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GD2 ganglioside on human T-lymphotropic virus type I-infected T cells: Possible activation of β-1,4-*N*-acetylgalactosaminyltransferase gene by p40^{tax}

(adult T-cell leukemia/trans-activation/glycosyltransferase)

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ABSTRACT **Ganglioside expression on adult T-cell leukemia (ATL) and human T-cell lymphotropic virus type I (HTLV-I)-infected cells was determined by using a panel of monoclonal antibodies. ATL lines and HTLV-I-infected cells specifically expressed GD2. Leukemia cells from ATL patients generally expressed low levels of GD2 but the percentage of GD2$^+$ cells increased up to 40–70% after *in vitro* culture in the presence of interleukin 2 for about a week. No other type of leukemia cells and normal peripheral T cells expressed GD2 during *in vitro* culture under the same conditions. The appearance of GD2 in the cultured ATL cells corresponded with the expression of p40^{tax}, a product of the HTLV-I gene. Peripheral lymphocytes infected with a p40^{tax}-expressing retroviral vector expressed high levels of GD2 in comparison with control lymphocytes containing the neomycin-resistance gene alone. The apparently increased levels of β-1,4-*N*-acetylgalactosaminyltransferase (GM2/GD2 synthase) mRNA in these cells were demonstrated by reverse transcription–polymerase chain reaction analysis. Concordance between mRNA expression for the HTLV-I *tax1/rex1* genes and the β-1,4-*N*-acetylgalactosaminyltransferase gene was also observed in uncultured ATL cells. These results suggest that high GD2 expression was due to neosynthesis from precursor GD3 by increased expression of this enzyme induced by p40^{tax} *in vitro* and *in vivo*.**

Dramatic changes in carbohydrate composition and metabolism have been observed in association with cellular differentiation, development, viral infection, and malignant transformation (1, 2). These carbohydrate structures characteristic of a particular cellular population can be generally classified into two types: (*i*) those arising from incomplete synthesis of normally existing carbohydrate chains and (*ii*) those arising from neosynthesis through activation of glycosyltransferases.

Studies of glycolipids in human leukemia cells have revealed the formation of tumor-associated carbohydrate markers characteristic of each specific type of leukemia. Globotriaosyl ceramide (Gb3) expression in Burkitt lymphoma has been described (3, 4). Specific expression of GD3‖ in myeloid and lymphoid leukemia cells was also reported (6, 7).

Two retroviruses that infect mainly leukocytes of human origin have been isolated and characterized in detail. One is the human immunodeficiency virus (HIV-1), which causes AIDS. Adachi *et al.* (8) reported the specific expression of Le^y antigen in a human T-cell line and peripheral lymphocytes infected with HIV. The other retrovirus is human T-cell lymphotropic virus type I (HTLV-I), which causes malignant transformation of T lymphocytes, resulting in adult T-cell leukemia (ATL) (9). No extensive studies on the expression of specific carbohydrate antigens in ATL cells and HTLV-I-positive (HTLV-I$^+$) cells have been reported although there are a few studies of a small number of ATL patients (10, 11).

In this study, we showed that the GD2 ganglioside was highly and specifically expressed in HTLV-I$^+$ cells and ATL cells. Significant levels of GD2 were not detected in any other type of leukemia or normal lymphocyte, indicating the relationship between GD2 and HTLV-I infection. Our analysis on p40^{tax}-expressing T cells and leukemia cells from ATL patients suggests that the HTLV-I p40^{tax} protein can induce the synthesis of GD2 through the activation of GM2/GD2 synthase.

MATERIALS AND METHODS

Cells and Cell Lines. HTLV-I-infected human T-cell lines used in this study include the interleukin 2 (IL-2)-independent lines HUT102 (9), MT-2 and MT-1 (12), TL-Su (13), and ATN-1 (14); and the IL-2-dependent lines Oka2, Oka3, Kaw2, Kaw3, 4070, Deg, Kih, Ish, and Ike. All lines were established from ATL patients except 4070 and Deg. 4070 was from peripheral blood mononuclear cells (PBMCs) of a HTLV-I carrier, and Deg was from a normal individual by coculture with TL-Su. All cell lines were CD4$^+$ except Kih, which was CD8$^+$.

