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Gangliosides GD1a and GM3 Induce Interleukin-10 Production by Human T Cells[1]

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Gangliosides are sialic acid-containing glycosphingolipids and exhibit various physiologic functions. Gangliosides GD1a and GM3 strongly induced interleukin-10 (IL-10) protein secretion and mRNA expression in T cells from normal human subjects while the other gangliosides were ineffective. IL-10 induction by both gangliosides was completely blocked by protein tyrosine kinase (PTK) inhibitors, herbimycin A, genistein, and tyrphostin AG 1288, but not by other signal transduction inhibitors. These results suggest that GD1a and GM3 may induce IL-10 production in T cells by regulating the PTK-dependent signaling pathway. These gangliosides may thus act as important immunoregulators via IL-10. © 1999 Academic Press

Key Words: **GD1a; GM3; T cell; IL-10; protein tyrosine kinase.**

Gangliosides are sialic acid-containing glycosphingolipids, and are constituents of the plasma membranes of various cells (1). Some gangliosides are also shed into the extracellular environment, and exhibit various physiologic functions (1). Exogenous gangliosides interact with plasma membranes and modulate transmembrane signaling systems, and thus regulate cell growth and differentiation (1, 2). Previous studies reported that various gangliosides either inhibited or enhanced humoral immune responses in mice and humans (3–8). Some of these effects were mediated by modulating the production of B cell stimulatory cytokines (3–5). Interleukin-10 (IL-10) is one of those cytokines and differentiate B cells into immunoglobulin-secreting cells (9) though it also has pleiotropic effects on other cell lineages (10). In the peripheral blood, IL-10 is produced by B cells themselves, and also by other accessory cells like T cells or monocytes, and thus augment immunoglobulin production of B cells through autocrine and paracrine mechanisms (9). Kimata and Yoshida reported that ganglioside GM2 inhibited immunoglobulin production of human lymphoblastoid B cell lines and *in vivo* activated B cells partially by inhibiting their autocrine IL-10 production (3). However, they did not examine the effects of gangliosides on the paracrine IL-10 production by accessory cells. In this study, I investigated the effects of individual gangliosides on IL-10 production by human T cells. I focused on the spontaneous IL-10 production by T cells from normal human individuals since the usage of stimuli may alter the cellular responses to gangliosides, and thus mask the original effects of gangliosides on the constitutive IL-10 production of non-immortalized cells. The results revealed the strong stimulatory effects of GM3 and GD1a. I further analyzed the involvement of signaling pathways in those gangliosides' effects.

MATERIALS AND METHODS

Reagents. Highly purified bovine brain gangliosides GM1, GM2, GM3, GD1a, GD1b, GT1b, and GQ1b were purchased from Sigma, Chemical Co. (St. Louis, MO). Staurosporine, herbimycin A, genistein, tyrphostin AG 1288, *N*-[2-((*p*-bromocinnamyl)amino)-ethyl]-5-isoquinolinesulfonamide (H-89) and *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7) were obtained from Calbiochem (La Jolla, CA).

Preparation of human T cells. Blood was taken from healthy volunteers who were informed of the objectives and methods of this study, and consented to participate. Peripheral blood mononuclear cells were isolated by centrifugation over Ficoll–Paque (Pharmacia, Uppsala, Sweden) as described (11), and were allowed to adhere to plastic dishes for 1 h at 37°C. From the non-adherent cells, $CD56^-$ cells were isolated by negative selection using immunomagnetic beads (Dynal, Great Neck, NY) as described (12), and were incubated with neuraminidase-treated sheep erythrocytes as described (13). From the rosette-forming cells, $CD14^-$ and $CD19^-$ cells were isolated by the immunomagnetic negative selection, and were used as T cells. This T cell population was >98% $CD3^+$, and the contamination of $CD14^+$, $CD19^+$, or $CD56^+$ cells was <2%.

[1] The nomenclature for gangliosides follows the system of Svennerholm (26).

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Abbreviations used: ELISA, enzyme-linked immunosorbent assay; IL-10, interleukin-10; PTK, protein tyrosine kinase; RT-PCR, reverse transcription polymerase chain reaction.

