T cell receptor-mediated stimulation of mouse thymocytes induces up-regulation of the GM2/GD2 synthase gene

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Abstract cDNA clones of the mouse GM2/GD2 synthase (EC 2.4.1.92) gene were isolated, and their analyses revealed that the protein has a type II transmembrane structure with 533 amino acids, which was very similar to the human homolog except for the mRNA size. The mRNA level in thymocytes dramatically increased after treatment with anti-CD3 monoclonal antibody, whereas it was not elevated when treated with prostaglandin E2. In situ hybridization showed an elevation of mRNA levels in medullar thymocytes, suggesting that T cell receptor-mediated signaling induces up-regulation of the GM2/GD2 synthase gene in mature thymocytes.

Key words: Ganglioside; Glycosyltransferase; Thymocyte; *N*-Acetylgalactosamine; T cell receptor

1. Introduction

Gangliosides have been studied as cellular receptors for toxins and viruses [1]. They are considered to be receptors for cell–cell or cell–extracellular matrix interactions, and modulators of receptors for cell growth factors [2]. They have also been analyzed as tumor specific antigens [3,4]. In normal tissues, gangliosides are abundant in brain tissues [1], and are also found in immune cells [5–7]. The lines of evidence have suggested that gangliosides on mouse lymphocytes are associated with their functional properties and play important roles in immunological reactions. However, the genetic control of these gangliosides is not well understood yet.

In the present study, we isolated cDNAs for the mouse GM2/GD2 synthase (EC 2.4.1.92) gene in order to analyze the regulation of the ganglioside synthesis in mouse immune cells. Since many gangliosides are synthesized via GM2 or GD2, the expression of this gene seemed critical for the metabolism of the major gangliosides. Alterations of mRNA levels and the enzyme activity in mouse thymocytes after stimulation with anti-CD3 monoclonal antibody (mAb) and prostaglandin E2 (PGE2) were analyzed. These results suggest that GM2/GD2 synthase is regulated at both gene transcriptional and epigenetic levels.

2. Materials and methods

2.1. Construction of cDNA expression libraries

A cDNA prepared from poly(A)$^+$ RNA of the mouse thymoma cell line EL4 was a gift from Dr. T. Taniguchi of Osaka University. The *Bst*XI adaptor was added to the cDNA, which was then ligated into the *Bst*XI site of the expression vector pCDM8 [8]. The library contained 2×10^7 independent colonies.

2.2. cDNA cloning and DNA sequencing

Transformed colonies were screened by replica plating method according to Hanahan and Meselson [9], using a labeled 1.3 kbp *Sma*I–*Eco*RI fragment of the human GM2/GD2 synthase clone pM2T1–1 [10] as a probe. The membranes were hybridized and washed at 60°C. Plasmid DNA from hybridization-positive clones was transfected into KF3027 cells using DEAE dextran [10]. After 64–72 h, GM2 expression was examined by the immunofluorescence (IF) assay using mAb 10–11 as described [10]. Positive clones were classified based on their cleavage profiles with *Eco*RI, *Bam*HI, *Hin*dIII, *Xho*I and *Sal*I endonucleases. Overlapping deletion subclones were constructed as previously described [10] in pUC19 in which the cDNA insert had been bidirectionaly subcloned. Sequencing was performed as described [10] by using a reverse primer.

2.3. Cell lines and tissues

B78 and KF3027 were mouse melanoma lines as reported [10]. MeWo was a human melanoma line provided by Dr. L.J. Old (Sloan-Kettering Cancer Center, New York). Mouse melanoma line JB-RH was given by Dr. P. Livingston (Sloan-Kettering Cancer Center). CMS-7 was a mouse fibrosarcoma line. WOP-3027 was a mouse fibroblast line provided by Dr. B Seed (Harvard University). NG108–15 was a mouse-rat hybrid line kindly given by Dr. A. Miyazaki in Tokyo University. They were cultured in Dulbecco's modified Eagle's minimal essential medium (D-MEM) supplemented with 10% fetal bovine serum.

2.4. Antibodies and flow cytometry

Anti-GM2 mAb 10–11 [11] was a gift from Dr. P. Livingston in Sloan-Kettering Cancer Center. A hamster anti-CD3 mAb (145–2C11) was provided by Dr. J.A. Bluestone, University of Chicago as reported previously [12]. Expression of gangliosides were analyzed with a FACScan (Becton-Dickinson) as previously described [10].

