Biochemical Analysis of Cytokine Effect on the
Glycosphingolipid Expression of Human Glioma Cell Lines

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ABSTRACT

We have already found that a human glioma cell line U118MG could modulate glycosphingolipid (GSL) expression via certain cytokines, though this effect is changeable and very difficult to reproduce even under consistent culture conditions. Consecutive analysis of cytokine effect using other 6 glioma (A172, T98G, KG-1C, U87MG, U138MG and U373MG) and one melanoma cell line (Mewo) failed to detect any GSL modulation. A total of ten cytokines were used in this experiment: IL-1beta, IL-2, IL-4, IL-6, IL-8, TNF-alpha, IFN-alpha, IFN-beta, IFN-gamma, and G-CSF, for treating KG-1C mixed glioma cells and 8 cytokines out of those for treating T98G glioblastoma cells. The rest of the cell lines were cultured with IFN-gamma and IL-4 because those two had once shown dramatic effect on GSL expression on U118MG cells. The fact that none of the 6 glioma cell lines responded to cytokines in terms of GSL modulation implies that cytokines do not play a significant role in regulating GSL expression in the glial cell lineage.

Key words: Glycosphingolipid, Cytokine, Glioma

INTRODUCTION

The cell surface is the site for the triggering various biological phenomena.

Abbreviations used in this text are as follows: IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; G-CSF, granulocyte-colony stimulating factor; FCS, fetal calf serum; PBS, phosphate buffered saline; TLC, thin layer chromatography; C/M/W, chloroform/methanol/water; CS, GalCer-1[3]-sulfate; CMH, GalCer or GlcCer containing alpha-hydroxy fatty acid; CDH, lactosylceramide; CTH, globotriaosylceramide; Gb_4Cer, globotetraosylceramide; nLc4Cer, neolactotetraosylceramide. Gangliosides are abbreviated according to the nomenclature of Svennerholm [1].

Carbohydrates are important constituents of the cell surface structure, which consist either of glycoprotein or glycosphingolipid (GSL). The composition of the GSLs varies with developmental processes including differentiation or dedifferentiation [2], and those molecules can serve as cell surface receptors for viruses or bacterial toxins [3] : together these facts strongly suggest that these molecules are as functionally active and equally important as other membrane-bound proteins. Modulation of the GSL profile in the process of malignant transformation has important implications, and may aid in understanding the altered behavior of transformed cells and the regulatory mechanisms of GSL expression. If external manipulation of GSL expression becomes possible, it could provide a tremendous boost to research as a tool for better understanding of regulatory mechanisms as well as biological functions of GSL and to immunotherapy by enabling more precise targeting of those molecules.

Immune cytokines are known to have pleiotropic action not only on immune cells but also on other somatic and neoplastic cells. They affect differentiation, growth and cell surface structures [4, 5]. We hypothesized that the expression of GSL in glioma cells could also be regulated by those active molecules. We first discovered that TNF-alpha, IFN-gamma, and IL-4 had unequivocal effects on the GSL profile of U118MG glioma cells, which clearly justified our hypothesis. However, U118MG cells changed their response to cytokines in repeated experiments and finally showed no response at all regardless of our attempts to reestablish reactivities. We report the results of a further trial on 6 other glioma cell lines and one melanoma cell line of the cytokine effect on GSL expression on glial cells.

MATERIAL AND METHODS

CELLS : Human glioma cell lines used were U87MG, U138MG, and U373MG, glioma cells purchased from American Type Culture Collection (ATCC) ; A172, T98G, and KG-1C glioma cells provided by Japanese Cancer Research Resources Bank (JCRB) ; and Mewo melanoma cells, also provided by JCRB. The cells were cultured in Dulbecco's MEM with 10% FCS supplemented with 0.1 mM of the non-essential amino acids, 2 mM L-glutamine, and 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 ml of the culture media.

