1.11
GxN	3.76	4.03
Gx2	3.25	2.83

[a] Values are expressed as micrograms of NeuAc per 1×10^7 cells. [b] Values are calculated from the total NeuAc and densitometric data by a CS-9000 chromatoscanner and expressed as micrograms of each ganglioside component per 1×10^7 cells.

summarized in Table 5, NFS60-L5 displayed almost the same level of GD1a as NFS60-H7 cells. Although the amount of each major component was slightly different in the two clones, they both expressed significant amounts of GD1a, in addition to GM1b and GD1α. Furthermore, all transfected cell lines producing various amounts of IL-3 showed the same ganglioside patterns as NFS60-H7 and exhibited GD1a as one of the three major components (data not shown). According to these data, GD1a was expressed in all IL-3-transfected NFS60 sublines in spite of their various IL-3-secreting capabilities to the extracellular medium. However, there seemed to be no relationship between IL-3-secreting capability and the amount of GD1a expression.

According to our results, not the secreted IL-3 but the intracellular IL-3 generated in the cells was thought to be critical for the autonomous cell growth ability of the IL-3 gene transfectants. Also, for ganglioside synthesis, the intracellular IL-3 produced in the cells would be enough to induce GD1a expression on the cell membrane. To further clarify whether or not the intracellular IL-3 generated by the transfected IL-3 gene should behave differently from exogenous IL-3 with respect to ganglioside biosynthesis, we conducted analyses of gangliosides by metabolic labeling with [^{14}C]galactose in the NFS60-I7 cells cultured in the presence of 300 units/mL rIL-3. As shown in Figure 7B, GD1a was detected at a significant concentration in NFS60-I7 cells stimulated by a large amount of rIL-3, although the percentage was lower than that of NFS60-H7 cells (17.7% and 27.6%, respectively). As the parental cells cultured with a low amount of IL-3 (i.e., 8% PWM-SCCM; concentration equivalent to about 5 units/mL rIL-3 as for cell growth-supporting capability) expressed only low levels of GD1a (5.6%), GD1a expression was caused by the exogenous IL-3 itself.

Relative Abundance of Each Ganglioside Component in NFS60 Cells. Gangliosides were extracted from cells harvested at the late-logarithmic growth phase, developed on HPTLC plates, and visualized by resorcinol hydrochloride spray followed by heating (Figure 8). It was true that GD1a was also present and detected from the metabolically labeled parental cells with [^{14}C]Gal (see Figure 4, lane a). However, the quantity of GD1a was very small because it could not be visualized by the resorcinol hydrochloride staining method, even if the ganglioside fraction from NFS60-I7 cells equivalent to total 5 μg of lipid-bound NeuAc was used (Figure 8, lane b). The data summarized in Table 5 are from the different HPTLC analyses of ganglioside patterns of the cells that were harvested at the late-logarithmic growth phase. As shown in Figure 8 and Table 5, the relative abundance of each ganglioside component is GD1a > GD1α > GM1b

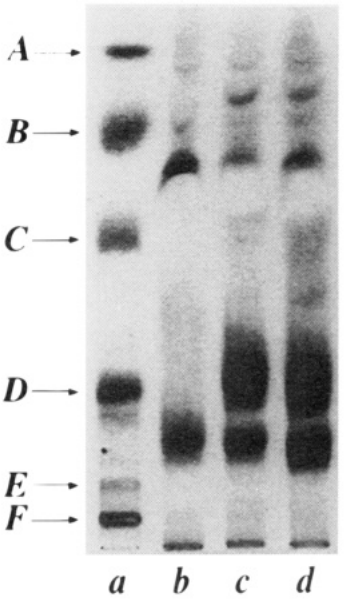

FIGURE 8: Ganglioside analysis from the parental and transfected cells harvested at the late-logarithmic growth phase. Gangliosides from NFS60-I7 (lane b), NFS60-L5 (lane c), and NFS60-H7 (lane d) cells were chromatographed in chloroform/methanol/0.5% aqueous $CaCl_2$ (50:50:10, v/v/v) and detected by resorcinol hydrochloride spray. The authentic gangliosides were developed in lane a; arrows: A GM3, B GM2, C GM1, D GD1a, E GD1b, and F GT1b. GM1b migrated between GM2 and GM1a, and the mobility of GD1α was between those of GD1a and GD1b.