2.5. Treatment of thymocytes in culture

Thymocytes from 4- to 6-week-old male BALB/c mice were prepared and cultured at a density of 2.5×10^7/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, 0.05 mM 2-mercaptoethanol. They were exposed to the anti-CD3 mAb at a dilution of 1:1000 or to 0.1 μM PGE2 for 1–12 h. PGE2 (Sigma) was dissolved in 95% ethanol and stored at −20°C.

2.6. Northern blotting

Total RNA and poly(A)$^+$ RNA from thymocytes and cell lines were prepared as described [10,13]. Separation and blotting of RNA were performed as described [10]. The membrane was prehybridized for 6 h

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) L25885.

Abbreviations: mAb, monoclonal antibody; IF, immunofluorescence; kbp(s), kilobasepair(s); PGE2, prostaglandin E2. Ganglioside nomenclature is based on that of Svennerholm [36]: GM2, GalNAcβ1→4 (NeuAcα2→3) Galβ1→4 Glc-Cer; GD2, GalNAcβ1→4 (NeuAcα2→8NeuAcα2→3) Galβ1→4 Glc-Cer.

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Mouse   MRLDRRALYALVLLLACASLGLLYSSTRNAPSLPNPLALWSPPQGPPRLDLLDLAPEPRY  60
Human   .W.G...C...........A...D..G.RL...P.A...S.R.PE.P.....  60

Mouse   AHIPVRIKEQVVGLLAQNNCSCESKGGSLPLPFLRQVRAVDLTKAFDAEELRAVSVAREQ  120
Human   ..............W.....S..G.....QK....I.......PA....A.AT...  120

Mouse   EYQAFLARSRSLADQLLIAPANSPLQYPLQGVEVQPLRSILVPGLSLQEASVQEIYQVNL  180
Human   .F....S..Q.P.....................................A..G..V.....  180

Mouse   SASLGTWDVAGEVTGVTLTGEGQPDLTLASPVLDKLNRQLQLVTYSSRSYQANTADTVRF  240
Human   T.....................A....V..G..Q..............T.....  240

Mouse   STKGHEVAFTILVRHPPNPRLYPPSSLPQGAEYNISALVIATKTFLRYDRLRTLIASIRR  300
Human   ..E...A....R...........G......Q.....................A..T....  300

Mouse   FYPTVTIVIADDSDKPERISDPHVEHYFMPFGKGWFAGRNLAVSQVTTKYVLWVDDDFVF  360
Human   ......V..........V.G.Y....L.....  360

Mouse   TARTRLEKLVDVLEKTPLDLVGGAVREISGYATTYRQLLSVEPGAPGLGNCFRQKQGFHH  420
Human   .............R.............F.................L..RR....  420

Mouse   ELVGFPSCVVTDGVVNFFLARTDKVRQVGFDPRLNRVAHLEFFLDFGLGFLRVGSCSDVV  480
Human   ......G.................E.......S.............S.....  480

Mouse   VDHASKVKLPWTAKDPGAETYARYRYPGSLDQSQVAKHRLLFFKHRLQCMTAE  533
Human   ......L.....SR.A.............E...M..............SQ  533
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Fig. 1. Deduced amino acid sequence of the mouse GM2/GD2 synthase cDNA clone pTm3–5. The amino acid sequence of human GM2/GD2 synthase is presented for comparison. The transmembrane domain is marked by a square. Potential *N*-glycosylation sites are underlined. Dots represent identical amino acids.

and hybridized with an *Xba*I fragment containing 2.1 kbp of mouse cDNA labeled by a random priming kit (Amersham) at 42°C for 12 h. The membrane was washed and exposed to a film as described [10]. The mouse 28 S tRNA-specific plasmid I-19/BS was provided by Dr. K. Abe (Kumamoto University, Kumamoto, Japan) and used as a control probe to correct for the amount of the applied RNA.

2.7. GM2/GD2 synthase assay

The membrane fraction of thymocytes were prepared as described by Thampoe et al. [14]. The enzyme assay was performed by duplicate samples as previously described [15].

