Complex Gangliosides as Cell Surface Inhibitors for the Ecto-NAD$^+$ Glycohydrolase of CD38†

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ABSTRACT: Leukocyte cell surface antigen CD38 is a single-transmembrane protein whose extracellular domain has catalytic activity for NAD$^+$ glycohydrolase (NADase). We previously reported that b-series gangliosides inhibit the NADase activity of the extracellular domain of CD38 expressed as a fusion protein [Hara-Yokoyama, M., Kukimoto, I., Nishina, H., Kontani, K., Hirabayashi, Y., Irie, F., Sugiya, H., Furuyama, S., and Katada, T. (1996) *J. Biol. Chem. 271*, 12951−12955]. In the present study, we examined the effect of exogenous gangliosides on the NADase activity of CD38 on the surface of retinoic acid-treated human leukemic HL60 cells and CD38-transfected THP-1 cells. After incubation of the cells with G_{T1b}, inhibition of NADase activity was observed. The time course of inhibition was slower than that of the incorporation of G_{T1b} into the cells, suggesting that incorporation into the cell membranes is a prerequisite for inhibition. Inhibition occurred efficiently when G_{T1b} and CD38 were present on the same cells (cis interaction) rather than on different cells (trans interaction). Although gangliosides may affect localization of cell surface proteins, indirect immunofluorescence intensity due to CD38 was not affected after G_{T1b} treatment. Comparison of the effect of G_{T1b} and G_{D1a} indicates that the tandem sialic acid residues linked to the internal galactose residue of the gangliotetraose core are crucial to the inhibition. These results suggest a novel role of complex gangliosides for the first time as cell surface inhibitors of CD38 through specific and cis interaction between the oligosaccharide moiety and the extracellular domain.

Gangliosides are lipid components of cell membranes. The polar part of gangliosides is a sialic acid-containing oligosaccharide moiety which is mainly extruded on the outer surface. Gangliosides have been considered to be involved in cell-to-cell interactions such as adhesion, and in morphogenesis processes (for review, see ref *2*). Gangliosides are also known to regulate the activities of various receptor-associated tyrosine kinases such as the receptors of insulin (*3*), epidermal growth factor (*4*), and nerve growth factor (*5*, *6*). Thus, gangliosides have been suggested to play a role as regulators of cell signaling. Recently, GM2/GD2[1] synthase gene (β1,4-*N*-actylgalactosaminyltransferase; EC 2.4.1.92) was disrupted in mice, which lack all complex gangliosides (*7*). This was expected to result in severe abnormalities of neuronal cell-to-cell interactions, as complex gangliosides are abundantly expressed in nerve tissues. However, the mutant mice developed normally without significant histological defects in the central nervous systems. Further study revealed axonal degeneration and myelination defects in the nervous system (*8*). On the other hand, the phenotypes of the homozygous mutant were found also in nonneuronal tissues. The mutant male mice were sterile and showed serious defects in the testis (*9*). In addition, the response of spleen T cells to interleukin 2 was impaired in the mutant mice (*10*). The underlying mechanisms have not been elucidated at present. Therefore, furher in vitro study of the roles of complex gangliosides is required to understand these phenotypes at the molecular level.

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[1] Ganglioside nomenclature is based on that of Svennerholm (*1*).

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In addition to its well-known role as a coenzyme, NAD^+ serves as a substrate of NAD^+ glycohydrolases, ADP-ribosyl cyclases, and ADP-ribosyltransferases. Accumulating evidence supports the physiological significance of NAD^+ metabolisms in mammalian cells. Cyclic ADP-ribose (cADPR),[2] formed from NAD^+ by ADP-ribosyl cyclase, is a potent Ca^{2+} mobilizer that increases the Ca^{2+} sensitivity of the ryanodine-sensitive Ca^{2+} release mechanism. Thus, cADPR acts as a second messenger, different from inositol 1,4,5-triphosphate, during Ca^{2+}-mediated responses (for review, see ref *11*). On the other hand, a glycosylphosphatidylinositol (GPI)-anchored ADP-ribosyltransferase responsible for the suppression of T cell receptor activation has been identified (*12*−*14*). Other related GPI-anchored ADP-ribosyltransferases have also been reported (*15, 16*), suggesting the involvement of cell surface NAD^+ metabolisms in cell signaling.

Lymphocyte cell surface antigen CD38 is a type II transmembrane protein composed of a short cytoplasmic domain, a transmembrane domain, and a large extracellular domain (for review, see refs *17*−*19*). The extracellular domain has the enzymatic activity of NAD^+ glycohydrolase (*20*), capable of both generating and degrading cyclic ADP-ribose (*21*−*23*). Consistent with the cADPR-producing activity of CD38, transfection or disruption of the CD38 gene in HeLa 3T3 cells or in mice, respectively, has been found to increase the intracellular Ca^{2+} concentration (*24*) or impair glucose-induced increase in the intracellular Ca^{2+} concentration (*25*). CD38-deficient mice show a complete loss of tissue-associated NAD^+ glycohydrolase activity (*26*). The requirement of CD38 in humoral immune responses (*26*) and in glucose-dependent insulin secretion (*25*) has been confirmed.