CYTOKINES : The cytokines used in this study were : human recombinant IL-1 beta (Otsuka Pharmaceutical Co, Ltd., Tokyo ; 100 U/ml), human recombinant IL-2 (Takeda Pharmaceutical Co., Osaka ; 4000 JRU/ml), IL-4 (Ono Pharmaceutical Co., Osaka ; 100 U/ml), IL-6 (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 ; 1000 U/m*l*), IFN-beta (Toray Industries, Inc., Tokyo ; 1000 U/m*l*), IFN-gamma (Ostuka Pharmaceutical Co., Tokyo ; 1000 U/m*l*) and TNF-alpha (Mochida Pharmaceutical Company, Tokyo ; 10 U/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 three cycles of washing with PBS and centrifugation. Total lipids of 170 to 540 mg in wet weight from 2 or 3 culture dishes were extracted with C/M/W (4 : 8 : 3, v/v/v) by 2 consecutive 24hr extractions followed by a final 2 hour-extraction at 40℃. The extracts were applied to a gel filtration column (Sephadex LH-20, Pharmacia, Sweden) for desalting. The 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), developed with C/M/W (60 : 25 : 4, v/v/v) for neutral fraction, and C/M/0.02%-CaCl$_2$ (55 : 45 : 10, v/v/v) for acidic fraction. Standard glycosphingolipids are as follows : CMH and CS were purified from porcine brain, CDH from equine erythrocytes, Gb$_4$Cer from human erythrocytes, and GM1, GD1a, GD1b, GT1b from human brain. GSLs developed on the plate were stained by Orcin-sulfate reagent.

<div align="center">RESULTS</div>

KG-1C cells : The neutral GSL of KG-1C cells were visualized on TLC as faint bands of CMH, CDH, CTH, and nLc4Cer as identified below the Gb4Cer standard. As shown in Fig. 1, among the 10 cytokines, TNF-alpha, G-CSF, IFN-beta, IL-2, IL-1beta, IFN-alpha, IFN-gamma, IL-8, IL-6 and IL-4, all failed to influence the neutral GSL composition. The acidic GSL were composed of almost solely GM3 and a small component as identified as GM2 in untreated KG-1C cells, which was not affected by any of the 10 cytokines (Fig. 2).

