Modulatory Effect of Cytokines on Glycolipid Expression of Human Glioma Cell Line U118MG

— Changeable response and gradual loss of sensitivity to cytokines of glioma cells in culture —

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ABSTRACT

The effect of immune cytokines on the composition of neutral and acidic glycolipids was analyzed. Untreated U118MG cells expressed four kinds of neutral glycolipid molecules (CMH, CDH, CTH, and Gb_4Cer), and two kinds of acidic glycolipid (GM3 and GM2) as its major components. Effect of immune cytokines on the composition of the glycolipid composition was analyzed. U118MG cells were grown subconfluently before a panel of cytokines (IL-1beta, 100U/m*l*; IL-2, 4000JRU/m*l*; IL-4, 100U/m*l*; IL-6, 100 ng/m*l*; IL-8, 100 ng/m*l*; IFN-alpha, 1000U/m*l*; IFN-beta, 1000U/m*l*; IFN-gamma, 1000U/m*l*; TNF-alpha 10U/m*l*, and G-CSF 2 ng/m*l*) were pulsed for 48 hrs. In the neutral glycolipids new bands supposed to be subspecies of CMH and CDH with different fatty acid chains were recognized in the cells treated with IFN-alpha, IL-4, IL-6 and IL-8, although no constitutional change of sugar moieties was noted. In the acidic ones, IFN-gamma and IL-4 treatment brought about remarkable changes in the glycolipid profile enhancing sulfatides, GM1, GD1a, GD1b, and GT1b of the acidc lipids, with or without GM2 and GM3 expression. TNF-alpha also induced

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Abbreviations:

IL, interleukin	IFN, interferon
TNF, tumor necrosis factor	G-CSF, granulocyte-colony stimulating factor
FCS, fetal calf serum	PBS, phosphate buffered saline
DMSO, dimethylsulfoxide	TLC, thin layer chromatography
C/M/W, chloroform/methanol/water	CS, GalCer-I^3-sulfate
CMH, GalCer or GlcCer containing alpha-hydroxy fatty acid	CDH, lactosylceramide
Gb_4Cer, globotetraosylceramide	CTH, globotriaosylceramide
	SPG, sialylparagloboside

Gangliosides are abbreviated according to the nomenclature of Svennerholm(1).

GM1. Repeated experiments with the same culture conditions revealed different responses to cytokines in the expression of subspecies of CMH and CDH, and significant changes in the acidic glycolipids were noted in IL-2, G-CSF, TNF-alpha and IL-4 treatment. The 3rd series of repeated experiments using TNF-alpha, IFN-gamma, IL-4 and IL-6 showed only induction of subspecies of CMH by IFN-gamma; no alteration of acidic glycolipid profile was noted. The 4th series of the same experiments in the same conditions resulted in no response. Raising the dose of cytokines, change of the passage of the cells, addition of DMSO (a potent differentiation factor) did not restore the sensitivity to the cytokines. Similar experiments using 6 other glioma cell lines and one melanoma cell line did not induce any glycolipid modulation. We believe that glioma cells have the potential to respond to certain cytokines to modulate their glycolipid expression under undetermined special conditions, and they are biologically unstable in terms of cytokine sensitivity in culture.

Key words: Glioma, Glycolipid, Cytokine

INTRODUCTION

Carbohydrate chains of glycolipids are exposed on the outer surface of the cell membrane, and are considered to be involved in various cell-surface phenomena. The facts that the composition of the glycolipids changes in various biological processes including differentiation or malignant transformation(2) and that those molecules serve as cell surface receptors or transducers of biological signals(3) strongly suggest that glycolipids perform essential functions and that their biosynthesis and expression must be precisely regulated. Little is known about the intracellular mechanisms or extrinsic factors that modulate them, however.

Immune cytokines are known to have pleiotropic actions not only on immune cells but also other somatic and neoplastic cells. They affect differentiation, growth and cell surface structures(4, 5). Recently, IL-4, IFN, and TNF have been found to influence glycolipid expression in melanoma and renal cell carcinoma cells(6, 7). It is conceivable that the expression of glycolipids in glioma cells may also be regulated, at least in part, by those extrinsic factors in the microenvironment. We demonstrated that certain cytokines can influence the glycolipid expression in a distinctive manner in glioma cells. However, repeated experiments have failed to show a reproducible cytokine effect. The results clearly showed the ability of glioma cells to respond to cytokines with glycolipid modulation and imply that it is a delicate phenomenon which is difficult to repro-

duce in ordinary culture conditions.