in the IL-3-transfected cells. Similarly, the parental cells displayed more GD1α than GM1b, and their levels were almost the same as the transfected cells (Figure 8, lane b). In contrast when the cells were harvested at the mid-logarithmic growth stage, the GD1a content in the IL-3-transfected cells was somewhat low and GM1b contents in the parental and transfected cells were very high (Figure 4). Thus, the relative abundance in this case was GM1b > GD1α > GD1a in the transfected cells and GM1b > GD1α in the parental cells. However, the absence of GD1a from the parental cells and the appearance of GD1a in the IL-3-transfected cells were consistent in spite of the time of cell harvest.

Regulation of the Metabolic Flow of Ganglioside Biosynthesis in NFS60 Cells. The ganglioside phenotype of the parental IL-3-dependent NFS60 cells, NFS60-I7, was characterized by the expression of "asialo" pathway-related gangliosides (GM1b and GD1α). In addition, the so-called "a" pathway-related component GD1a was expressed in IL-3-independent transfectant, NFS60-H7. To analyze key factors that control the glycolipid biosynthesis system, we determined the ganglioside glycosyltransferase activity levels of each step of the pathway leading to the biosynthesis of "a" series gangliosides. The ganglioside synthetic activities in the parental NFS60-I7 cells and transfected subline NFS60-H7 were compared, as shown in Figure 9. GM2 synthase, GM1a synthase, and GD1a synthase did not show significant changes between two cell lines, that is, these syntheses were not influenced by IL-3 gene transfection. However, GM3 synthase was distinctively and remarkably up-regulated in the IL-3-transfected cells. The mean enzyme activities of GM3 synthase from the parental and transfected cells were 24.93 ± 5.12 ($n = 3$) and 93.39 ± 12.33 pmol/h/mg of protein ($n = 3$), respectively ($p < 0.001$). This GM3 synthase is the most upstream key enzyme for the synthesis

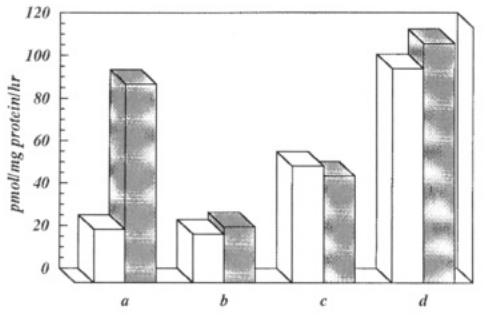

FIGURE 9: Activities of glycolipid glycosyltransferases from the parental NFS60-I7 and the IL-3-transfected NFS60-H7 cells. GM3, GM2, GM1a, and GD1a synthases were assayed as described in Materials and Methods. Open columns: activities from NFS60-I7 cells. Closed columns: activities from NFS60-H7 cells. Each pair of columns a, b, c, and d represent GM3, GM2, GM1a, and GD1a synthases, respectively. The results shown in this figure are from one set of determinations out of two independent assays using replicate cell cultures.

of "a" pathway gangliosides. The notable increase in the activities of this sialyltransferase resulted in ganglioside GD1a expression for this cell line and a shift in metabolic flow, i.e., from the "asialo" pathway alone to the "asialo" pathway plus "a" pathways. Ganglioside GD1a expression as a consequence of IL-3 transfection seems to be controlled by the activity of a single glycosyltransferase, GM3 synthase.

DISCUSSION

It has been reported that gangliosides and their related compounds are associated with transmembrane signaling of tyrosine kinase receptor family members. However, similar associations of glycosphingolipids with signal transduction of the other families of cytokine receptors have not been well investigated yet. Moreover, even hematopoietic factor-associated glycosphingolipids have not been analyzed and characterized. In the present study, we selected IL-3-dependent cell line NFS60-I7 and the IL-3 gene as a model for analyzing hematopoietic factor-associated glycolipids. By IL-3 cDNA transfection into NFS60-I7, the gene was presumably integrated into chromosomes and expressed constitutively, while the endogenous IL-3 gene remained unexpressed. The transfected cell lines acquired autonomous growth without exogenous IL-3 and extracellularly secreted various amounts of IL-3.