T98G cells : The neutral GSL of T98G cells were composed of CMH, CDH, CTH and the fourth component below the Gb4Cer which was believed to be nLc4Cer. TLC immunostaining using KH2 monoclonal antibody which detects nLc4Cer showed that the fourth component was actually nLc4Cer from its posi-
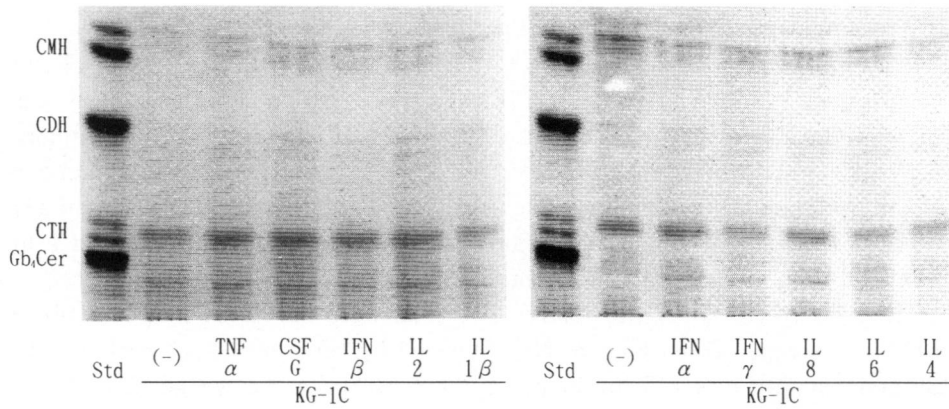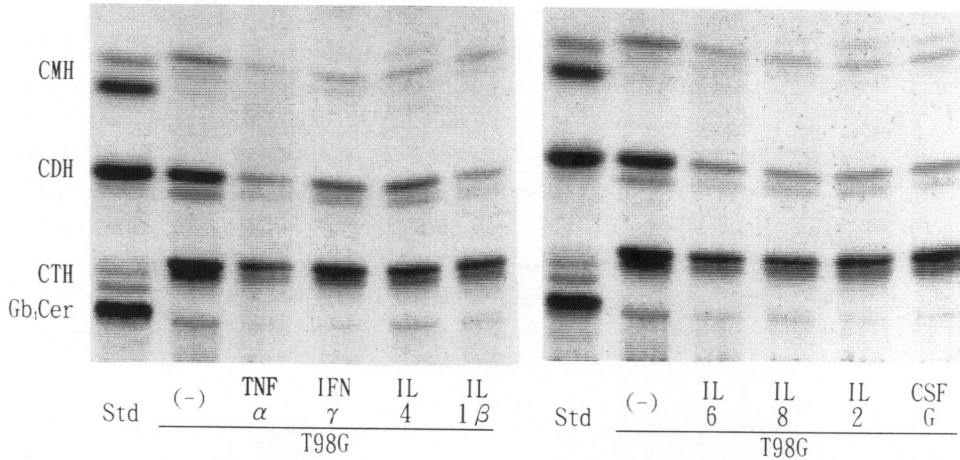
Fig. 1 Thin layer chromatogram of neutral glycolipids of KG-1C cells treated with 10 cytokines. The final concentration of cytokines were: human recombinant IL-1 beta, 100 U/m*l*; human recombinant IL-2, 4000 JRU/m*l*; IL-4, 100 U/m*l*; IL-6, 100 ng/m*l*; IL-8, 100 ng/m*l*; recombinant G-CSF, 2 ng/m*l*; IFN-alpha, 1000 U/m*l*; IFN-beta, 1000 U/m*l*; IFN-gamma, 1000 U/m*l*; and TNF-alpha, 10 U/m*l*. Those cytokines were added to 2 plates each when the cells grew to subconfluency, and the culture was continued for the next 48 hrs. Glycolipids applied on the TLC were equal to that derived from 50 mg wet weight of cells. Developing solvent was C/M/W (60 : 25 : 4, v/v/v). Glycolipids were visualized by Orcin-sulfate reagent.
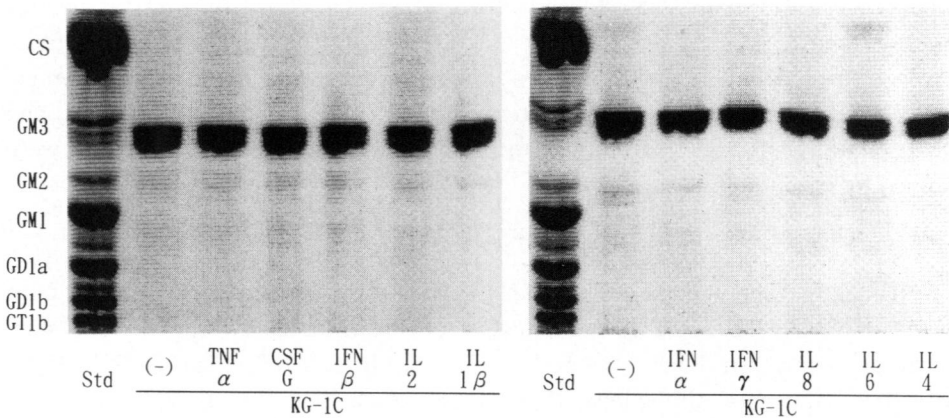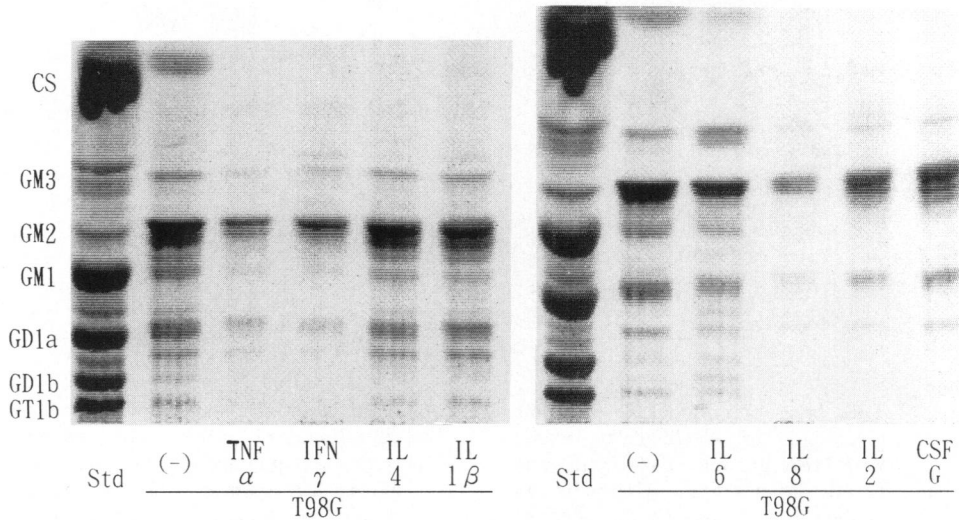