MATERIAL AND METHODS

CELLS: Human glioma cell line U118MG was purchased from American Type Culture Collection (ATCC), MD, and was cultured from passage 445 to 448 in Dulbecco's MEM with 10% FCS supplemented with 0.1 mM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate without addition of antibiotics at 37℃ with 5%CO_2 atmosphere; the cells were maintained in 15-cm diameter culture dishes (Nunc, Sweden) with 50 m*l* of the culture media. The number of cells at confluence was $10^7 \pm 10\%$/dish with more than 95% viability as determined by trypan blue dye exclusion test. Other cell lines used were U87MG, U138MG, and U373MG glioma cells purchased from ATCC; A172, T98G, and KG1C glioma cells and Mewo melanoma cells were provided by Japanese Cancer Research Resources Bank (JCRB), Tokyo; Mewo melanoma cells were also provided by JCRB.

CYTOKINES: The cytokines used in this study were: human recombinant IL-1 beta (Otsuka Pharmaceutical Co, Ltd., Tokyo; 100U/m*l*), human recombinant IL-2 (Takeda Pharmaceutical Co., Osaka; 4000JRU/m*l*), IL-4 (Ono Pharmaceutical Co., Osaka; 100U/m*l*), IL-6 (Toray Industries, Inc., Tokyo; 100 ng/m*l*), IL-8 (Toray Industries, Inc., Tokyo; 100 ng/m*l*), recombinant G-CSF (Chugai Pharmaceutical Co. Ltd., Tokyo; 2 ng/m*l*), IFN-alpha (Otsuka Pharmaceutical Co., Tokyo; 1000U/m*l*), IFN-beta (Toray Industries, Inc., Tokyo; 1000U/m*l*), IFN-gamma (Otsuka Pharmaceutical Co., Tokyo; 1000U/m*l*) and TNF-alpha (Mochida Pharmaceutical Company, Tokyo; 10U/m*l*). These were added to 2 plates each at the indicated final concentrations when the cells grew to subconfluency, and the culture was continued for the next 48 hrs. The concentrations of cytokines had been determined from specific lot data of each reagent to obtain the maximum biological activities.

EXTRACTION OF GLYCOLIPIDS: The cells were harvested from the dishes with a cell scraper (3086 Falcon, NJ) followed by washing with PBS three times by centrifugation. Total lipid of 2×10^7cells about 200mg wet weight, was extracted with C/M/W (4:8:3, v/v/v) by 2 consecutive 24 hr extractions followed by a final 2 hr extraction at 40℃. The extracts were applied to a gel filtration colum (Sephadex LH-20, Pharmacia, Sweden) for desalting. Total glycolipid collected was further applied to an ion-exchange column (Sephadex A-25, acetate form, Pharmacia, Sweden) to separate neutral and acidic fractions. The acidic fraction was again desalted with the gel filtration column.

THIN LAYER CHROMATOGRAPHY: Both neutral and acidic fractions of each sample were chromatographed on a high performance TLC plate (Merck,

Silica Gel 60, Germany). They were developed with C/M/W (60 : 25 : 4, v/v/v) for the neutral fraction and with C/M/0.2%-$CaCl_2$ (55 : 45 : 10, v/v/v) for the acidic fraction. Standard glycolipids are as follows: CMH and CS were purified from porcine brain, CDH from equine erythrocytes, Gb_4Cer from human erythrocytes, and GM1, GD1a, GD1b, GT1b from human brain. Glycolipids developed on the plate were stained by Orcin-sulfate reagent.

RESULTS

EFFECT OF CYTOKINES ON THE MORPHOLOGY, DIFFERENTIATION AND THE VIABILITY OF U118MG: U118MG cells were mainly spindle-shaped but partly polygonal, and did not show any morphological changes after addition of cytokines. The cells were uniformly spindle-shaped when they became confluent. U118MG cells were strongly positive for GFAP and moderately positive for S100 protein as determined by immunostaining technique. They were not affected by cytokine treatment, so that the effect of cytokines on differentiation could not be evaluated (data not shown). The viability of the cells with or without cytokine treatment was more than 95% by trypan blue dye exclusion test.