By analyzing and comparing gangliosides of IL-3 gene-transfected NFS60-H7 cells with the IL-3-dependent parental NFS60-I7 cells, we found an IL-3-associated ganglioside pattern in NFS60-H7. In NFS60-I7 cells, the major components were Gx1 and Gx2. However, the third major component, GxN, was significantly expressed in NFS60-H7 cells. By using HPTLC and immunostaining with an anti-GgOse4Cer and anti-GM1a antibodies after *C. perfringens* neuraminidase treatment, Gx1 was considered to be IV-NeuAc-GgOse$_4$Cer (GM1b) or IIINeuAc-GgOse$_4$Cer (GM1α). Gx2 was presumed to be IV(NeuAc)$_2$-GgOse$_4$Cer (GD1c) or IVNeuAc,IIINeuAc-GgOse$_4$Cer (GD1α), and GxN was speculated to be IVNeuAc,IINeuAc-GgOse$_4$Cer (GD1a). These data were further confirmed by FAB-MS of the isolated gangliosides and/or by immunostaining with MoAbs recognizing a terminal GM1b-specific sugar sequence, a terminal NeuAcα2→3Galβ1→3GalNAcβ1→ sequence, and a GD1α-specific oligosaccharide terminal sequence. By these analyses, Gx1 was identified as IV^{3}NeuAc-GgOse$_4$-Cer (GM1b) instead of GM1α, Gx2 was IV^{3}NeuAc,III^6-NeuAc-GgOse$_4$Cer (GD1α) instead of GD1c, and GxN was determined as IV^{3}NeuAc,II^{3}NeuAc-GgOse$_4$Cer (GD1a).

We conducted two sets of transfection and selection procedures: (1) the transfection of pcD-MCGF, followed by selection with IL-3 independency, and (2) the transfection of pCR3-MCGF, followed by selection with G418 resistance. They both reached the same findings that the IL-3 gene-transfected cells exhibited autonomous cell growth and that GD1a was expressed as a major component of gangliosides in addition to GM1b and GD1α. Because ganglioside GD1a was expressed after acquiring autonomous cell growth ability and after progression of the tumor stage in NFS60 cells, GD1a found in IL-3 gene-transfected cells could be considered one of the tumor-associated gangliosides. There were few reports that GD1a was a tumor-associated ganglioside. In mouse lymphoma cell line, so-called "a"-series gangliosides including GD1a were expressed as high metastatic potential markers (Murayama et al., 1986). Furthermore, GD1a as well as GD1b and sialylparagloboside was reported in v-fes gene-transfected cells (Nakaishi et al., 1988). In our case, however, the ganglioside expressed after progression of the tumor stage by IL-3 gene transfection was only GD1a. Thus, GD1a is a unique ganglioside in our NFS60 cell system.

While IL-3 producibility to the culture medium was shown to correlate with the expression level of the IL-3 message (Figure 1 and Table 3), autonomous cell growth potential was revealed to have little connection with extracellular IL-3 production (Figure 2 and Table 3). In addition, it was striking that anti-IL-3 antiserum could not neutralize the growth of the transfectant NFS60-H7 and NFS60-L5 cells (Figure 3). We postulate from these findings that, not the secreted IL-3, but the intracellular IL-3 produced in the cells would be enough for the autonomous cell growth of the IL-3 gene-transfected NFS60 cells. Lang et al. reported that the introduction of the GM-CSF gene into GM-CSF-dependent FDC-P1 cells resulted in their autonomous growth even in the presence of the antiserum (Lang et al., 1985). Moreover, Dunbar et al. showed that COOH-terminal-modified IL-3 was retained intracellularly and stimulated autocrine cell growth (Dunbar et al., 1989). They indicated that autocrine growth can occur as a result of the intracellular action of a growth factor and that autocrine growth factor might act independently on its normal receptor in the transfectants. Our findings were in good agreement with theirs.

Likewise, ganglioside GD1a expression was shown to have little correlation with IL-3 producibility (Tables 3 and 5 and Figure 8). Presumably, not the secreted IL-3 but the intracellular IL-3 produced in the cells would be critical for GD1a expression in the IL-3 gene-transfected NFS60 cells. Furthermore, it was demonstrated that extracellular rIL-3 induced ganglioside GD1a expression in the parental NFS60-I7 cells (Figure 7B). Although the intensity of GD1a was less than that from NFS60-H7 cells, at least 300 units/mL rIL-3 would be enough for GD1a induction in NFS60-I7 cells. The mechanisms of GD1a induction by IL-3, however, remain uncertain and require further study.