Fig. 2 Thin layer chromatogram of acidic glycolipids of KG-1C cells treated with 10 cytokines as described in Fig. 1. Glycolipids applied were equal to that derived from 100 mg wet weight of cells. Developing solvent was C/M/W containing 0.02% of $CaCl_2$ (55 : 45 : 10). Glycolipids were visualized by Orcin-sulfate reagent.

tive reaction against this antibody (data not shown). Eight cytokines were used for this cell line, TNF-alpha, IFN-gamma, IL-4, IL-1beta, IL-6, IL-8, IL-2 and

G–CSF, which resulted in all negative responses (Fig. 3). The acidic GSL consisted of multiple components: GM3, CM2, GM1, GD3, GD1a GD2, GD1b, and
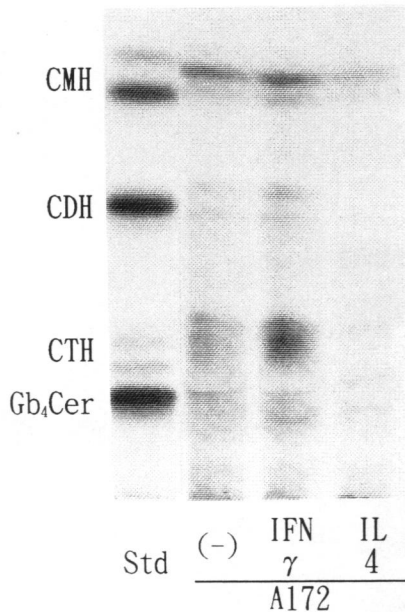

Fig. 3 Thin layer chromatogram of neutral glycolipids of T98G cells treated with 8 cytokines. The final concentration of cytokines was the same as described in Fig. 1. Glycolipids applied on the TLC were equal to that derived from 50 mg wet weight of cells. Developing solvent was C/M/W (60 : 25 : 4, v/v/v). Glycolipids were visualized by Orcin-sulfate reagent.
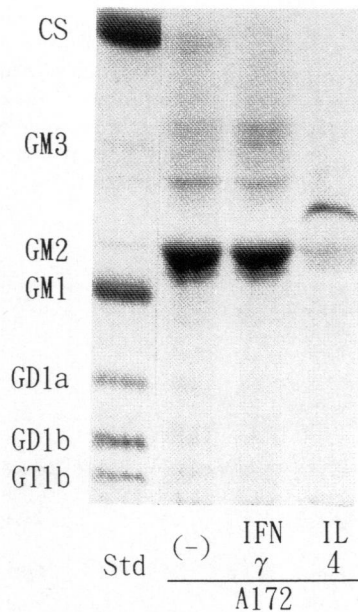

Fig. 4 Thin layer chromatogram of acidic glycolipids of T98G cells treated with 8 cytokines. Glycolipids applied were equal to that derived from 100 mg wet weight of cells. Developing solvent was C/M/W containing 0.02% of $CaCl_2$ (55 : 45 : 10). Glycolipids were visualized by Orcin-sulfate reagent.

GT1b, as listed from the top of TLC. As in the neutral GSL treated by cyto-kines, acidic components did not show any recognizable changes by the 8 cyto-kines (Fig. 4).