EFFECT OF CYTOKINES ON THE NEUTRAL GLYCOLIPID COMPOSITION OF U118MG: The neutral glycolipids of U118MG were CMH, CDH, CTH and Gb_4Cer (Fig. 1). There was no change in glycolipid components with density of U118MG cells. The relative glycolipid composition was not affected by the 10 cytokines tested in this study. However, IFN-gamma and IL-4 enhanced the production of all glycolipids as well as the appearance of an additional pair of bands below the original CMH and CDH bands, which we suspected to contain hydroxylated fatty acids because of their slightly more hydrophilic nature. IL-6 generated hydroxy-CDH, while IL-8 produced hydroxy-CMH.

EFFECT OF CYTOKINES ON THE ACIDIC GLYCOLIPID COMPOSITION OF U118MG: The acidic glycolipids of U118MG were composed mainly of GM3 and GM2 judging from their relative mobilities on TLC. Sulfatide (CS), GD2 and GD3 as minor components were also faintly detected as minor components on the TLC (Fig. 2). Of the 10 cytokines tested, three remarkably modulated glycolipid profile. TNF-alpha induced GM1 expression, IFN-gamma stimulated the expression of CS, GM1, GD1a, GD1b, and GT1b in addition to GM2 and GM3, and IL-4 induced CS, GM1, GD1a, GD1b, GT1b while with decreasing GM2 and GM3 expression.

REPEATED EXPERIMENTS USING U118MG: In the 2nd series of experiments IL-1beta, IL-2, G-CSF, IFN-alpha, IFN-gamma, IL-4 and IL-8 induced an appearance of an additional band in the neutral fractions. CDH was

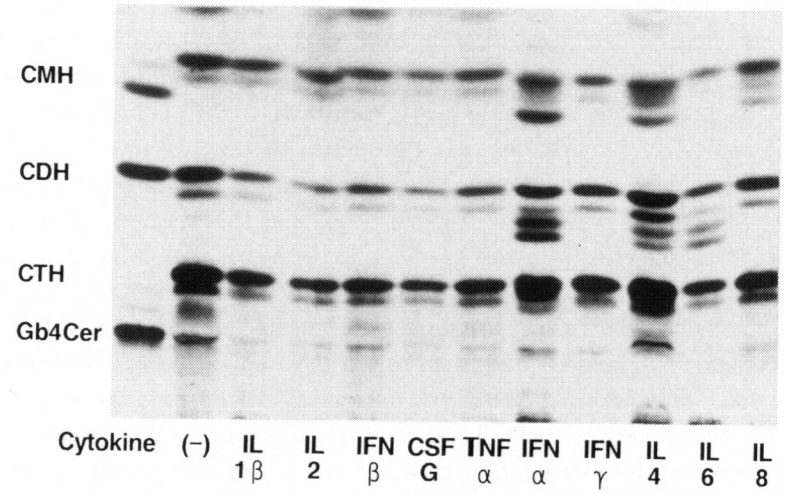

Fig. 1 The 1st series of thin layer chromatograms of neutral glycolipids of U118MG treated with 10 cytokines. Glycolipids applied on the TLC were equal to that derived from 4×10^6 cells (40 mg wet weight). Developing solvent was C/M/W (60 : 25 : 4, v/v/v). Glycolipids were visualized by Orcin-sulfate reagent.