Monoclonal Antibodies (mAbs). mAbs used in this study were as follows: anti-GD2 ganglioside, mAb 3F8 (mouse IgG3) (15); anti-GD3, mAb R24 (mouse IgG3) (16); anti-GM2, mAb 10-11 (mouse IgM) (17); anti-GM3, mAb M2590 (mouse IgM) (Meiji Seika, Tokyo) (18); anti-HTLV-I env, mAb REY-7 (rat IgG) (19); anti-HTLV-I p40^{tax}, mAb Lt4 (mouse IgG) (20); anti-CD4, OKT4; anti-CD8, OKT8; and anti-CD3, OKT3 (the last three mAbs were obtained from American Type Culture Collection).

Serological Analysis. The expression of antigens on leukemia cells, leukemic cell lines, and lymphocytes was measured by flow cytometry (FCM) using a FACScan (Becton Dickinson).

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Abbreviations: HTLV-I, human T-cell lymphotropic virus type I; ATL, adult T-cell leukemia; ALL, acute lymphoblastic leukemia; IL, interleukin; r, recombinant; mAb, monoclonal antibody; RT–PCR, reverse transcription–polymerase chain reaction; GalNAc-T, β-1,4-*N*-acetylgalactosaminyltransferase; PBMC, peripheral blood mononuclear cell; FCM, flow cytometry; PBL, peripheral blood lymphocyte; neo, neomycin resistance.

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‖Gangliosides are designated according to the nomenclature of Svennerholm (5).

For immunofluorescence assay of $p40^{tax}$, mAb Lt4 and fluorescein isothiocyanate-labeled second antibody were used.

Western Blot Analysis. The $p40^{tax}$ protein was detected by Western blot analysis by the method of Towbin *et al.* (21). The poly(vinylidene difluoride) membrane (Immobilon; Millipore) with transferred proteins was incubated with mAb Lt4 and was stained using the VectaStain ABC kit (Vector Laboratories), and then $p40^{tax}$ was detected with the ECL detection system (Amersham). Densitometric analysis of the $p40^{tax}$ bands was performed by using the Unigraphy UHG-101 (Unique Medical, Tokyo).

Introduction and Expression of $p40^{tax}$ in Normal Peripheral Blood Lymphocytes (PBLs). $p40^{tax}$ was expressed in normal PBLs by a retroviral vector. The construction of the $p40^{tax}$-expressing retroviral vector DGL-Tax1 and a control vector, DGL, lacking the Tax1-coding region, has been described (22), and its schematic structure is shown in Fig. 3*A*.

Infection of PBLs with the retroviral vector was carried out as described (22). Briefly, normal PBLs that had been stimulated by phytohemagglutinin and expanded by addition of recombinant (r) IL-2 were infected with DGL-Tax1 by cocultivation with γ-irradiated (2000 rad; 1 rad = 0.01 Gy) virus-producing cells and selected with G418.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction (RT–PCR). To analyze the expression of UDP-GalNAc:GM3/GD3 β-1,4-*N*-acetylgalactosaminyltransferase (GalNAc-T; EC 2.4.1.92) and HTLV-I tax1/rex1 (23) mRNAs, a RT–PCR analysis was performed. We have reported (24) the cDNA cloning of the GalNAc-T gene.

Total RNA was prepared and single-strand cDNA was synthesized with a $(dT)_{14}$ primer as described (25). The PCR primers used for the GalNAc-T gene were a M2T-S1 sense primer (GalNAc-T cDNA clone M2T1-1, nt 142–161), 5′-AGGCCCGGGCTGCCAGATCT-3′, and a M2T-AS2 antisense primer (nt 677–696), 5′-GGTCTGGAAGCTTCGGCTGC-3′. RT–PCR conditions for *tax1/rex1* gene have been described (25). The primers used were a pX-1 sense primer, 5′-TACCTGAGGGCCCCATCCACGCCGGTTGA-3′, and a pX-2 anti-sense primer, 5′-ACACAGTCTCGAGACACGTAGACTGGG TAT-3′.