A172 cells: The neutral GSL of A172 cells were composed of CMH, CDH, CTH and a broad or split fourth band which was believed to consist of Gb4Cer and nLc4Cer. The cytokines used for this cell line were IFN-gamma and IL-4, which had previously shown remarkable effect on GSL composition of U118MG cells (14). As shown in Fig. 5, these two cytokines did not change the GSL profile of A172 cells. The acidic GSL components were GM3, GM2, LM1, GD1a, GD1b, and GT1b, among which GM2 was the most predominant component. Neither IFN-gamma nor IL-4 was ineffective to alter the GSL profile. On TLC of IL-4 treated A172 cells the band consistent with GM2 was elevated in a con-
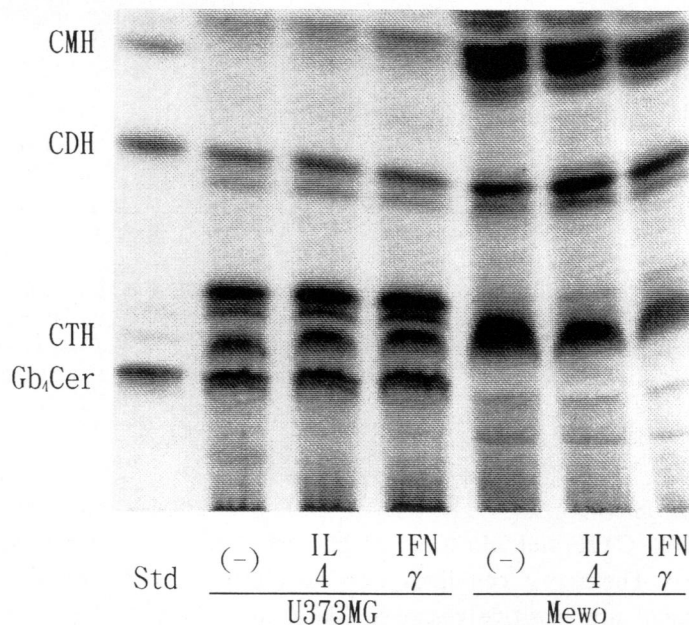

Fig. 5 Thin layer chromatogram of acidic glycolipids of A-172 cells treated with IFN-gamma (1000 U/ml) and IL-4 (100 U/ml). Glycolipids applied were equal to that derived from 100 mg wet weight of cells. Developing solvent was C/M/W containing 0.02% of CaCl$_2$ (55 : 45 : 10). Glycolipids were visualized by Orcin-sulfate reagent.

Fig. 6 Thin layer chromatogram of acidic glycolipids of A-172 cells treated with IFN-gamma (1000 U/ml) and IL-4 (100 U/ml). Glycolipids applied were equal to that derived from 100 mg wet weight of cells. Developing solvent was C/M/W containing 0.02% of CaCl$_2$ (55 : 45 : 10). Glycolipids were visualized by Orcin-sulfate reagent.

vex shape, but this is often seen as an artifact from salt contamination in the specimen (Fig. 6).

U373MG and Mewo cells: The neutral GSL of U373MG cells consisted of CMH, CDH, CTH which was recognized as three split bands probably due to the different length of fatty acid chains, and Gb4Cer. In another experiment, CTH of U373MG was recognized as two bands as usually seen in other glioma cell lines so that subspecies of GSL component may appear even in the consistent culture condition. The GSL of the Mewo melanoma cell line were CMH, CDH, CTH, a fourth component which was believed to be nLc4Cer, and an unidentified

Fig. 7 Thin layer chromatogram of neutral glycolipids of U373MG and Mewo cells treated with IFN-gamma (1000 U/m*l*) and IL-4 (100 U/m*l*). Glycolipids applied were equal to that derived from 50 mg wet weight of cells. Developing solvent was C/M/W containing 0.02% of CaCl$_2$ (55 : 45 : 10). Glycolipids were visualized by Orcin-sulfate reagent.

fifth band. Above two cell lines were also treated with IL-4 and IFN-gamma, which had no effect on the GSL composition (Fig. 7). The acidic GSLs of U373MG were GM3, GM2, GM1, GD3, GD1a, GD1b and GT1b from top to bottom of the TLC, while those of Mewo were GM3 (the most predominant component), GM2, GM1, GD3 (the second predominant component), GD1a, GD1b, and GT1b. As in neutral GSL, neither IL-4 or IFN-gamma influenced the GSL
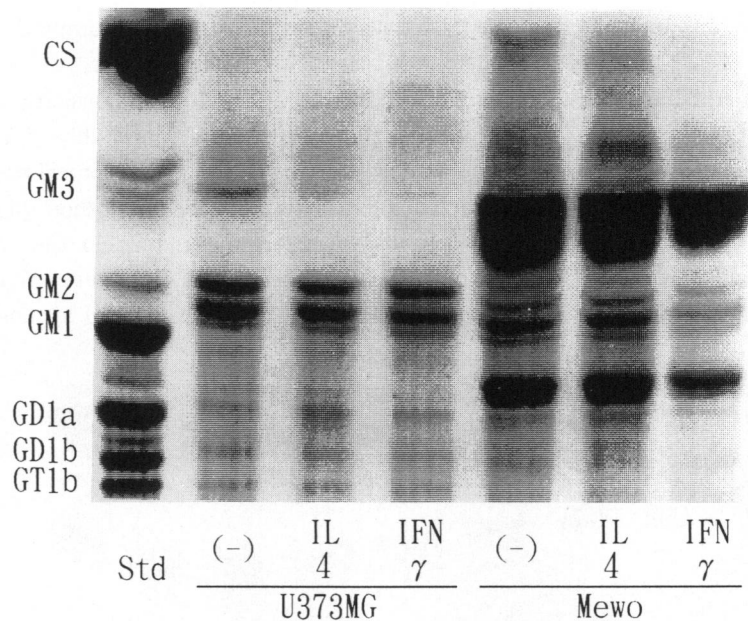
Fig. 8 Thin layer chromatogram of acidic glycolipids of U373MG and Mewo cells treated with
IFN-gamma (1000 U/ml) and IL-4 (100 U/ml). Glycolipids applied were equal to
that derived from 100 mg wet weight of cells. Developing solvent was C/M/W contain-
ing 0.02% of CaCl$_2$ (55 : 45 : 10). Glycolipids were visualized by Orcin-sulfate re-
agent.