We previously reported for the first time the inhibitory effect of b-series gangliosides on enzymes acting on NAD^+, ADP-ribosyltransferases, NAD^+ glycohydrolases, and ADP-ribosyl cyclases, including CD38 (*27, 28*). The tandem sialic acid residues linked to the internal galactose residue are crucial to this inhibition. The lactonization of ganglioside suggests that the negative charges in the carboxyl groups of the sialic acid residues are involved in the inhibition. Thus, we have proposed that two carboxyl groups of the tandem sialic acid residues mimic the diphosphate moiety of NAD^+ and that in this way b-series gangliosides act as inhibitors of NAD^+-metabolizing enzymes (*27, 28*).

When we previously examined the effect of gangliosides on CD38 activities, a recombinant fusion protein of the extracellular domain of CD38 with maltose binding protein (MBP−CD38) was used. Therefore, the present study was undertaken to investigate whether gangliosides can play a regulatory role on native CD38 present in the cell surface membrane. We examined the effect of gangliosides on CD38 expressed in HL60 cells, since expression of CD38 can be induced in HL60 cells by retinoic acid (*29*). It had been previously confirmed that NAD^+ glycohydrolase activity in retionic acid-differentiated HL60 cells is attributed to CD38 (*20*). In addition, no complex gangliosides occur endogenously in HL60 cells, rendering them ideal for evaluation of the effect of exogenous higher gangliosides on CD38. In particular, our system enables us to compare the effect of G_{T1b} and G_{D1a} to evaluate the involvement of the tandem sialic acid residues. Interpretation may be complicated if cells or transfectants that express G_{T1b} endogenously are used, since such cells also bear a series of precursor gangliosides.

Our results demonstrate that exogenous complex gangliosides inhibit CD38 NAD^+ glycohydrolase activity on the cell surface. Several lines of evidence suggest that this inhibition is due to interaction between the oligosaccharide moiety of gangliosides and the extracellular domain of CD38 on the same cell surface (cis interaction). Such cell surface inhibition of the ecto-enzyme represents a new concept to explain the physiological roles of complex gangliosides. Possible structural and functional aspects of the interaction are discussed.

EXPERIMENTAL PROCEDURES

Cell Culture. HL60 cells and THP-1 cells were cultured in RPMI-1640 containing 10% heat-inactivated fetal calf serum and 200 μg/mL kanamycin at 37 °C in 95% air and 5% CO_2. HL60 cells were treated with 1 μM retinoic acid (RA-HL60 cells) for 1 or 2 days as described previously (*30*). Mouse hybridoma HB136 cells, which produce an anti-CD38 monoclonal antibody (HB7, subclass IgG1), were obtained from the American Type Culture Collection. Surface expression of CD38 after the retinoic acid treatment for 2 days was about 10^5 molecules per RA-HL60 cell based on the binding of [^{125}I]anti-CD38 monoclonal antibody (HB7).

Establishment of CD38 Transfectant. Human full-length CD38 cDNA was recovered from pCDM:CD38 (kindly supplied by Dr. David G. Jackson) by *Hin*dIII and *Xho*I digestion, and then ligated into the expression vector pMKITNeo, resulting in pMKITNeo:CD38. pMKITNeo:CD38 and pMKITNeo (for control) were linearized with *Sma*I digestion and transfected into THP-1 cells using a standard electroporation procedure. The transfected cells were selected with G418 (0.6 mg/mL, Sigma), and G418-resistant cells were isolated. Expression of CD38 was monitored by Western blotting, immunofluorescence, and NAD^+ glycohydrolase activity. CD38-transfected THP-1 cells were routinely maintained under G418 (0.2 mg/mL) selection.

Materials. [*carbonyl*-^{14}C]NAD^+ (41 mCi/mmol, 1.2 mM) was purchased from Amersham LIFE SCIENCE (U.K.). Another mouse anti-CD38 monoclonal antibody (HB7) was ^{125}I-iodinated with chloramine T and used to estimate the surface expression of CD38. E-RDF medium was purchased from Kyokuto Pharmaceutical Corp. (Tokyo, Japan). The medium is completely serum-free and contains insulin and transferrin. Gangliosides were purified from total brain gangliosides as described previously (*31*−*33*). The oligosaccharide moiety of G_{T1b} was prepared using endoglycoceramidase (Takara, Japan) as described previously (*28*).

Ganglioside Treatment. RA-HL60 cells were washed 3 times with serum-free medium (E-RDF) and suspended in E-RDF at a cell density of 5×10^5 cells/mL or 2×10^6 cells/mL. Stock solutions of gangliosides in methanol were dried and dissolved in E-RDF just before use. After addition of the ganglioside solution to the cell medium, the cells were incubated at 37 °C for the indicated times. For measurement of NAD^+ glycohydrolase activity, the reaction was started

[2] Abbreviations: cADPR, cyclic ADP-ribose; GPI-anchored, glycosylphosphatidylinositol-anchored; MBP, maltose-binding protein; RA-HL60 cells, retinoic acid-treated HL60 cells; MβCD, methyl-β-cyclodextrin.

without washing the cells. The viability of cells was more than 99% after the treatment based on trypan blue staining. We observed a slight aggregation of the cells after incubation with G_{T1b} when HL-60 cells were treated with retinoic acid for 2 days. On the other hand, such aggregation did not occur at all when cells treated with retinoic acid for 1 day were used. The difference may be because some adhesion molecules are not yet expressed after 1 day treatment. However, there were no essential differences in the inhibitory effect of G_{T1b} in RA-HL60 cells between the two conditions in terms of time course, does-dependency, influence of MβCD, and the order of the potency of various gangliosides (Figures 1, 3, 6, and 7).