Both GM1b and GD1α were first described in rat ascites hepatoma AH7974F (Matsumoto et al., 1981; Taki et al., 1986). It is postulated that GD1α is biosynthesized by the transfer of NeuAc from CMP-NeuAc to position III of GM1b in an α2→6 linkage (Hirabayashi et al., 1990), and GM1b

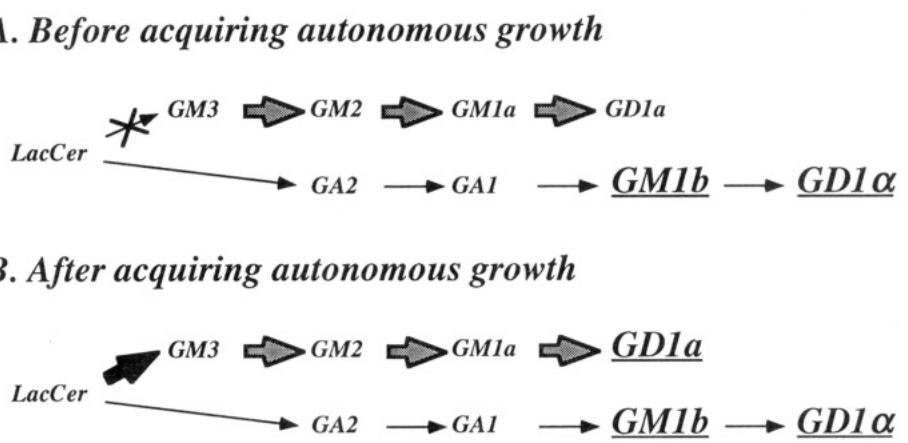

FIGURE 10: Shift in ganglioside biosynthetic pathway in NFS60 cells before and after acquisition of autonomous growth by IL-3 gene transfection. A gray, thick arrow indicates high glycosyltransferase activity, an arrow with a X sign indicates inactivated GM3 synthase, and a solid, thick arrow indicates up-regulation of GM3 synthase. (A) Before acquisition of autonomous cell growth in NFS60-I7, only GM1b and GD1α were the major gangliosides. In spite of the high activities of GM2, GM1a, and GD1a synthases, GD1a could not be synthesized because of GM3 synthase inactivation. (B) After acquisition of autonomous cell growth in NFS60-H7 cells, GD1a was expressed as another major ganglioside in addition to GM1b and GD1α, which were already expressed in the parental cells. Together with the high activities of GM2, GM1a, and GD1a synthases, IL-3 gene introduction resulted in the up-regulation of GM3 synthase and GD1a production as an IL-3-associated ganglioside.

is synthesized from $GgOse_4Cer$ (GA1) by NeuAc transfer to position IV in α2→3 linkage (Dasgupta et al., 1990). Thus, GM1b and GD1α were end products in the so-called "asialo" or "α" pathway. On the other hand, GD1a is biosynthesized from II^3NeuAc-$GgOse_4Cer$ (GM1a) by the transfer of NeuAc residue to position IV of GM1a in an α2→3 linkage. According to our data in this study, the progression of tumor stage by the acquisition of autonomous cell growth ability resulted in dramatic changes in cell surface gangliosides and their biosynthetic pathways. Namely, ganglioside biosynthesis in NFS60 cells shifted from the so-called "asialo" pathway alone to the "asialo" plus "a" pathways after IL-3 gene transfection and acquisition of autonomous cell growth (Figure 10).

We demonstrated that this shift in metabolic flow was a consequence of the up-regulation of a single glycosyltransferase, GM3 synthase, which is the most upstream key enzyme for the synthesis of ganglio series ganglioside biosynthesis. The activities of the sialyltransferase that synthesizes GD1a directly and the other downstream enzymes were almost the same level between the parental and transfected cells. Namely, in NFS60-I7 cells, which do not express IL-3, the activities of GD1a synthase and the previous step transferases, GM1a synthase and GM2 synthase, remained high enough to catalyze their reactions. However, GD1a could not be synthesized because the activity of the most upstream GM3 synthase was at a low level, in spite of the high activities of the downstream glycosyltransferases. On the other hand, the introduction of IL-3 gene into NFS60 cells seems to result in an elevation of GM3 synthase activity, and this up-regulation, together with the activities of downstream glycosyltransferases, causes the remarkable expression of GD1a.