Southern Blot Analysis of RT–PCR Products. The RT–PCR products separated by PAGE were transferred onto nylon filters (GeneScreen*Plus*; DuPont). For the detection of PCR products of GalNAc-T gene, filters were hybridized with a probe at 60°C for 15 hr. The probe was labeled with ^{32}P by using the random-priming labeling kit (Amersham). For the detection of the *tax1/rex1* gene, an oligonucleotide probe, pX-PX (TCCCAGGGTTTGGACAGAGTCTT), was used as described (25).

RESULTS

Ganglioside Expression on Human Leukemia Lines. The results of ganglioside expression on various human leukemia cell lines are summarized in Table 1. Among 14 HTLV-I^+ lines, 11 lines showed a high percentage of $GD2^+$ cells and one line had a moderately high percentage. The percentage of GD2-expressing cells also represents the level of GD2 expression as shown in Fig. 1*B*. More than half expressed a significant amount of GD3. On the other hand, of 7 T-cell acute lymphoblastic leukemia (T-ALL) lines, only 3 weakly expressed GD2. Other leukemia lines did not express these gangliosides. These results were also confirmed by TLC and immunostaining (data not shown).

Ganglioside Expression on Uncultured and Short-Term Cultured Leukemia Cells from Patients. Expression of gangliosides on uncultured leukemia cells was analyzed (Table 2). Seven of 13 ATL patients had weak to moderate expression of GD2, and 11 patients also expressed moderate to low levels of GD3. Seven T-ALL patients did not express GD2, whereas they expressed moderate to high levels of GD3. Common ALL (c-ALL) and acute myelocytic leukemia (AML) cells did not express GD2.

Table 1. Expression of gangliosides on cultured cell lines

Cells		Ganglioside expression			
Type	Name	GM3	GM2	GD3	GD2
ATL and HTLV-1$^+$	HUT102	−	+++	−	−
	MT-1	−	−	++	+
	MT-2	−	−	−	+++
	ATN-1	−	+	−	+++
	TL-Su	−	++	±	++
	Oka2	−	+	++	+++
	Oka3	−	+	+	++
	Kaw2	−	++	++	+++
	Kaw3	−	+	+++	+++
	4070	−	+	++	+++
	Deg	−	+	+	+++
	Kih	−	+	+++	+++
	Ike	−	−	++	+++
	Ish	−	++	−	−
T-ALL	CCRF-CEM	−	−	−	−
	Jurkat	−	+++	−	±
	CCRF-HSB-2	−	++	−	+
	P-12/Ichikawa	−	−	−	−
	MOLT-3	−	−	−	−
	MOLT-4	−	−	+	+
	RPMI 8402	−	−	−	−
Myeloid	U937	−	−	−	−
	HL-60	−	+	−	−
	K-562	−	+	−	−
B-ALL	Raji	−	−	−	−
	Daudi	−	−	−	−
	Ara-10	−	+	−	−
Null-ALL	NALM-6	−	−	−	−

Ganglioside expression measured by FCM was classified as follows: −, no positive cells; ±, <5% positive cells; +, 5–40% positive cells; ++, 40–70% positive cells; +++, >70% positive cells.

Ganglioside expression of leukemia cells cultured in the presence of rIL-2 at 1 unit/ml was analyzed. Many ATL cells had a dramatic increase in GD2 expression, although cells

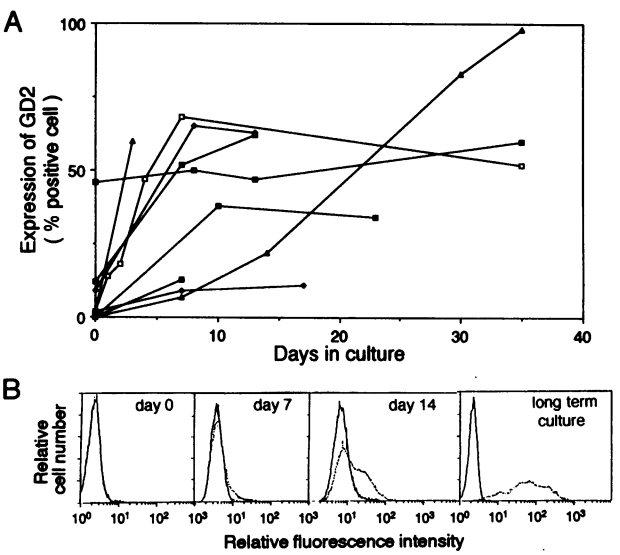

FIG. 1. Expression of GD2 during the culture of ATL cells in the presence of rIL-2. (*A*) Percent of $GD2^+$ cells in FCM. mAb 3F8 was used. The various symbols represent individual cases. (*B*) An example of FCM during culture. The experimental results are shown by dotted lines. Controls are indicated by solid lines.