Lipid Analysis. The cells were washed 3 times with PBS. Lipids were extracted by chloroform/methanol at sequential ratios of 2:1, 1:1, and then 1:2, and the extracts were combined. For the analysis of gangliosides, the lipids were developed on a precoated high-performance thin-layer chromatography plate (Silica Gel 60; E. Merck, Darmstadt, Germany) with chloroform/methanol/12 mM $CaCl_2$ (5:4:1, v/v/v). The gangliosides were visualized with the resorcinol/HCl reagent. Quantification of ganglioside was done through color development using the resorcinol/HCl reagent using *N*-acetylsialic acid as a standard. Cholesterol was measured using a cholesterol oxidase-based assay kit (Boehringer-Mannheim GmbH, Germany).

Measurement of NAD$^+$ Glycohydrolysis. To measure the NAD$^+$ glycohydrolysis catalyzed by RA-HL60 cells, the reaction was started by the addition of 2.5 μL of 240 μM [*carbonyl*-^{14}C]NAD$^+$ to 50 μL of the cell suspension (5×10^5 or 1×10^6 cells/mL). After incubation for 5 min at 37 °C, aliquots (10 μL) were withdrawn and spotted onto Whatmann 3MM paper. The paper was developed, and the radioactivities of [^{14}C]NAD$^+$ and [^{14}C]nicotinamide were measured as described previously (*28*). The ratio of the radioactivity of [^{14}C]nicotinamide to the sum of radioactivities of [^{14}C]nicotinamide and the remaining [^{14}C]NAD$^+$ was calculated. The breakdown of original [^{14}C]NAD$^+$ was separately evaluated and subtracted from all the values.

Flow Cytometric Analysis. The cells were washed with PBS once and incubated with anti-CD38 monoclonal antibody (T16) or control mouse immunogloblin [20 μg (4×10^6 cells)$^{-1}$ (80 μL)$^{-1}$] on ice for 2 h. The cells were then washed with PBS and further incubated with fluorescein isothiocyate-labeled anti-mouse IgG (DAKO, Denmark) on ice for 30 min. Fluorescence intensity was measured with CytoACE-150 (Jasco, Japan). Vital cells were gated on the bases of forward angle light scatter and 90° light scatter parameters.

Cyclodextrin Treatment. RA-HL60 cells were washed 3 times with E-RDF and suspended in E-RDF at a cell density of 2×10^7 cells/mL. The cells were incubated for 1 h at 37 °C in the absence or presence of 5−10 mM methyl-β-cyclodextrin (Sigma) in E-RDF. The viability of cells was more than 90% after the treatment based on trypan blue staining. Flow cytometric analysis did not show any decrease in the surface expression of CD38 by methyl-β-cyclodextrin treatment.

RESULTS

Effect of G_{T1b} and Related Gangliosides on NAD$^+$ Glycohydrolysis by RA-HL60 Cells. The NAD$^+$ glycohydrolase activity of MBP−CD38 is inhibited by b-series gangliosides, especially by $G_{Q1b\alpha}$, G_{Q1b}, and G_{T1b} (*28*). In the present study, we mainly focused on the effect of G_{T1b} on the NAD$^+$ glycohydrolase activity of CD38 expressed on the surface of RA-HL60 cells. First, the cells were incubated with G_{T1b} or structurally related gangliosides for 3 h, and then NAD$^+$ glycohydrolysis was measured. As shown in Figure 1, NAD$^+$ glycohydrolysis was decreased after incubation with G_{T1b}. A similar decrease was also observed in the case of G_{D1b}. On the other hand, the effects of G_{D1a} and G_{D3} were smaller than that of G_{T1b}, and G_{M1b} had no significant effect. The result may suggest an inhibitory effect of gangliosides on cell surface CD38 that depends on the oligosaccharide moieties. However, to evaluate the action of exogenous gangliosides, it is necessary to investigate which ganglioside state is responsible for the effect, namely, present in the medium or incorporated into the membranes.

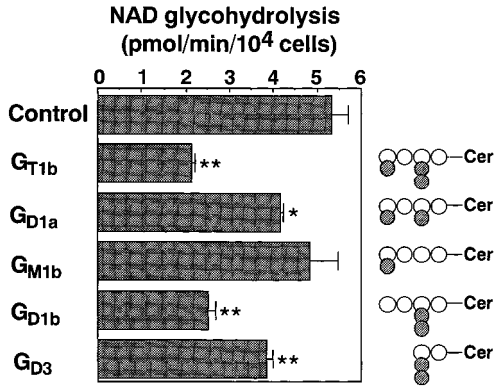

FIGURE 1: Effect of GT1b and structurally related gangliosides on NAD$^+$ glycohydrolysis catalyzed by RA-HL60 cells. HL60 cells treated with retinoic acid for 1 day (5×105 cells/mL) were incubated with 50 μM gangliosides for 3 h, and NAD$^+$ glycohydrolysis was measured as described under Experimental Procedures. Values are means ± SD from triplicate assays. *, $p < 0.05$; **, $p < 0.01$. Essentially the same result was obtained in HL60 cells treated with retinoic acid for 2 days. The filled circles indicate the sialic acid residues.