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Enzyme-linked immunosorbent assay (ELISA) for IL-10. T cells (2×10^5/200 μl/well) were cultured in triplicate in round bottom 96-well tissue culture plates with or without gangliosides at indicated doses in the culture medium at 37°C in an atmosphere of 5% CO_2 in air for 24 h (unless otherwise indicated). Since the high concentration of serum hinders the ganglioside binding to the cell membrane (14–17), we used serum-free Hymedium 920 (Kohjin Bio, Tokyo, Japan) containing 250 mg/liter human albumin, and this level of albumin is appropriate for the cell maintenance and ganglioside binding (15–17). The composition of amino acids, minerals, or vitamins in Hymedium 920 is similar to that in RPMI 1640 (Life Technologies, Grand Island, NY), except that the former medium contains 0.48 mg/liter thymidine, 2 mg/liter human transferrin, 0.2 μg/ml 3,3′,5-triiodo-L-thyronine, and 60 mg/liter kanamycin. Gangliosides were dissolved in absolute ethanol and diluted at least 1000-fold to the required concentrations in the culture medium. Control cultures contained ethanol at the highest concentration used in the experimental cultures, and this level of ethanol was not toxic to PBMC. The culture supernatants were then harvested and stored at −70°C until used. The activity of IL-10 in the culture supernatants was measured by an ELISA kit (Endogen Inc., Cambridge, MA) according to the manufacturer's instruction. The detection limit for IL-10 was 3 pg/ml.

Reverse-transcription polymerase chain reaction (RT-PCR). T cells (3×10^6 cells/3 ml/well) were incubated in 12-well plates for 4, 8, and 12 h in the presence or absence of gangliosides at 10 μM, and total cellular RNA was extracted using mRNA purification kit (Pharmacia) according to the manufacturer's instruction. cDNA was made from RNA samples as described (18). PCR was initiated in the thermal cycler programmed for 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles as described (18). The following primer sequences were used: 5′AGAGCGCCAGATCCGATTTT-3′ and 5′-ATCAAGGCGCATGTGAACTC-3′ as IL-10 antisense and sense primers, 5′-GGTCTCAAACATGATCTGGG-3′ and 5′-GGGTCAGA-AGGATTCCTATG-3′ as β-actin antisense and sense primers, respectively. The PCR products were analyzed by electrophoresis on 2.5% agarose gels and stained with ethidium bromide. The PCR product size was 295 and 268 bp for IL-10 and β-actin, respectively.

Statistical analyses. For the data in Fig. 1, one-way analysis of variance with Dunnet's multiple comparison test was used. For the data in Fig. 3, one-way analysis of variance with Scheffe's multiple comparison test was used. A value of $P < 0.05$ was considered significant.

RESULTS

Dose dependency for the effects of individual gangliosides on IL-10 protein secretion by human T cells. As shown in Fig. 1, GD1a and GM3 strongly induced IL-10 secretion in T cells though only marginal level of IL-10 (5 pg/ml) was secreted in control. The stimulatory effects of both gangliosides were revealed at 0.1 μM, increased dose-dependently, and were maximized at 10 μM. On the other hand, the other gangliosides GM1, GM2, GD1b, GD2, GD3, GT1b, and GQ1b were ineffective for IL-10 secretion. The results were similar after 48 and 72 h of culture (data not shown). After 24, 48, and 72 h of culture, all the gangliosides (0.01 to 100 μM) did not significantly change the proliferation and viability of T cells as examined by ^{3}H-thymidine uptake and trypan blue exclusion test, respectively (data not shown). Since 10 μM seems optimal concentration for IL-10 induction by GD1a and GM3, this concentration is used in subsequent experiments.

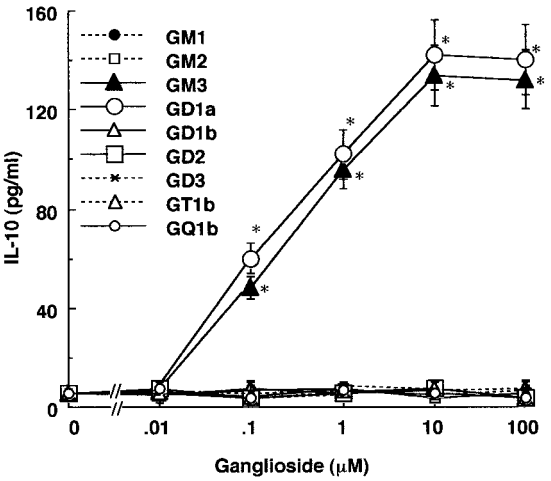

FIG. 1. Dose dependency for the effects of various gangliosides on IL-10 protein secretion. T cells from one healthy donor were cultured in triplicate for 24 h in the presence or absence of indicated doses of gangliosides. The culture supernatants were assayed for IL-10 by ELISA. Values are the mean ± SD of triplicate cultures. *$P < 0.001$ versus control cultures. The data are representative of five separate experiments using T cells from five different donors.