Table 2. Expression of gangliosides on uncultured cells

Cells		Ganglioside expression			
Type	Name	GM3	GM2	GD3	GD2
ATL	Oga	−	−	++	+
	Tas	−	−	++	++
	Kih	−	−	+	+
	Kun	−	−	+	−
	Ohs	−	−	±	±
	Ura	−	−	+	+
	Kaw	−	−	+	++
	Kit	−	−	++	±
	Ike	−	−	−	−
	3491	ND	ND	++	+
	D276	ND	ND	+	−
	Ish	ND	ND	++	+
	Tan	ND	ND	+	−
T-ALL	Nod	−	−	+	−
	Suz	−	−	±	−
	Ito	−	−	+	−
	Ogat	−	−	+	−
	And	−	−	++	−
	Nii	−	−	+++	−
	Kata	−	−	+	−
c-ALL	Yam	−	−	±	−
	Kat	−	−	−	−
	Nis	−	−	±	−
	Kon	−	−	−	−
	Oka	−	+	+	−
	Say	−	−	−	−
AML	Kiy	−	−	−	−
	Ina	−	−	−	−
	Sak	−	−	±	−

Expression of gangliosides was classified as described in Table 1. ND, not done.

from two patients showed a mild increase and cells from one patient expressed GD2 well (Fig. 1*A*). Most samples were 40–70% $GD2^+$ at day 7 of culture. In contrast, leukemia cells other than ATL (i.e., T-ALL, c-ALL, and AML) did not express GD2 during culture (data not shown).

GD3 expression on ATL cells did not increase significantly during culture (data not shown). An example of the time course of GD2 expression is shown in Fig. 1*B*.

Expression of GD2 and the HTLV-I Gene. The majority of normal lymphocytes could hardly be induced to express GD2 by various stimulatory reagents including concanavalin A, phytohemagglutinin, immobilized anti-CD3 mAb, phorbol ester, IL-1, or some combination (data not shown). The expression of HTLV-I gene and GD2 was analyzed during the culture *in vitro* of two ATL samples. In sample 1 (Fig. 2 *A* and *B*), the expression of HTLV-I env protein analyzed by FCM quickly reached a plateau on day 1. The expression of GD2 and $p40^{tax}$ reached a plateau at day 2. In sample 2 (Fig. 2 *C* and *D*), the increase in expression was slower but otherwise similar to sample 1. Therefore, HTLV-I gene expression, especially $p40^{tax}$, was presumed to be associated with GD2 expression.

GD2 Expression on Peripheral T Lymphocytes Expressing HTLV-I $p40^{tax}$. To elucidate the effect of $p40^{tax}$ protein on GD2 expression in T lymphocytes, a retroviral vector containing the Tax1 coding region, DGL-Tax1, was constructed to infect normal lymphocytes (Fig. 3*A*). As shown in Fig. 3*B*, lymphocytes with $p40^{tax}$ expressed much more GD2 than the control lymphocytes with the neomycin-resistance (neo) gene alone, although both of them expressed fairly high levels of GD3, the precursor of GD2. Similar results were obtained in two individuals. These results were consistently obtained using lymphocytes cultured for 3, 3.5, and 4.5 months after infection with retroviral vectors.

Expression of the GalNAc-T mRNA in HTLV-I^+ Cells and Peripheral T Lymphocytes Expressing HTLV-I $p40^{tax}$. The mRNA expression of GalNAc-T gene was examined by RT–PCR analysis. As shown in Fig. 4, lymphocytes with $p40^{tax}$ clearly showed the 555-bp band expected from the GalNAc-T cDNA sequence whereas control cells with the neo gene alone and normal PBLs cultured with IL-2 showed very faint bands. These results are compatible with GD2 expression described above.