Time Course of Incorporation of G_{T1b} into RA-HL60 Cells and Its Effect on NAD$^+$ Glycohydrolysis. To evaluate the action of exogenous gangliosides, we examined the time course of incorporation of G_{T1b} and its effect on NAD$^+$ glycohydrolysis. After RA-HL60 cells were incubated with G_{T1b}, lipids were extracted from the cells and analyzed by thin-layer chromatography (Figure 2B), and the amount of G_{T1b} in the extracts was measured (Figure 2A). Incorporation of G_{T1b} reached a plateau level after1 h, and G_{T1b} was stable up to 5 h.

As shown in Figure 3A, G_{T1b} had no effect when NAD$^+$ glycohydrolysis was measured immediately after the addition of G_{T1b} (time zero). The incorporation of G_{T1b} at time zero was eliminated by trypsin treatment (data not shown). Thus, G_{T1b} that is present in the medium or peripherally associated with the membranes does not affect NAD$^+$ glycohydrolysis. As the time course of the inhibitory effect of G_{T1b} was slower than that of its incorporation (compare Figures 2A and 3A), it is suggested that the incorporation of G_{T1b} into the cell membranes is a prerequisite for the inhibitory effect on cell surface CD38.

Access of the oligosaccharide moiety of G_{T1b} to CD38 may be hindered by the ceramide moiety when G_{T1b} is present in

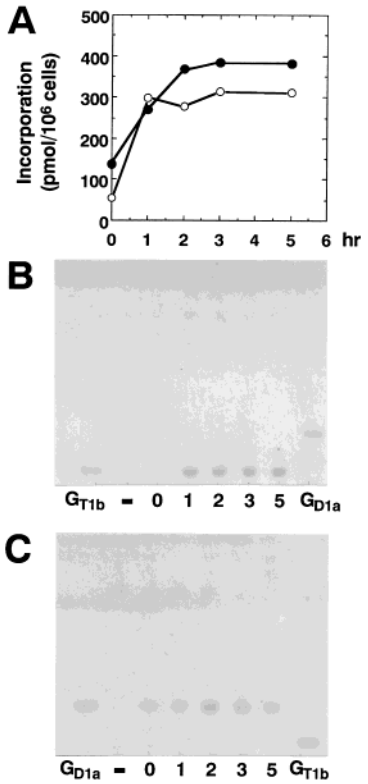

FIGURE 2: Time course of incorporation of G_{T1b} and G_{D1a} into RA-HL60 cells. HL60 cells treated with retinoic acid for 1 day (2×10^6 cells/mL) were incubated with 50 μM G_{T1b} or G_{D1a} at 37 °C for the indicated times. The lipids were then extracted as described under Experimental Procedures. (A) Incorporation of G_{T1b} (open circles) and G_{D1a} (filled circles) was quantified through color development using the resorcinol/HCl reagent. (B, C) Lipids equivalent to 10^6 cells after incubation with G_{T1b} (B) and G_{D1a} (C) for the indicated times (hours) were analyzed on TLC. Lipids from cells incubated in the absence of gangliosides were also analyzed (−, in panels B and C). The gangliosides were visualized by the resorcinol/HCl reagent. Standard G_{T1b} (300 pmol) and G_{D1a} (450 pmol) were spotted on both sides.

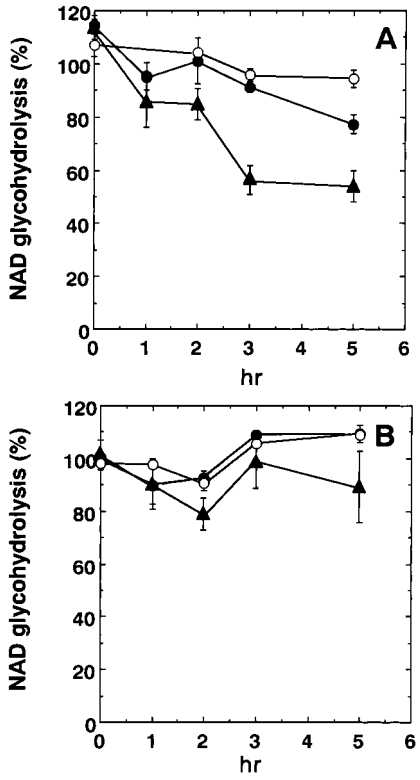

FIGURE 3: Time course of the inhibitory effect of G_{T1b} and G_{D1a} on NAD^+ glycohydrolysis in RA-HL60 cells. HL60 cells treated with retinoic acid for 1 day (1×10^6 cells/mL) were incubated with 10 μM (○), 20 μM (●), or 50 μM (▲) G_{T1b} (A) or G_{D1a} (B) at 37 °C. NAD^+ glycohydrolysis was measured as described under Experimental Procedures. The values are means ± SD from triplicate assays, expressed as percentages of the control values in the absence of the gangliosides at each indicated time, namely, 8.0 and 7.7 pmol min^{-1} $(10^4$ cells$)^{-1}$ (time zero), 5.7 and 6.0 pmol min^{-1} $(10^4$ cells$)^{-1}$ (1 h), 5.8 and 6.0 pmol min^{-1} $(10^4$ cells$)^{-1}$ (2 h), 5.5 and 6.0 pmol min^{-1} $(10^4$ cells$)^{-1}$ (3 h), and 5.8 and 6.4 pmol min^{-1} $(10^4$ cells$)^{-1}$ (5 h), for G_{T1b} and G_{D1a}, respectively. Essentially similar results for G_{T1b} were obtained in HL60 cells treated with retinoic acid for 2 days.