2.8. Treatment of mice with anti-CD3 mAb in vivo

Four- to six-week-old male BALB/c mice were administered with 25 μl of ascites containing anti-CD3 mAb diluted in 175 μl of D-MEM intravenously, then sacrificed at the indicated times. Thymuses were embedded in O.C.T. compound and rapidly frozen on dry ice.

2.9. Preparation of RNA probes for in situ hybridization

Mouse GM2/GD2 synthase cDNA (pTm3–5) was transcribed from the T3 or T7 RNA polymerase promoters of Bluescript II KS$^-$ (Stratagene) to yield sense or antisense probes. The transcription reactions in 25 μl contained 1 μg of linearized template DNA, 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 30 mM dithiothreitol, 10 U of RNase inhibitor (Takara Shuzo), 1 mM each of ATP, CTP and GTP, 0.65 mM UTP, 0.4 mM digoxigenin-labeled UTP (Boehringer Mannheim) and 20 U of T3 or T7 RNA polymerase (Stratagene). The RNA probes were degraded by alkaline hydrolysis to an average length of 150 nucleotides [16].

2.10. In situ hybridization

In situ hybridization of mRNA in thymus was performed as previously described [17]. Hybridization proceeded at 60°C for 12–16 h in hybridization buffer containing 2.5 μg/ml of digoxigenin-labeled riboprobes. After washing and blocking, the slides were incubated with sheep anti-digoxigenin Fab-fragments conjugated to alkaline phosphatase (Boehringer Mannheim). Finally, the slides were incubated with the substrate, then mounted with PermaFluor (Lipshaw, Detroit, MI).

3. Results

3.1. Isolation and characterization of mouse GM2/GD2 synthase cDNAs

Approximately 2×10^6 independent recombinants from the

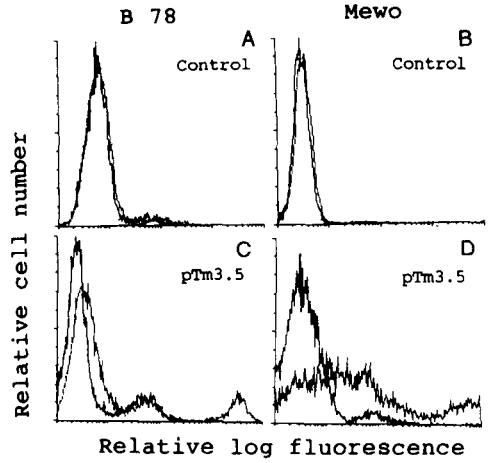

Fig. 2. GM2 expression on mouse B78 and human MeWo cell lines transfected with pTm3–5. Transient transfection and flow cytometry of GM2 expression were performed as described in Materials and Methods. Bold lines indicate the profile of the controls stained with FITC-conjugated goat anti-mouse IgM alone. A and B, transfection with vector alone. C and D, transfection with pTm3–5.

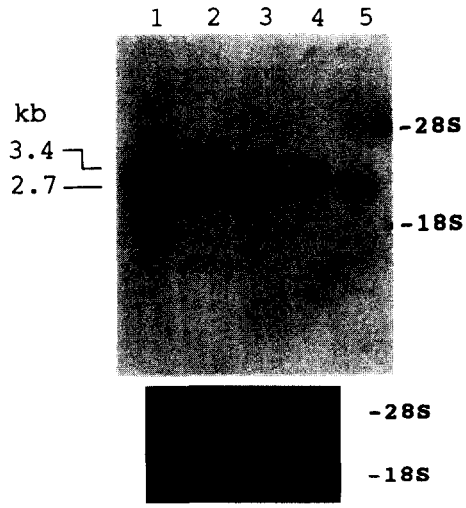

Fig. 3. Northern blots of various cell lines. Poly(A)$^+$ mRNA (10 μg) from 5 cell lines was separated in a 1.25% agarose-formaldehyde gel, then hybridized with a ^{32}P-labeled pTm3–5 insert probe. The mRNAs were derived from EL4 (lane 1), JB-RH (lane 2), CMS-7 (lane 3), NG108–15 (lane 4) and WOP-3027 (lane 5). Ethidium bromide staining of the gel is shown at the bottom.

EL4 cDNA library were screened and thirty one hybridization positive clones were isolated, then transfected into KF3027 cells. Twelve GM2-positive clones containing a >1.5 kb insert were placed into 5 groups based on their cleavage profiles with several endonucleases. Clone pTm3–5, one of the major group was characterized in detail.