Expression of HTLV-I tax1/rex1 and GalNAc-T mRNAs in Uncultured PBMCs of ATL Patients. RT–PCR was performed to examine the expression of tax1/rex1 and GalNAc-T mRNAs in uncultured PBMCs obtained from six ATL patients, and the results were compared with GD2 expression. The tax1/rex1 mRNA is generated by a two-step splicing (double splicing) and has two overlapping open reading frames termed *tax1*, encoding $p40^{tax}$, and *rex1*, encoding $p27^{rex}$ and $p21^{x\text{-}III}$ (26). We have found (25) a single-spliced mRNA of *pX*

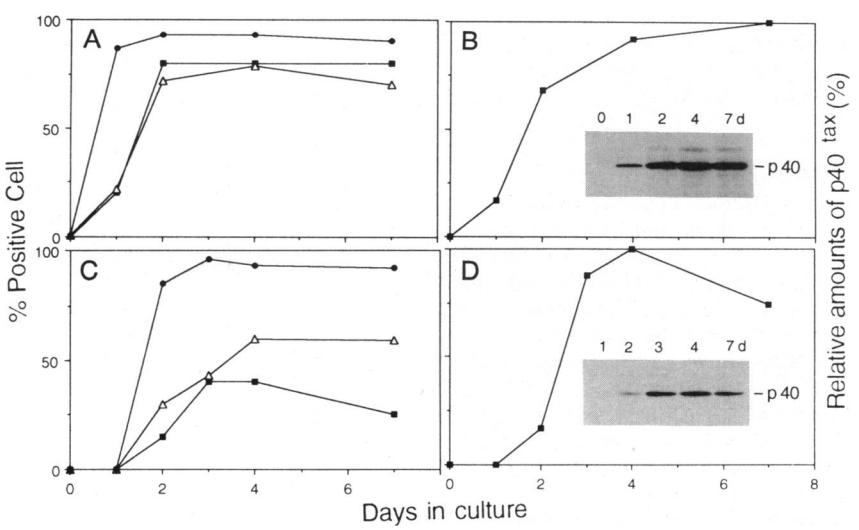

FIG. 2. GD2 and HTLV-I gene expression in ATL cells during culture *in vitro*. Leukemia cells from two ATL patients were cultured in the presence of rIL-2 (1 unit/ml). Expression of HTLV-I env protein (●) and GD2 (△) was measured as the percentage of positive cells by FCM. For $p40^{tax}$ (■) expression, samples were stained with mAb Lt4. Stained cells were scored by counting >200 cells in three fields (*A* and *C*). Western blot analysis using lysates of 5×10^4 cells was performed (*B* and *D*). The bands shown in *B* and *D* (*Insets*) were quantified with a densitometer and data are presented as percent maximum levels during culture.