the medium. This situation can explain why it is necessary for G_{T1b} to be incorporated into the cell membranes for inhibition. To examine this possibility, we prepared the oligosaccharide moiety of G_{T1b} by endoglycoceramidase cleavage. As shown in Figure 4, the oligosaccharide moiety of G_{T1b} had almost no effect on NAD^+ glycohydrolysis, as well as on the inhibitory effect of G_{T1b}. Therefore, the entire structure of G_{T1b} that can be integrated in the lipid bilayer is necessary for the inhibitory effect.

Flow Cytometric Analysis of RA-HL60 Cells after Ganglioside Treatment. Internalization of the surface protein by exogenous gangliosides has been reported. For example, indirect immunofluorescence intensity due to CD4 on the lymphoma cell line MOLT-3 cells or human peripheral blood lymphocytes was reduced to approximately 10% after incubation with G_{M1a} (*34*) or G_{M3} (*35*), respectively. Thus, we investigated whether gangliosides induce such a decrease in immunofluorescence intensity due to CD38 in RA-HL60 cells. After incubation of RA-HL60 cells with G_{T1b} or G_{M1a} for 3 h, the cells were labeled with anti-CD38 monoclonal antibody (T16) or control mouse IgG and analyzed by flow cytometry. Inhibition of NAD^+ glycohydrolysis by G_{T1b} and G_{M1a} was 45% and 25%, respectively. As shown in Figure 5, the fluorescence intensity spectrum was not affected by G_{T1b} or G_{M1a} treatment. In addition, we observed no apparent differences in anti-CD38 antibody-staining patterns among RA-HL60 cells with and without G_{T1b} or G_{M1a} treatment in confocal microscopy (data not shown). We therefore suggest that the internalization of CD38 is not responsible for the inhibitory effect of gangliosides. Rather, inhibition is probably due to the interaction between gangliosides and CD38 on the cell surface.

Interaction between G_{T1b} and CD38 on the Same Cell Surface. The interaction of gangliosides with CD38 can occur either on the same cell (cis interaction) or between different cells (trans interaction). To examine this point, G_{T1b} was incorporated into RA-HL60 cells as well as into HL60 cells. Uptake of G_{T1b} was comparable in both cells (Figure 6A). In HL60 cells, expression of CD38 is very low, and NAD^+ glycohydrolase activity is negligible (*20*). As shown in Figure 6A, NAD^+ glycohydrolysis by RA-HL60 cells was appreciably inhibited after incubation of the cells with G_{T1b}. By contrast, NAD^+ glycohydrolysis by RA-HL60 cells was almost unaffected in the presence of HL60 cells that incorporated G_{T1b}. These results suggest that the action of G_{T1b} on CD38 on the same cell surface is more effective than that on CD38 on the other cells.

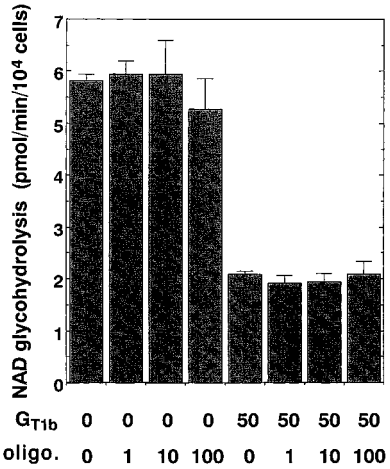

FIGURE 4: Effect of the oligosaccharide moiety of G_{T1b} on the NAD^+ glycohydrolysis in RA-HL60 cells either in the absence or in the presence of G_{T1b}. HL60 cells treated with retinoic acid for 1 day (5×10^5 cells/mL) were incubated for 3 h without or with 50 μM GT1b. The oligosaccharide moiety of G_{T1b} was then added at final concentrations of 1, 10, or 100 μM, and NAD^+ glycohydrolysis was measured as described under Experimental Procedures. Values are means ± SD from triplicate assays.