profile (Fig. 8).

U138MG and U87MG cells: The neutral GSL of U138MG cells were composed
of CMH, CDH, CTH and nLc4Cer, which were almost identical with that of
U87MG cells. These two cell lines were similarly treated with IL-4 and IFN
-gamma without any positive response (data not shown). The acidic GSLs of
these two cell lines were composed from GM3, GM2, GM1, GD3, GD1a, GD1b
and GT1b; GM3 and GM2 were the major components. Neither L-4 nor IFN-
gamma affected the GSL expression in these cells (data not shown).

DISCUSSION

The biological actions of immune cytokines are not restricted to the immune
system; it has been long pointed out that the nervous system also utilizes
immune cytokines as communication signals within its system. As the central
nervous system has been regarded as an immunologically privileged site, it seems
contradictory to the traditional concept that immune cytokines are produced in
the nervous system This led us to wonder if the central nervous system may

form its own immune-like system using immune cytokines. In fact, it has been revealed that astrocytes actually can function as antigen presenting cells by expressing class II MHC antigens in response to such cytokines as IL-1, TNF-alpha, IFN-alpha, and IFN-gamma [6]. Astrocytes themselves seem to be capable of producing a variety of cytokines such as TNF-alpha, IFN-gamma, G-CSF, GM-CSF, IL-6 and IL-8 as suggested by recent polymerase chain reaction technique [7, Nitta, T., personal communication]. It is highly likely that cytokines play a key role in regulating astrocyte functions; if so, cytokines may modulate cell surface antigens to alter their functions including GSLs, which are richest in the nervous system.

It is well known that immune cytokines can influence the expression of cell surface antigens. IFN-gamma stimulates induction of MHC antigens or tumor antigens [8, 9, 13]; it also enhances cell adhesion molecules such as ICAM-1 [10]. TNF-alpha as well as IL-1beta similarly induces ELAM-1 molecules, which may play some physical role in triggering the immune reaction [11]. Certain cytokines have been found to enhance GSL antigens; IFN-alpha causes sialyl Lewis-X amplification [12], and IL-4 and IL-6 stimulate Forssman antigen expression on macrophages [13]. Yates, *et al.* [14, 15] examined the effect of IFN-beta on GSL composition of glioma cells without any remarkable change but fatty acid alteration. This is the first report describing the result of GSL analysis of 6 glioma cell lines treated with multiple cytokines.

Contrary to our expectation of positive cytokine effect on GSL expression on gliomas, no glioma cells examined in this study reacted to 2 to 10 cytokines. We previously reported elsewhere that U118MG cells had shown dramatic GSL modulation in responding to TNF-alpha, IL-4 and IFN-gamma [16, Yamaki, *et al.*, Neuroimmunol. Res. in press]. Unfortunately, the results were not reproducible and U118MG cells ultimately lost their sensitivity to cytokines so that further study was not possible. This report described the results of the same study using other glioma cell lines. Considering the completely negative result in this study we must conclude that glioma cells have the potential to respond to cytokines to modulate GSL expression, but only under special conditions.

ACKNOWLEDGMENT

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