We recently observed one example that the glycosphingolipid biosynthesis is controlled by the most upstream glycosyltransferase activation during cellular differentiation (Nakamura et al., 1992). In addition, Holmes et al. reported a similar case associated with oncogenesis (Holmes et al., 1987). Further, the synthesis of glycolipid HNK-1 antigens, the ligands of L- and P-selectins (Needham & Schnaar, 1993), was reported to be developmentally regulated by the activity of *N*-acetylglucosaminyltransferases (Chou & Jungalwala, 1993). In the present study, it was shown that the metabolic flow of glycosphingolipid biosynthesis was controlled by the single, most upstream glycosyltransferase, not only in lacto series glycosphingolipids but also in ganglio series glycolipids. Namely, the tumor-associated expression of ganglioside GD1a in IL-3-transfected NFS60 cells seems to be controlled by the activity of a single glycosyltransferase, GM3 synthase. However, further work might be required to reveal the molecular mechanism of GD1a expression controlled by GM3 synthase.

It is noteworthy that the ganglioside patterns of the parental and IL-3-transfected NFS60 cells seem to be dependent upon the time of cell harvest. Although the major components exist constantly, the relative abundance of each component seems to vary from mid-logarithmic stage to late-logarithmic stage. The relative abundance was GM1b > GD1α in the parental cells and GM1b > GD1α > GD1a in the transfected cells, when the cells were harvested at the mid-logarithmic stage. On the other hand, at the late-logarithmic stage, the abundance changed to GD1α > GM1b in the parental cells and GD1a > GD1α > GM1b in the transfected cells. Together with the IL-3-associated expression of GD1a, the ganglioside phenotype seems to be dependent on autonomous cell growth capability and cell growth stage. Thus, it is of great interest to clarify whether there would be some interactions between cell surface gangliosides and the cell growth machinery in NFS60 cells.

GD1a has been reported as a differentiation marker of murine T helper lymphocyte subpopulation T_H2 by Ebel et al. (1992). The functional role of these subtype-specific gangliosides was mentioned to be under consideration: involvement in cell−cell interactions and homing processes, as reported for the other systems (Springer, 1990; Feizi, 1991), or association with the cellular activity of the T_H2 cells. Together with our present report, it is strongly suggested that GD1a plays an important role in murine immune and hematopoietic cells.

In some cell systems, cell surface gangliosides are considered as modulators of the activity of functional membranous proteins. Such an interaction with the family

of tyrosine kinase receptors has been described (Bremer et al., 1984, 1986; Hanai et al., 1988; Nojiri et al., 1991). Because similar associations of glycosphingolipids with signal transduction of the other families of cytokine receptors have not been well investigated yet, whether GD1a plays any functional role in the activity of IL-3- or IL-3 receptor-related proteins (e.g., IL-3 signaling) in NFS60 cells should be elucidated. Further, receptors of IL-3, GM-CSF, and IL-5 share the same β-subunit, and signaling through them has also been thought to share common pathways. Thus, whether GD1a is not only an IL-3-associated ganglioside but also a GM-CSF-, IL-5-, and EPO-associated ganglioside remains to be clarified. The experiments to examine these possibilities are currently underway in our laboratory.

ACKNOWLEDGMENT

We are indebted to Dr. Ken'ichi Arai, Dr. Yoshio Hirabayashi, Dr. Tadashi Tai, Dr. Sudo, and Dr. J. N. Ihle for generous gifts of materials. We thank Dr. Masatsugu Ohta in our laboratory for his helpful advice and for preparing PWM-SCCM and Dr. Ikuo Kawashima for helping us stain GD1a by MoAb GMR17. We thank Dr. Toshio Suda (Kumamoto University, Kumamoto, Japan) for taking the initiative in this work; Dr. Masao Iwamori (University of Tokyo, Tokyo, Japan), Dr. Kumi Sakoe, Dr. Yasuhito Terui, Dr. Takao Sakai, Dr. Yusuke Furukawa, and Dr. Seiichi Kitagawa in our laboratory for their valuable comments; and Ms. Yayoi Tadenuma and Ms. Jinko Yamanoi-Saito for their technical and secretarial assistance.

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BI942200R