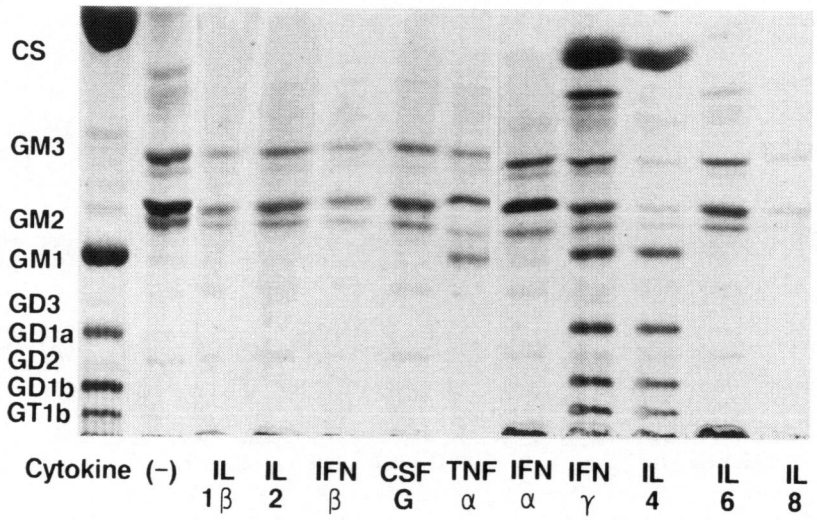

Fig. 2 The 1st series of thin layer chromatograms of acidic glycolipids of U118MG treated with 10 cytokines. Glycolipids applied were equal to that derived from 4×10^6 cells (40 mg wet weight). Developing solvent was C/M/W containing 0.2% of $CaCl_2$ (55 : 45 : 10, v/v/v). Glycolipids were visualized by Orcin-sulfate reagent.

significantly changed only by IL-8 treatment (Fig. 3). In the acidic fraction, sulfatide was clearly detected in cells treated with IL-1beta, IL-2, G-CSF and TNF-alpha. Cells treated with IL-1beta, IL-2 and TNF-alpha expressed long sugar chain gangliosides including GM1, GD1a, GD1b and GT1b; G-CSF enhanced GM1 and GD1a remarkably. IL-4 amplified the expression of GD2 and GD3 (Fig. 4). The above responses were different from the first series of experiments. The 3rd experiments were done using TNF-alpha, IFN-gamma, IL-4 and IL-6, which had striking effects in the previous experiments, resulting in no response except for induction of an additional band in CMH by IFN-gamma (Fig. 5). No changes were observed in the 4th series of the same experiments. The dose of IFN-alpha and IFN-gamma were then raised ten-fold to 10000U/m*l* and the dose of IL-4 was increased to 1000U/m*l*, but no positive response was obtained. DMSO, routinely used for cell freezing, is a potent cell differentiation reagent and was presumed to be a possible contaminant in the initial culture. We added DMSO at 0.5% in the culture with IFN-gamma or IL-4, which resulted in no response. Another seed of U118MG cells was purchased from ATCC, and was repeatedly cultured from passage 443 to 445 to examine for IFN-gamma and IL-4 effect, but no change in glycolipid composition was seen.

REPEATED EXPERIMENTS USING OTHER CELL LINES: The six other glioma cell lines U87MG, U138MG, U373MG, A172, T98G and KG1C, and the melanoma cell line Mewo were also examined for their cytokine sensitivities

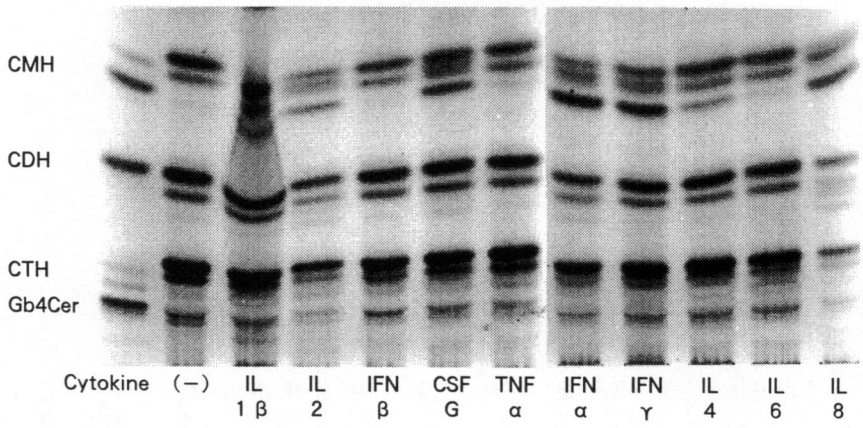

Fig. 3 The 2nd series of thin layer chromatograms of neutral glycolipids of U118MG treated with 10 cytokines. Glycolipids applied on the TLC were equal to that derived from 4×10^6 cells (40 mg wet weight). Developing solvent was C/M/W (60 : 25 : 4, v/v/v). Glycolipids were visualized by Orcin-sulfate reagent.