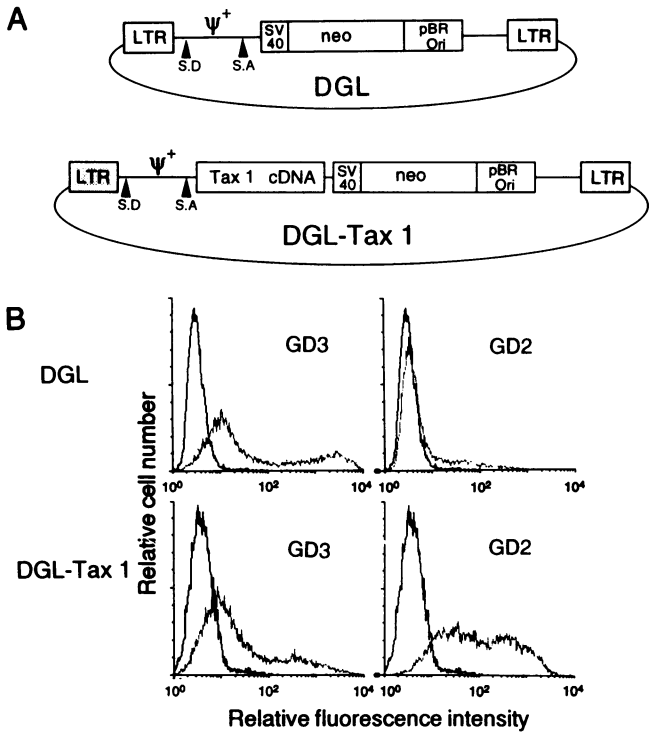

FIG. 3. GD3 and GD2 expression on normal PBLs infected with the recombinant retroviral vector. (*A*) Constructions of p40^{tax}-expressing retroviral vector, DGL-Tax1, and DGL control vector. SV40, simian virus 40 early region promoter; pBRori, pBR322 origin of replication; S.A., splice acceptor site; S.D., splice donor site; ψ^+, extended packaging signal. (*B*) Expression of GD3 and GD2 on PBLs infected with DGL-Tax1 or DGL. mAb R24 (GD3) or mAb 3F8 (GD2) was used. The experimental results are shown by light lines. Control staining patterns are shown by bold lines.

gene with the capability to produce p21$^{X\text{-}III}$ alone. The tax1/rex1 mRNA (343-bp band) was detected in four of six samples and a single-spliced HTLV-I pX mRNA (151-bp band) was detected in one sample (Fig. 5*A*). GalNAc-T mRNA was detected in the four samples that were also positive for tax1/rex1 mRNA (Fig. 5*B*). Two cases with the strong expression of GalNAc-T also showed high GD2 expression (Fig. 5*B*). GalNAc-T mRNA was not detected in normal PBMCs

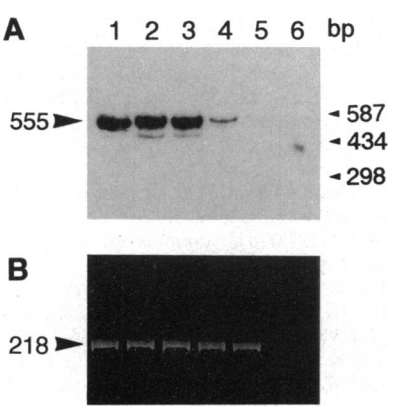

FIG. 4. Southern blot analysis of RT–PCR products. (*A*) Southern blot of the GalNAc-T gene. Twenty-six cycles of PCR were performed. The probe was the *Xba* I fragment of GalNAc-T cDNA clone pM2T1-1. (*B*) RT–PCR products after 21 cycles of β-actin. A BA-1 sense primer, 5′-TACATGGCTGGGGTGTTGAA-3′, and a BA-2 anti-sense primer, 5′-AAGAGAGGCATCCTCACCCT-3′, were used. Lanes: 1, IMR-32 (neuroblastoma as a positive control); 2, MT-2; 3, DGL-Tax1-infected PBLs; 4, DGL-infected PBLs; 5, normal PBL cultured with rIL-2; 6, human genomic DNA.

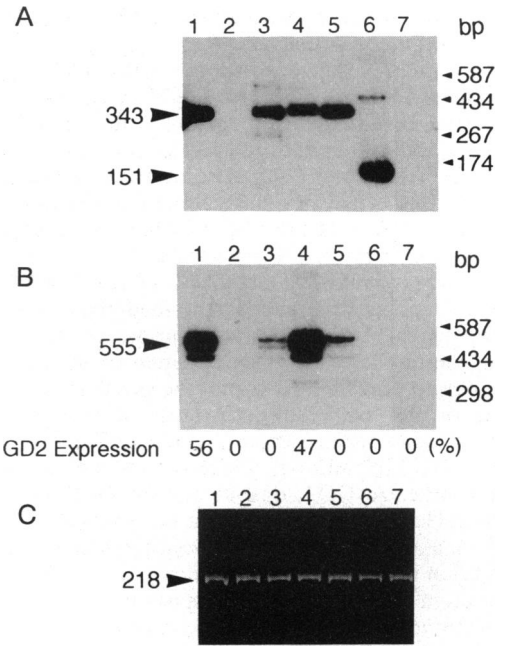

FIG. 5. Detection of HTLV-I tax1/rex1 and GalNAc-T mRNA in uncultured PBMCs from ATL patients. (*A*) Southern blot of *tax1/rex1* genes. (*B*) Southern blot of the GalNAc-T gene. Thirty-five cycles of PCR for *tax1/rex1* and 26 cycles for GalNAc-T were performed. Percent of GD2$^+$ cells by FCM is shown. (*C*) RT–PCR products after 21 cycles of β-actin. Lanes: 1–6, acute-type ATL patients; 7, normal individual.

even by 40 cycles of PCR. Thus, expression of GalNAc-T mRNA, tax1/rex1 mRNA of HTLV-I, and GD2 showed an apparent concordance in uncultured ATL cells as well.