3.2. *Nucleotide sequence and predicted amino acid sequence*

The complete nucleotide sequence of clone pTm3–5 (2,118 bp) was determined and revealed that a continuous open reading frame of 1599 bp (533 amino acids) was present with no poly A signal or poly A tail. The sequence homology with the human cDNA[a] [10] was 84.8% and 86.9% at the nucleotide and the amino acid level, respectively (Fig. 1). Inspection and a hydropathy analysis [18] of the predicted protein sequence suggested that it has a similar structural organization to those of known glycosyltransferases [19,20]. There is a single hydrophobic segment (18 amino acids) near the amino terminus. Three potential sites for N-linked glycosylation were also found at the identical sites as in the human homolog. Flow cytometry revealed that both B78 and MeWo transiently transfected with pTm3–5 expressed high levels of GM2 (Fig. 2).

3.3. *Expression of GM2/GD2 synthase mRNA in cell lines*

Northern blotts with poly(A)$^+$ RNA from mouse-derived cells revealed a major mRNA band at 2.7 kb and additional 3.4 kb band as shown in Fig. 3. No high molecular weight mRNA as found in human cells [10] was detectable.

3.4. *Expression of mouse GM2/GD2 synthase gene and enzyme activity in thymocytes stimulated with anti-CD3 mAb or PGE2*

Thymocytes cultured with anti-CD3 mAb showed an elevated level of GM2/GD2 synthase mRNA 8 h after addition of the mAb. After 12 h, the mRNA level markedly increased (Fig. 4). On the other hand, PGE2 induced a mild increase 12 h after stimulation. The enzyme activities in the thymocytes stimulated

[a]The original nucleotide sequence in this reference has been corrected (Genbank/EMBL Data Bank accession number M83651).

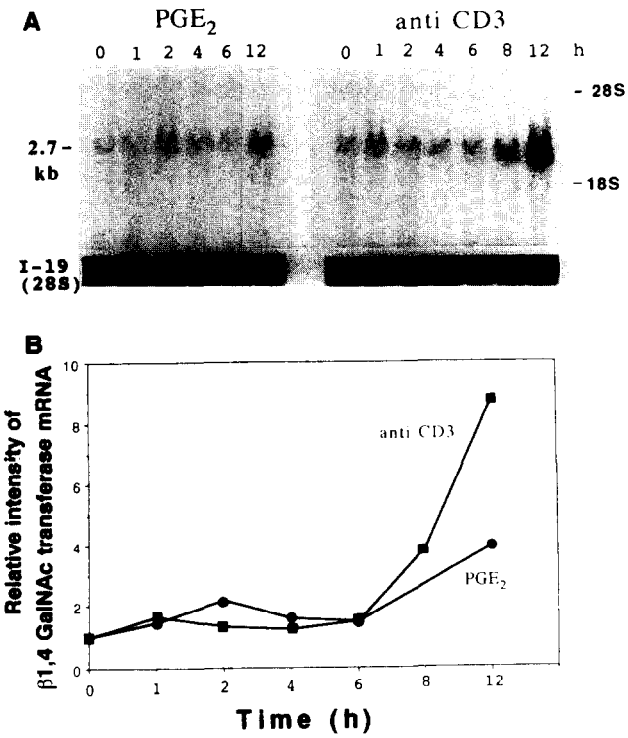

Fig. 4. Expression of GM2/GD2 synthase gene during the stimulation of thymocytes with PGE2 or anti-CD3 mAb. A, Northern blot of total RNA extracted from thymocytes cultured with PGE2 (left) or anti-CD3 mAb (right). 40 μg of each RNA was separated and blotted as in Fig. 3. The relative intensities of bands measured using a Bio-Imaging Analyzer BAS 2000 (Fuji) were plotted in B after correction using the 28S rRNA bands as described in section 2. ■, samples treated with anti-CD3 mAb. ●, PGE2-treated samples.