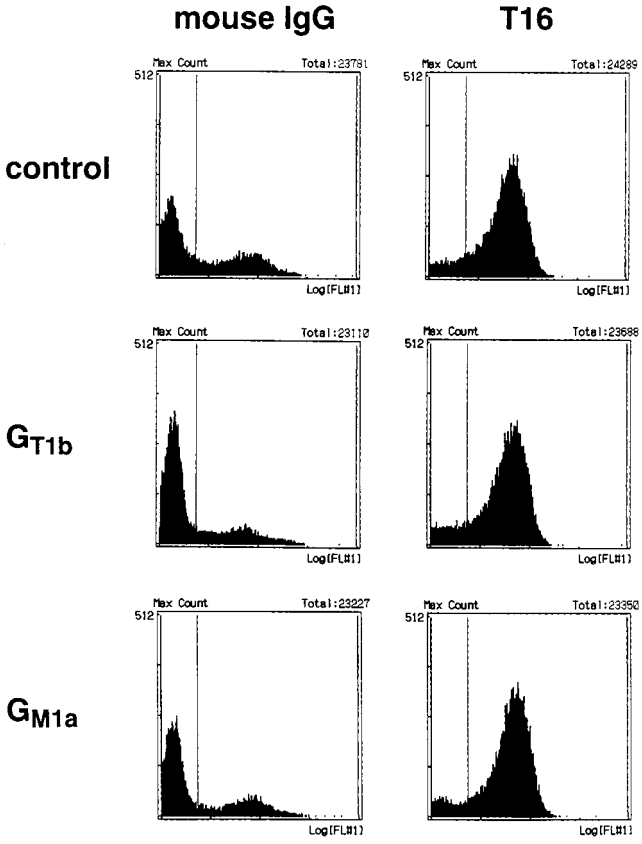

FIGURE 5: Lack of effect of exogenous gangliosides on internalization of CD38. HL60 cells treated with retinoic acid for 2 days (4×10^6 cells/mL) were incubated with 50 μM G_{T1b} or G_{M1a} for 3 h at 37 °C. The cells were stained and subjected to flow cytometry as described under Experimental Procedures. This result is representative of three independent experiments.

As retinoic acid can induce expressions of various proteins other than CD38, G_{T1b} on RA-HL60 cells may be associated with proteins that are missing in HL60 cells. Thus, we used CD38-transfected THP-1 cells (SCl-65 and SCl-94) as shown in Figure 6B. A small amount of NAD^+ glycohydrolysis by

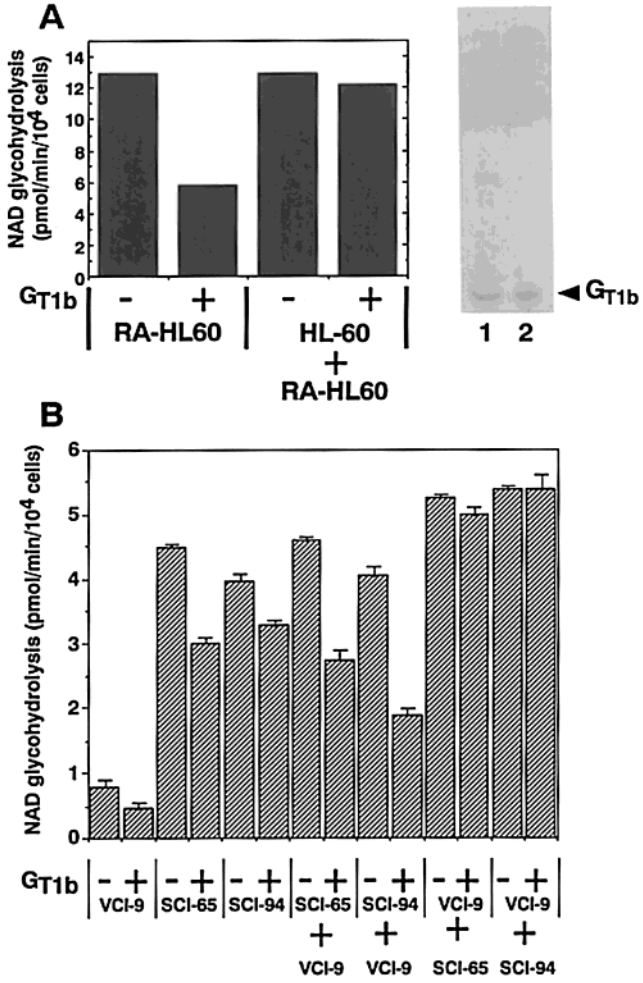

FIGURE 6: Inhibitory effect of G_{T1b} when G_{T1b} is incorporated into CD38-expressing cells. (A) Either RA-HL60 treated with retinoic acid for 2 days or HL60 cells (1×10^5 cells/mL, 50 μL each) were incubated without and with 50 μM G_{T1b} for 3 h at 37 °C, followed by the addition of an equal volume of E-RDF or RA-HL60 cells (1×10^5 cells/mL), respectively. Lipids equivalent to 10^6 cells from RA-HL60 cells (lane 1) or HL-60 cells (lane 2) were analyzed on TLC. The gangliosides were visualized by the resorcinol/HCl reagent. (B) THP-1 cells untransfected (VCl-9) or transfected with CD38 (SCl-65 and SCl-94) were incubated without and with 50 μM G_{T1b} for 3 h at 37 °C (1×10^6 cells/mL, 50 μL each). Then an equal volume of E-RDF or THP-1 cells (either CD38 transfected or untransfected, 1×10^6 cells/mL) was added. In both panels A and B, the NAD^+ glycohydrolysis reaction was started by adding 5 μL of 240 μM [^{14}C]NAD^+. The release of [^{14}C]nicotinamide during 15 min incubation at 37 °C was measured as described under Experimental Procedures. Values are means from duplicate assays in (A) and means ± SD from triplicate assays in (B).

untransfected THP-1 cells (VCl-9) was due to endogenous NADase activity, which we could not completely suppress with antisense CD38 (data not shown). NAD^+ glycohydrolysis by CD38-transfected THP-1 cells was inhibited by G_{T1b} treatment both in the absence and in the presence of VCl-9. In contrast, G_{T1b} treatment on VCl-9 did not affect the NAD^+ glycohydrolysis by SCl-65 as well as that by SCl-94. This result confirms that the effect of G_{T1b} in THP-1 cells is more efficient when G_{T1b} and CD38 are present on the same cell surface than on the other cells.