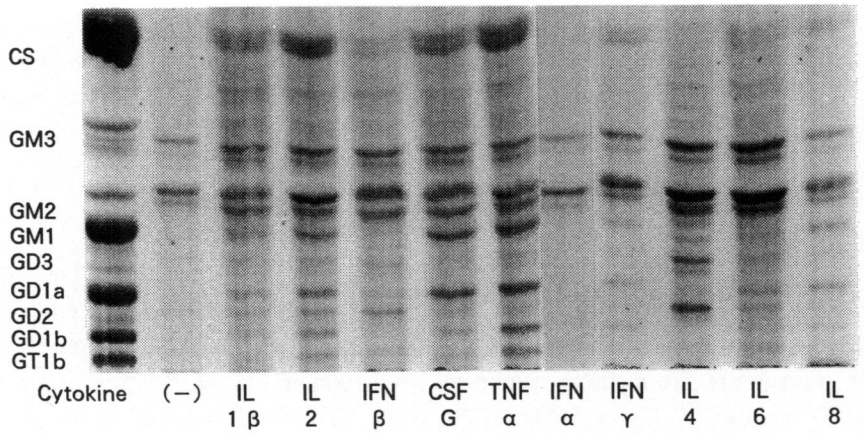

Fig. 4 The 2nd series of thin layer chromatograms of acidic glycolipids of U118 treated with 10 cytokines. Glycolipids applied were equal to that derived from 4×10^6 cells (40 mg wet weight). Developing solvent was C/M/W containing 0.2% of $CaCl_2$ (55 : 45 : 10, v/v/v). Glycolipids were visualized by Orcin-sulfate reagent.

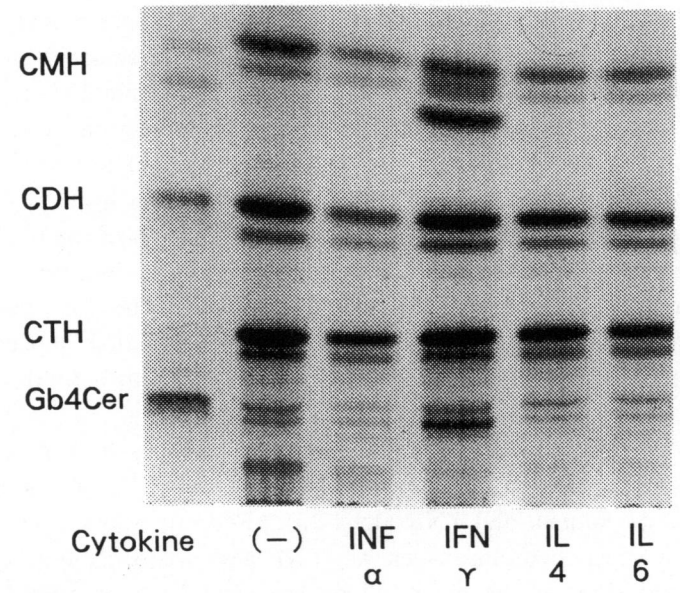

Fig. 5 The 3rd series of thin layer chromatograms of neutral glycolipids of U118MG treated with 4 cytokines. Glycolipids applied were equal to that derived from 4×10^6 cells (40 mg wet weight). Developing solvent was C/M/W (65 : 25 : 4, v/v/v). Glycolipids were visualized by Orcin-sulfate reagent.

in terms of glycolipid modulation. All were treated with TNF-alpha, IFN-gamma, and IL-4 in the same method as U118MG. U373MG cells showed prominent development of cellular process morphologically by IFN-gamma without glycolipid modulation. No other significant change was recognized in other cells. KG1C cells were treated with all the 10 cytokines, with negative response.

DISCUSSION

Neutral glycolipids of U118MG cells were composed of CMH, CDH, CTH, and also minor amounts of Gb_4Cer; acidic ones consisted of GM2 and GM3 additionally with sulfatide, GD2 and GD3. The acidic glycolipid composition showed slight alterations from those reported by Fredman *et al.*(8), indicating that U118MG included 3'-LM1 (SPG), GM2, GM3, GD2, GD3 and LD1 in decreasing order of quantity. We did not recognize SPG as the most predominant component, nor could we identify the band corresponding to LD1. This difference might have derived from the amount of the cells used, the culture conditions, or the passage of U118MG cells used; they used 4 passage levels between 562 and 573 contrary to 445 and 448 in the present experiment.