DISCUSSION

Kuriyama *et al.* (10) and Suzuki *et al.* (11) reported that the GD3 ganglioside was the characteristic component in ATL cells. In their study, only ATL cells expressed GD3, whereas T-ALL lines and normal T cells did not. GD3 is expressed, however, in 10–15% of human normal peripheral T lymphocytes, and the proportion of GD3$^+$ lymphocytes increased after stimulation with mitogens and with anti-GD3 mAb (27, 28). We made a similar observation in normal lymphocytes stimulated with a variety of reagents. In contrast, GD2 was detected only on ATL and HTLV-I$^+$ cell lines in our analysis. GD2 could, therefore, be considered to be a specific surface marker of ATL and HTLV-I$^+$ T cells.

Two possibilities are conceivable for the mechanism by which human T lymphocytes express GD2. One is that GD2 expression results from the malignant transformation of mature T cells by HTLV-I infection. The other is that GD2$^+$ lymphocytes represent a minor T-cell subset that is difficult to enrich *in vitro* by current manipulation.

Based on the facts that GD2 is specifically expressed in HTLV-I$^+$ cells and that it is not detectable in stimulated lymphocytes, it seems likely that GD2 expression is induced by products of the HTLV-I gene, such as p40^{tax}. This tax protein is a potent transactivator of viral long terminal repeat-directed transcription and also of a variety of cellular genes for such compounds as granulocyte–macrophage colony-stimulating factor, IL-3, and IL-4 (29), c-fos (30), and IL-2 and IL-2 receptor α chain (26). Our results showed that the appearance of p40^{tax} correlated well with GD2 expression in ATL cells cultured *in vitro* and also in T lymphocytes infected with a p40^{tax}-expressing retrovirus vectors.

The results of RT–PCR analysis showed that HTLV-I$^+$ cells and peripheral T cells, both expressing p40^{tax}, express GalNAc-T mRNA that corresponds with GD2 expression. This suggests the possibility that p40^{tax} activates the expression of GalNAc-T responsible for the synthesis of GD2 from GD3. In addition, uncultured ATL cells also expressed p40^{tax}, GalNAc-T, and GD2 in some patients, suggesting a trans-activating effect of p40^{tax}. Although the intensities of tax1/rex1 mRNA bands in Fig. 5*A*, lanes 3–5, were almost equal, GalNAc-T mRNA levels varied. These results suggest that the trans-activation of GalNAc-T gene by p40^{tax} may not be direct. Other cellular genes described above may also be activated by p40^{tax} through or with various cellular factors. If so, intervening factors present between them might modify their accurate correlation. It may be possible to determine whether or not p40^{tax} directly acts on the promoter of GalNAc-T gene when genomic GalNAc-T DNA becomes available. HUT102 and Ish, which do not express GD3, also cannot synthesize GD2, despite the presence of abundant p40^{tax} and GalNAc-T mRNA (data not shown).

GD2 is a disialylganglioside existing mainly in normal human brain (31). It is also specifically expressed in human tumors of neuroectodermal origin such as neuroblastoma (15), melanoma (32, 33), and astrocytoma. Thurin *et al.* (34) reported that GD2 was expressed in melanoma cells only at the advanced stage (vertical phase) with metastatic potential. On the other hand, Cheresh *et al.* (35) demonstrated that GD2 and GD3 play an important role in the attachment of melanoma cells to solid substrata. These results indicate that GD2 on ATL cells may play a significant biological role in the attachment to vascular endothelium resulting in infiltration into various tissues such as skin, one peculiar clinical manifestation of ATL. Since GD3 mediates T-cell activation and IL-2 production (27, 28), the potential role of GD2 in transducing signals to regulate T-cell activation is also conceivable and deserves further investigation.

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