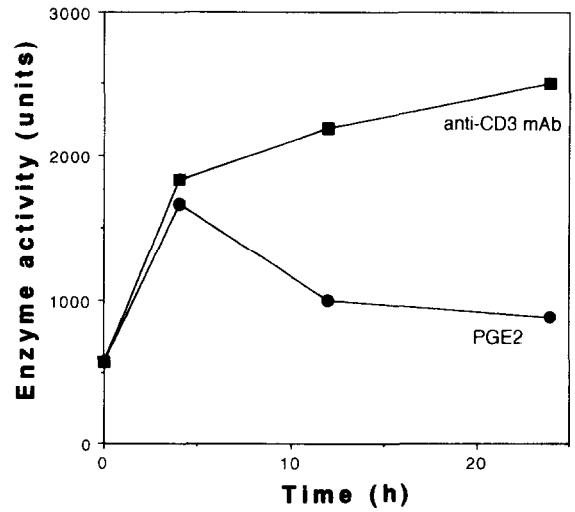

Fig. 5. GM2/GD2 synthase activity in thymocytes incubated with PGE2 or anti-CD3 mAb. Thymocytes were treated as in Fig. 4, and their enzyme activities were measured as described in section 2. ●, PGE2 and ■, anti-CD3 mAb.

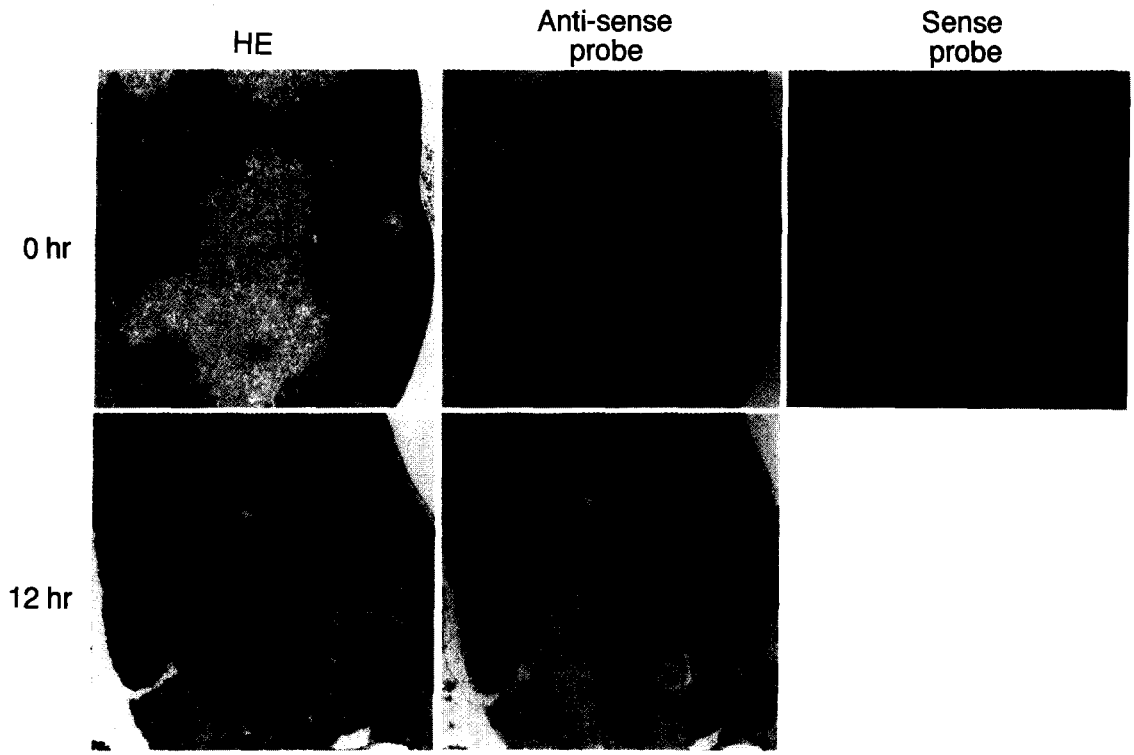

Fig. 6. Up-regulation of GM2/GD2 synthase mRNA in thymocytes treated with anti-CD3 mAb. mRNA expression was examined by in situ hybridization using the antisense RNA probe as described in section 2 (B,E). The sense RNA probe was used as a negative control (C). HE, hematoxylin-eosin staining (A,D). m, medulla; c, cortex.

by these two reagents altered in a different manner as shown in Fig. 5. Although there was a mild increase of enzyme activity in both preparations at an early phase (4 h), only anti-CD3 stimulation elevated it more after 12 h.