Induction of IL-10 mRNA expression by GD1a and GM3. As shown in Fig. 2, IL-10 mRNA expression is induced in T cells treated for 4 h with GD1a and GM3 (10 μM) whereas that is not detected in controls and in T cells treated with the other gangliosides (not shown). The expression of IL-10 mRNA was also demonstrable after 8 and 12 h of incubation with GD1a and GM3 (data not shown).

The involvement of signal transduction pathways in IL-10 secretion induced by GD1a and GM3. To evaluate signal transduction pathways involved in IL-10 induction by GD1a and GM3, we preincubated T cells with several signal transduction inhibitors, and then stimulated them with these gangliosides, and examined the influence of these inhibitors. As shown in Fig. 3, Herbimycin A, a protein tyrosine kinase (PTK) inhibitor, completely inhibited both GD1a and GM3 effects on IL-10 secretion. In contrast, protein kinase C inhibitor staurosporine, protein kinase A inhibitor H-89, and Ca^{2+}/calmodulin-dependent enzyme inhibitor W-7 did not block the effects of these gangliosides. Figure 4 shows the dose dependency for the inhibitory effects of herbimycin A; IC_{50} for the inhibition of IL-10 induction by GD1a and GM3 were 0.18 and 0.15 μM, respectively. Other PTK inhibitors, genistein and AG 1288, also blocked IL-10 induction by both gangliosides, although on a molar basis less efficiently than herbimycin A; IC_{50} for GD1a and GM3 were 20 and 18 μM in genistein, and 18 and 12 μM in AG 1288, respectively. These PTK inhibitors also inhibited IL-10 mRNA induction by GD1a and GM3 as examined by RT-PCR (data not shown). These results suggest that PTK-dependent signaling pathway may be involved in

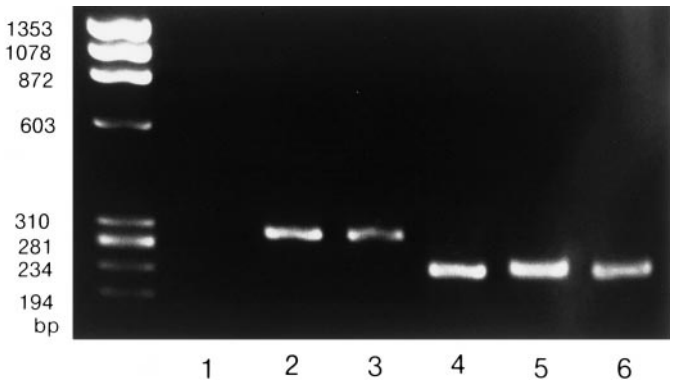

FIG. 2. RT-PCR analysis for the effects of GD1a and GM3 on IL-10 mRNA expression in T cells. T cells were cultured for 4 h with medium alone (lanes 1 and 4), 10 μM GD1a (lanes 2 and 5), or 10 μM GM3 (lanes 3 and 6), and RNA was extracted. RT-PCR products for IL-10 and β-actin are shown in lanes 1 to 3 and lanes 4 to 6, respectively. A size marker is shown in the far left lane. The results are representative of four separate experiments using T cells from four different donors.

the GD1a and GM3-induced IL-10 production in T cells.

DISCUSSION

This study demonstrated that GD1a and GM3 induced IL-10 mRNA expression and protein secretion in T cells. These results indicate that GD1a and GM3 may enhance humoral immunity via IL-10 induction. These gangliosides may also act as suppressors for cell-mediated immunity since IL-10 inhibits the production of Th1 cytokines like interferon-γ or IL-2, and also suppresses macrophage activity (10).

PTK activation is required for the IL-10 induction by GM3 and GD1a. Previous study also reported that GD1a down-modulated CD4 expression on T cells partially via PTK (19). Though precise mechanisms how these gangliosides promote PTK activity in T cells are unknown, it is suggested that they may interact with T cell surface proteins linked to src family nonreceptor PTK (20, 21). Especially GM3 is a major ganglioside in T cell plasma membranes and is enriched in the detergent-resistant areas of the membranes (membrane microdomains) (20, 21). Several reports suggest that GM3 in these microdomains may form complexes with CD4 (20) and/or with glycosylphosphatidylinositol-anchored glycoproteins like CD59 (21), which are both closely associated with nonreceptor PTK such as $p56^{lck}$. It is thus hypothesized that exogenous GM3 and/or GD1a may also bind to these nonreceptor PTK-linked proteins in the membrane microdomains, and trigger early tyrosine phosphorylation and thus induce downstream signals leading to the expression of IL-10 genes. These gangliosides may also interact with other T cell surface activation molecules associated with nonreceptor PTK, i.e., CD3/T cell receptor complex, CD8 molecule, or IL-2 receptor β chain (22). Thus the target molecules for GM3 and GD1a should further be elucidated. On the other hand, it is reported that these gangliosides inhibit several growth factor receptor-associated PTK; GM3 and GD1a inhibited tyrosine phosphorylation of epidermal growth factor (23) and platelet-derived growth factor receptors (24), respectively. It is thus rather implausible that these growth factor receptor-associated PTK may be involved in IL-10 induction by these gangliosides, however, this possibility should also be investigated.