Comparison of the Effect of G_{T1b} and That of G_{D1a}. To investigate the involvement of the tandem sialic acid residues in the inhibitory effect of G_{T1b}, we compared the effect of G_{T1b} with that of G_{D1a}. A structural difference between G_{T1b}

and G_{D1a} is the presence of an additional sialic acid residue in G_{T1b}.

Incorporation of G_{D1a} was saturated after 2 h, and the amount of incorporation of G_{D1a} was comparable to that of G_{T1b} (Figure 2). When the cells were treated with trypsin before lipid extraction, the incorporation of both G_{T1b} and G_{D1a} was 150 pmol per 10^6 cells. However, the effect of G_{D1a} on NAD^+ glycohydrolysis was appreciably smaller than that of G_{T1b} (Figure 3B). This suggests that the tandem sialic acid residues are important for the inhibitory effect of G_{T1b}.

It is unlikely that the incorporated gangliosides distribute uniformly over the cell membrane, most probably due to the presence of microdomains on the cell surface (for review, see ref *36*). The formation of microdomains is explained by segregation of sphingolipids and the cholesterol-enriched lipid phase from the phospholipid environment (*37*). In fact, Simons et al. have reported that 75% of the exogenous G_{M1a} was present in such microdomains when Madin−Darby canine kidney cells were incubated with tritiated G_{M1a} (*38*). It is expected that such trapping of gangliosides in microdomains affects the inhibitory effect of gangliosides. Thus, it is possible that the difference in the effect of G_{T1b} and G_{D1a} on NAD^+ glycohydrolysis arises from differences in their surface localization.

To manipulate the localization of gangliosides with the microdomains, we used methyl-β-cyclodextrin (MβCD) that has been used to disrupt the microdomains (*39, 40*) by depletion of endogenous cholesterol (*41, 42*). In RA-HL60 cells, the cellular cholesterol (3.4 nmol/10^6 cells) was reduced to 2.0 and 1.1 nmol/10^6 cells after incubation with 5 and 10 mM methyl-β-cyclodextrin for 1 h, respectively (more than 40% and 65% decrease, respectively). The incorporation of G_{T1b} was 350 and 450 pmol/10^6 cells, and that of G_{D1a} was 530 and 630 pmol/10^6 cells, without and with the MβCD treatment, respectively. As shown in Figure 7A, the inhibitory effect of G_{T1b} was increased in the MβCD-treated cells. Although the inhibitory effect of G_{D1a} was also slightly observed in the MβCD-treated cells, the effect was significantly smaller than that of G_{T1b} irrespective of cholesterol depletion (Figure 7B). These results suggest that the requirement of the tandem sialic acid residues for the potent inhibitory effect of G_{T1b} is not due to a cholesterol-dependent surface localization of G_{T1b}.

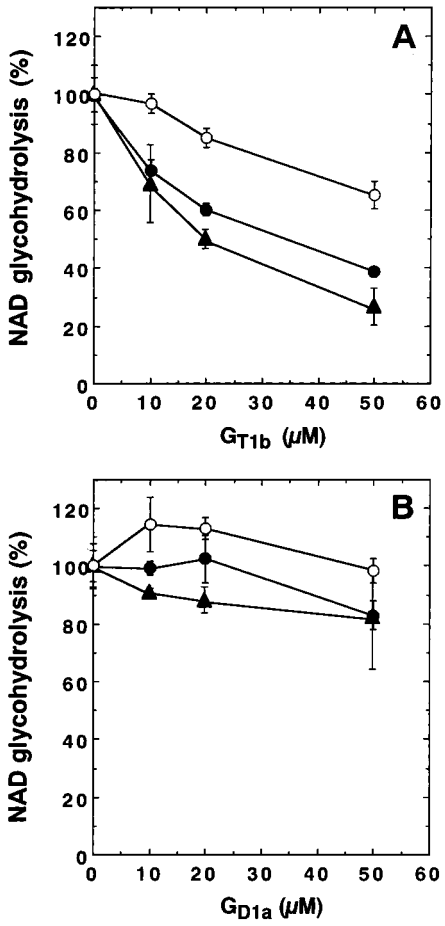

FIGURE 7: Effect of G_{T1b} and G_{D1a} on NAD^+ glycohydrolysis in cholesterol-depleted RA-HL60 cells. HL60 cells treated with retinoic acid for 1 day (5×10^5 cells/mL) were incubated without (○) or with 5 mM MβCD (●) or 10 mM MβCD (▲) as described under Experimental Procedures. The cells were then incubated with G_{T1b} (A) or G_{D1a} (B), and NAD^+ glycohydrolysis was measured as described under Experimental Procedures. Values are means ± SD from triplicate assays, expressed as percentages of the control values in the absence of the gangliosides for each concentration of MβCD, which were 5.2 and 5.4 pmol min^{-1} $(10^4$ cells$)^{-1}$ (in the absence of MβCD), 8.1 and 7.6 pmol min^{-1} $(10^4$ cells$)^{-1}$ (5 mM MβCD), and 8.6 and 9.3 pmol min^{-1} $(10^4$ cells$)^{-1}$ (10 mM MβCD), for G_{T1b} and G_{D1a}, respectively. Essentially similar results for G_{T1b} were obtained in HL60 cells treated with retinoic acid for 2 days.