3.5. *mRNA expression of GM2/GD2 synthase gene in the thymus*

In situ hybridization of GM2/GD2 synthase gene in thymus revealed that the expression was confined to the medulla and scarce in the cortex. When treated with anti-CD3 mAb, mRNA expression in the medulla increased markedly at 12 h as shown in Fig. 6.

4. Discussion

Levels of ganglioside synthases are strictly regulated in the development of the brain as reported by Yu et al. [21]. Changes of gangliosides on immune cells are also considered to be associated with their differentiation and activation [22,23]. Generally it is believed that pre-T cells undergo positive/negative selection in the thymus, resulting in the generation of functional CD4- or CD8-type T cells existing in the thymic medulla [24,25]. In this study, we focused on enzyme activity and relative mRNA level of GM2/GD2 synthase in developing thymocytes after treatment with two reagents which are considered to stimulate thymocytes through different signaling pathways [26,27]. The anti-CD3 mAb binds to the T cell receptor complex and triggers mature T cell activation and proliferation mimicking antigen stimulation via protein tyrosine kinases, a Ca^{2+} increase [28,29] and C kinase [30]. On the other hand, PGE2 increases intracellular cAMP [31], which is reportedly produced in thymic stroma cells [32]. In NG108–15 cells, it quickly elevates GM2 synthase activity through phosphorylation of the relevant proteins by A kinase [33].

In order to analyze the regulation of ganglioside synthesis in mouse thymocytes under these conditions, mouse GM2/GD2 synthase cDNAs were isolated. They showed very high homology with human homolog [10], suggesting that this enzyme is required during development to maintain the structure, resulting in the correct localization, high stability, substrate specificity and other properties. Using an obtained cDNA clone as a probe, alterations of the gene expression as well as the enzyme activity in mouse thymocytes were analyzed.

Stimulations through cAMP and T cell receptor resulted in different responses in terms of GM2/GD2 synthase gene expression and activity. Enzyme activity in thymocytes increased 4 h after exposure to either reagent, whereas the mRNA level did not. The elevation of the enzyme activity at 4 h might be due to post-translational regulations, probably phosphorylation of the enzyme itself or of relevant proteins as reported previously [34]. Actually, transient elevation of cAMP was confirmed in thymocytes 30 min after exposure to PGE2 (data not shown). On the other hand, thymocytes treated with anti-CD3 mAb showed increasing activity of GM2/GD2 synthase even at 24 h. It seemed to be associated with the increased mRNA level as shown in Fig. 4. In situ hybridization clearly demonstrated the actual increase of the mRNA level in the medullar thymocytes. This up-regulation of GM2/GD2 synthase mRNA after culture for 8–12 h with anti-CD3 mAb may occur via many cellular factors involved in T cell activation. Recently, Yoshimura et al. reported that cytotoxic T cells induced against FBL-3N leukemia expressed high levels of GD2 and GM2/

GD2 synthase mRNA after repeated in vitro stimulation, but concanavalin A-treated lymphocytes did not [35]. Taken together, T cell receptor-mediated signaling seems to be essential for the transcriptional regulation of GM2/GD2 synthase gene in mature T cells.

The significance of the up-regulation of GM2/GD2 synthase in medullar thymocytes is unknown. The marked elevation of the mRNA level by anti-CD3 stimulation might result in a dynamic change of glycolipid expression on T cells, which may be associated with a functional property of the T cells. Changes of glycolipid profiles and their meanings remain to be investigated.