Modulation of cell surface antigens by immune cytokines has been well acknowledged along with their pleiotropic activities. Certain modulation such as induction of MHC antigens as well as certain tumor-associated antigens(9, 10), or cell adherent molecules such as ICAM-1 by IFN-gamma(11), or ELAM-1 by TNF-alpha and IL-1beta(12), should be important for causing immune reaction or inflammation. Cytokines can also enhance glycolipid antigens; e.g., sialyl-Lewis X antigen by IFN-alpha(13), or Forssman antigen by IL-4 or -6 on certain types of cells(14), which may only suggest that those molecules are also functionally active. We found dramatic changes in glycolipid profiles with elongation of sugar chain of gangliosides in U118MG. The fact that glycolipid antigens of gliomas receive such strong cytokine regulation implies that those mysterious molecules are closely concerned with biological activities of the cells stimulated by certain cytokines.

Astrocytes may play an important role as accessory immune cells such as antigen presenting cells in the CNS responding to certain cytokines such as IL-1, IFN-alpha and -gamma, and TNF-alpha(15). Recently gliomas have been found to produce various cytokines such as TNF-alpha, IFN-gamma, G-CSF, GM-CSF, IL-6 and IL-8, as demonstrated by the expression of mRNA(16) (Nitta, T., personal communication, 1992). These in no doubt that cytokines play an important role in expressing astrocyte functions; therefore, it is also conceivable that they can influence the carbohydrate structures on the cell surface. Yates *et al.*(17, 18) examined the effect of IFN-beta on glycolipid profile of glioma cells

and found no constitutional change of carbohydrates; however, there has been no report describing other cytokine effects on the glycolipid expression of glioma cells.

Turning to other types of cells, Furukawa *et al.*(19) demonstrated increased GD3 in melanocytes by TNF-alpha with some morphological change. In melanoma cells, IL-4 alone and in combination with IFN or TNF was reported to increase the GM3/GD3 ratio expression(6), which may have some relation with differentiation into less metastatic cell types. In renal cell carcinomas, IL-4 and IFN-gamma caused an increase in GD2 and GD3 expression; those cytokines also modulated HLA class I and HLA-DR expression, which may be related with immunological recognition by the host(7). There has been no report that cytokines are capable of causing such a drastic change of glycolipid composition as initially seen in IFN-gamma or IL-4 treatment in this experiment. This change might have been specific for gliomas, since the brain where the glioma occurs is abundant with complex glycolipids which could well have the ability to synthesize those molecules.

We subsequently failed to reproduce the drastic change of glycolipid profile of U118MG cells seen in the initial experiment. The cells showed different responses to cytokines in the 2nd experiment, and gradually lost sensitivities to cytokines in the following experiments. The same passage, which once showed positive response to cytokines, did not show the same response. Other passage levels or another seed obtained from ATCC did not reproduce the data either. Using freshly prepared cytokines and changing the dose of cytokines also yielded no responses. Addition of DMSO, which was a possible contaminant in the initial experiment and a potent differentiation reagent, did not recover the positive effect. We must come to the conclusion that glioma cells are unstable in their biological characteristics; there are reports describing chromosomal changes in permanent cell lines(20), changes in expression of growth factor receptor(21) or cytokine expression(22) after multiple passages. We presume U118MG cells were transiently susceptible to cytokine stimulation, but rapidly lost the sensitivity due to their biological instability in the culture conditions. The above data still suggest that glioma cells are potentially responsive to cytokines, and glycolipid modulation can be mediated through external signals such as cytokines.