It is unknown why only GD1a and GM3 were effective for IL-10 induction and the other gangliosides were ineffective. The immunoregulatory activity of gangliosides may be related to their carbohydrate structure; GD1a and GM3 contain NeuAcα23Galβ1 at the non-reducing end of the sugar chain (15) and this may be essential for the IL-10 induction. However, this structure seems not to be decisive since GT1b, having this structure, did not induce IL-10 production. Another determining factor may reside in the ceramide residues such as the ratio of 18-carbon to 20-carbon sphingosine (25). The precise relationship between

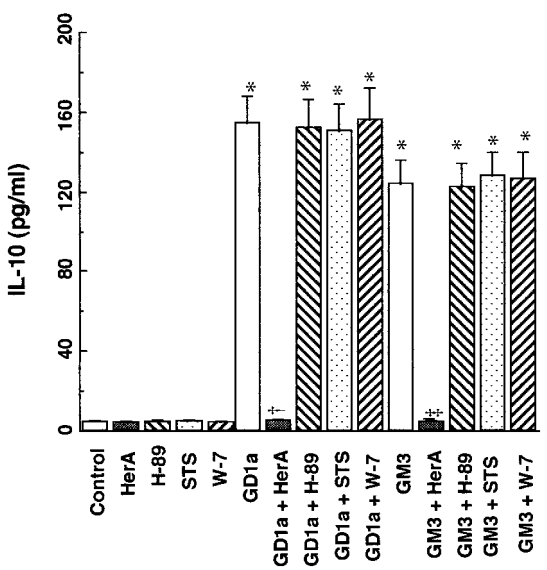

FIG. 3. The effects of signal transduction inhibitors on IL-10 secretion induced by GD1a and GM3. T cells from 1 healthy donor were preincubated for 1 h with medium alone or with medium containing signal transduction inhibitors before the addition of GD1a or GM3 (10 μM). After 24 h, the culture supernatants were assayed for IL-10 by ELISA. The inhibitors were herbimycin A (HerA) (1 μM), H-89 (0.1 μM), staurosporine (STS) (1.5 nM), and W-7 (50 μM). The concentration of each inhibitor was determined to specifically inhibit the aimed signal transduction pathway as recommended by the manufacturer. Values are the mean $\pm$ SD of triplicate cultures. *$P < 0.001$ versus control cultures. †$P < 0.001$ versus cultures with GD1a alone. ‡$P < 0.001$ versus cultures with GM3 alone. The data are representative of five separate experiments using T cells from five different donors.

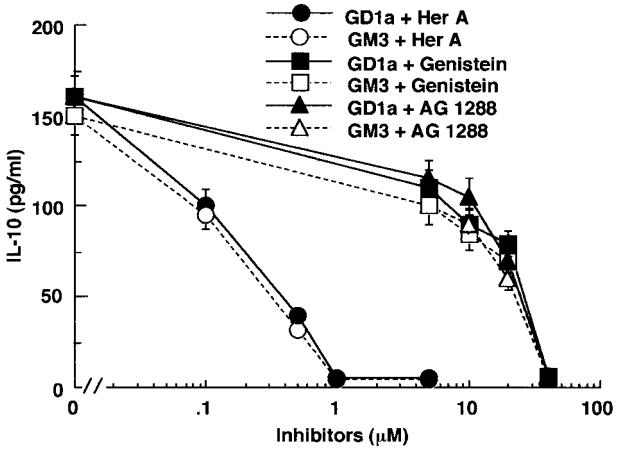

FIG. 4. Dose dependency for the effects of PTK inhibitors on IL-10 secretion induced by GD1a and GM3. T cells from 1 healthy donor were preincubated for 1 h with either herbimycin A (HerA), genistein, or AG 1288 at the indicated doses, and stimulated with GD1a or GM3 (10 μM). After 24 h, the culture supernatants were assayed for IL-10 by ELISA. Values are the mean ± SD of triplicate cultures. The basal IL-10 secretion in the absence of gangliosides was 5 pg/ml. The data are representative of five separate experiments using T cells from five different donors.

ganglioside structure and immunoregulatory activity remains to be elucidated.

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