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References

[1] Wiegandt, H. (1985) in: Glycolipids (Wiegandt, H., Ed.) pp. 199–260, Elsevier, New York.
[2] Hakomori, S. (1990) J. Biol. Chem. 265, 18713–18716.
[3] Furukawa, K. and Lloyd, K.O. (1990) in: Human Melanoma. From Basic Research to Clinical Application (Ferrone, S., Ed.) pp. 15–30, Springer, Heidelberg.
[4] Hakomori, S. and Kannagi, R. (1983) J. Natl. Cancer Inst. 71, 231–251.
[5] Schwarting, G.A. and Gajewski, A. (1983) J. Biol. Chem. 258, 5893–5898.
[6] Muthing, J., Egge, H., Kniep, B. and Muhladt, P.F. (1987) Eur. J. Biochem. 163, 407–416.
[7] Horikawa, K., Yamasaki, M., Iwamori, M., Nakakuma, H., Takatsuki, K. and Nagai, Y. (1991) Glycoconj. J. 8, 354–360.
[8] Seed, B. and Aruffo, A. (1987) Proc. Natl. Acad. Sci. USA 84, 3365–3369.
[9] Hanahan, D. and Meselson, M. (1980) Gene 10, 63–67.
[10] Nagata, Y., Yamashiro, S., Yodoi, J., Lloyd, K.O., Shiku, H. and Furukawa, K. (1992) J. Biol. Chem. 267, 12082–12089.
[11] Natoli, E.J., Livingston, P.O., Pukel, C.S., Lloyd, K.O., Wiegandt, H., Szalay, J., Oettgen, H.F. and Old, L.J. (1986) Cancer Res. 46, 4116–4120.
[12] Leo, O., Foo, M., Sachs, D.H., Samelson, L.E. and Bluestone, J.A. (1987) Proc. Natl. Acad. Sci. USA 84, 1374–1378.
[13] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[14] Thampoe, I.J., Furukawa, K., Vellvé, E. and Lloyd, K.O. (1989) Cancer Res. 49, 6258–6264.
[15] Yamashiro, S., Ruan, S., Furukawa, K., Tai, T., Lloyd, K.O., Shiku, H. and Furukawa, K. (1993) Cancer Res. 53, 5395–5400.
[16] Cox, K.H., Deleon, D.V., Angerer, L.M., Angerer, R.C. (1984) Dev. Biol. 101, 677–679.
[17] Yamamoto, A., Atsuta, M. and Hamatani, K. (1992) Biochem. Function 10, 71–77.
[18] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[19] Paulson, J.C. and Colley, K.J. (1989) J. Biol. Chem. 264, 17615–17618.
[20] Joziasse, D.H. (1992) Glycobiology 2, 271–277.
[21] Yu, R.K., Macala, L.J., Taki, T., Weinfeld, H.M. and Yu, F.S. (1988) J. Neurochem. 50, 1825–1829.
[22] Hersey, P., Schibeci, S.D., Townsend, P., Burns, C. and Cheresh, D.A. (1986) Cancer Res. 46, 6083–6090.
[23] Welte, K., Miller, G., Chapman, P.B., Yuasa, H., Natoli, E., Kunicka, J.E., Cordon-Cardo, C., Buhrer, C., Old, L.J. and Houghton, A.N. (1987) J. Immunol. 139, 1763–1771.
[24] Blackman, M., Kappler, J. and Marrack, P. (1990) Science 248, 1335–1341.
[25] Ramsdell, F. and Fowlkes, B.J. (1990) Science 248, 1342–1348.
[26] McConkey, D.J., Hartzell, P., Amador-Perez, J.F., Orrenius, S. and Jondal, M. (1989) J. Immunol. 143, 1801–1806.
[27] Smith, C.A., Williams, G.T., Kingston, R., Jenkinson, E.J. and Owen, J.J.T. (1989) Nature 337, 181–184.
[28] Clevers, H., Alarcon, B., Wileman, T. and Terhorst, C. (1988) Annu. Rev. Immunol. 6, 629–662.
[29] Crabtree, G.R. (1989) Science 243, 355–361.
[30] Weiss, A. and Imboden, J.B. (1987) Advances in Immunol. 41, 1–38.
[31] McConkey, D.J., Orrenius, S. and Jondal, M. (1990). J. Immunol. 145, 1227–1230.
[32] McCormack, J.E., Kappler, J., Marrack, P. and Westcott, J.Y. (1991) J. Immunol. 146, 239–243.
[33] Scheideler, M.A. and Dawson, G. (1986) J. Neurochem. 46, 1639–1643.
[34] Bunnell, B., Adams, D.E. and Kidd, V.J. (1990) Biochem. Biophys. Res. Commun. 171, 196–203.
[35] Yoshimura, A., Takamiya, K., Kato, I., Nakayama, E., Shiku, H. and Furukawa, K. (1995) Scand. J. Immunol., in press.
[36] Svennerholm, L. (1963) J. Neurochem. 10, 613–623.