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REFERENCES

1. SVENNERHOLM L. Ganglioside designation. Adv Exp Med Biol 1980, 125 : 11.
2. HAKOMORI S-I. Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. Annu Rev Biochem 1981, 50 : 733-764.
3. FISHMAN PH. Overview : Gangliosides as cell surface receptors and transducers of biological signals. In : LEDEEN RL, HOGAN EL, TETTAMANTI G, YATES AJ and YU RK, editors. New trends in ganglioside research. Padova, Liviana Press, 1988, 183-201.
4. GREEN AR. Peptide regulatory factors : Multifuntional mediators of cellular growth and differentiation. Lancet 1989, 1 : 705-707.
5. GREEN AR. Peptide regulatory factors : Interleukins and immune system 2. Lancet 1989, 1 : 1003-1005.
6. HOON DS, BANEZ M, OKUN E, MORTON DL, IRIE RF. Modulation of human melanoma cells by interleukin-4 and in combination with gamma-interferon or alpha-tumor necrosis factor. Cancer Res 1991 ; 51 : 2002-2008.
7. HOON DS, OKUN E, BANEZ M, IRIE RF, MORTON DL. Interleukin-4 alone and with gamma-interferon or alpha-tumor necrosis factor inhibits cell growth and modulates cell surface antigens on human renal cell carcinomas. Cancer Res 1991;51 : 5678-5693.
8. FREDMAN P, MANSSON J-E, BIGNER S, WIKSTRAND CJ, BIGNER DD, SVENNERHOLM L. Gangliosides in the human glioma cell line U-118 MG grown in culture or as xenografts in nude rats. Biochim Biophys Acta 1990, 1045 : 239-244.
9. GREINER JW, GUADAGNI F, NOGUCHI P, PESTKA S, COLCHER D, FISHER PB, SCHLOM J. Recombinant interferon enhances monoclonal antibody-targeting of carcinoma lesions *in vivo*. Science 1987, 235 : 895-898.
10. WEBER JS and ROSENBERG SA. Modulation of murine tumor major histocompatibility antigens by cytokines *in vivo* and *in vitro*. Cancer Res 48, 5818-5824, 1988.
11. FADY C, GARDNER AM, GERA JF, LICHTENSTEIN A. Interferon-induced increase in sensitivity of ovarian cancer targets to lysis by lymphokine-activated killer cells : Selective effects on HER2/neu-overexpressing cells. Cancer Res 1992, 52 : 764-769.
12. BEVILACQUA MP, POBER JS, MENDRICK DL, COTRAN RS, GIMBRONE MA Jr. Identification of an inducible endothelial-leukocyte adhesion molecule. Proc Natl Acad Sci USA 1987, 84 : 9238-9242.
13. OKADA Y and TSUJI T. Neoexpression of sialyl oligomeric Lewis X by Kupffer cells in chronic hepatitis. Lancet 1990, 335 : 1302-1306.
14. KLEIST RV, SCHMITT E, WESTERMANN J, MÜHLRADT PF. Modulation of Forssman glycolipid expression by murine macrophages : Coinduction with class II MHC antigen by the lymphokines IL4 and IL6. Immunobiology 1990, 180 : 405-418.
15. HERTS L, MCFARLIN DE, WAKSMAN BH. Astrocytes : auxiliary cells for immune responses in the central nervous system ? Immunol Today 1990, 11 : 265-268.
16. NITTA T, SATO K, ALLEGRETTA M, BROCKE S, LIM M, MITCHELL DJ, STEINMAN L. Expression of granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) genes in human astrocytoma cell lines and in glioma specimens. Brain Res 1992, 571 : 19-25.

17. YATES AJ, STEPHENS RE, MARKOWITZ DL, ELDER P, SIRINEK LP, WHISLER RL. Resistance to natural killer cytolysis and neutral glycolipid composition of cultured human glioma and fetal brain cells. J Neuropathol Exp Neurol 1985, 44: 371-383.
18. YATES AJ, MARKOWITZ DL, STEPHENS RE, PEARL DK, WHISLER RL. Growth inhibition of cultured human glioma cells by beta interferon is not dependent on changes in ganglioside composition. J Neuropathol Exp Neurol 1988, 47: 119-127.
19. FURUKAWA K, ARITA Y, SATOMI N, EISINGER M, LLOYD KO. Tumor necrosis factor enhances GD3 ganglioside expression in cultured human melanocytes. Arch Biochem Biophys 1990, 281: 70-75.
20. BIGNER SH, BJERKKVIG R, LAERUM OD, MUHLBAIER LH, BIGNER DD. DNA content and chromosomes in permanent cultured cell lines derived from malignant human gliomas. Anal Quant Cytol Histol 1987, 9: 435-444.
21. NISTE'R M, WEDELL B, BETSHOLTZ C, BYWATER M, PETTERSSON M, WESTERMARK B, MARK J. Evidence of progressional changes in the human malignant glioma line U-343MGa: Analysis of karyotype and expression of genes encoding the subunit chains of platelet-derived growth factor. Cancer Res 1987; 47: 4953-4960.
22. LICHTOR T, DOHRMANN GJ, GURNEY ME. Cytokine gene expression by human gliomas. Neurosurgery 1990, 26: 788-793.