DISCUSSION

In the present study, we clearly demonstrated that exogenous gangliosides inhibit NAD^+ glycohydrolase activity on the cell surface CD38. We showed the following four characteristics of the inhibitory effect of G_{T1b} on the cell surface CD38. First, G_{T1b} that is incorporated into the cell membranes is responsible for the inhibition. Second, G_{T1b} does not induce the internalization of CD38. Third, the inhibition occurs when G_{T1b} and CD38 are present on the surface of the same cells. Fourth, the tandem sialic acid residues of the oligosaccharide moiety of G_{T1b} are crucial to the inhibition. On the basis of these results, we outline the inhibition process as access of the oligosaccharide moiety of G_{T1b} integrated in the lipid bilayer with its ceramide moiety to the extracellular domain of CD38 on the same cell surface. Such cell surface inhibitors of ecto-enzymes have not been confirmed to date. The present study adds the evidence obtained in our previous study that complex gangliosides act as a regulator of cell surface NAD^+ metabolisms.

Recognition of the oligosaccharide moiety of gangliosides on the cell surface by proteins on the other cells has been investigated to explain cell-to-cell communication events. For example, gangliosides serve as receptors for bacterial toxins or ligands of adhesion molecules (*2, 8, 43*). However, in the present study, the inhibitory effect of gangliosides was not due to cell aggregation or adhesion of RA-HL60 cells. Taking these results together with those with CD38-transfectecd THP-1 cells (Figure 6), it is unlikely that the inhibitory effect of G_{T1b} primarily depends on cell-to-cell interaction.

Cholesterol depletion has been demonstrated to impair microdomain-mediated processes (*41, 42*). In the present study, we observed the enhancement of the inhibitory effect of G_{T1b} in MβCD-treated RA-HL60 cells (Figure 7A). Beside the increase of the incorporation of gangliosides, two other possibilities may explain this result. One is that the inhibition does not occur within the microdomains. This view is

consistent with the finding that CD38 was mostly recovered in the detergent-soluble fractions after RA-HL60 cells were lysed with Triton X-100, which implies that CD38 is largely excluded from the microdomains (data not shown). Cholesterol depletion can cause release of G_{T1b} from the microdomains and thus increases the effective concentration of G_{T1b} in the inhibition of CD38. The other possibility is that cholesterol competes with G_{T1b} for access to CD38 and the decrease of cholesterol favors the inhibitory effect of G_{T1b}. As the addition of ceramide decreased the inhibitory effect of G_{T1b} on NADase activity in the RA-HL60 cell lysate (data not shown), the ceramide moiety of G_{T1b} is probably involved in the interaction with CD38 and competes with cholesterol within the lipid bilayer. Although further study is needed to elucidate the precise mechanism, the sensitivity of the inhibitory effect to the change in membrane composition verifies that the inhibition is indeed a membrane-associated event.

We previously reported that the tandem sialic acid residues of gangliosides are necessary for the inhibition of MBP−CD38 (*28*). In the present study, we showed that the tandem sialic acid residues of G_{T1b} are also required for the inhibition of intact CD38 on RA-HL60 cells (Figures 3 and 7). To date, it has been suggested that complex gangliosides are endogenous ligands for myelin-associated glycoprotein and a sialic acid residue on the terminal galactose of a gangliotetraose core is necessary for the interaction (*8*, *43*). The present study suggests an entirely different structural requirement of complex gangliosides. To better understand the structural aspects of this inhibition, it is important to know whether the tandem sialic acid residues of G_{T1b} can access the NAD^+ binding site of CD38 on the cell surface.

As shown in Figure 8A, the two carboxyl groups in the tandem sialic acid residues of G_{T1b} are located about 15−20 Å above the membrane surface when NeuAcα2→8NeuAcα2→3Galβ1→4Glc residues are extended (indicated by arrows). On the other hand, the catalytic site of CD38 is considered to be about 20 Å distant from the membrane surface, on the basis of the crystal structure of ADP-ribosyl cyclase, a CD38 homologue (*44*). A catalytically important glutamic acid residue in CD38 (Glu146) is conserved in ADP-ribosyl cyclase (Glu98) (*45*, *46*) and is located near the cavity about 20 Å above the membrane surface (Figure 8B). Our proposal is that two carboxyl groups of the tandem sialic acid residues of b-series gangliosides mimic the diphosphate moiety of NAD^+. Although the binding site for the diphosphate moiety of NAD^+ has not been identified, the site is probably close to a catalytic site. Thus, if the two carboxyl groups of G_{T1b} are accessible to the diphosphate binding site of CD38, such lateral access covers the catalytic pocket of CD38 (*46*) and hinders the binding of NAD^+ (Figure 8C).

Recently, we succeeded in the crystallization of MBP−CD38 complexed with G_{T1b} (*47*). Information on the tertiary structure of the complex will provide a more precise understanding of the mechanism of the inhibitory effect of gangliosides.

Spleen is known to be rich in NAD^+ glycohydrolase activity, and the activity is reduced to less than 1% in the CD38 knockout mice (*26*). Consistent with our proposal that gangliosides act as cell surface inhibitors of CD38, the neuraminidase treatment of mouse splenic cells increased the NAD^+ glycohydrolase activity (data not shown).

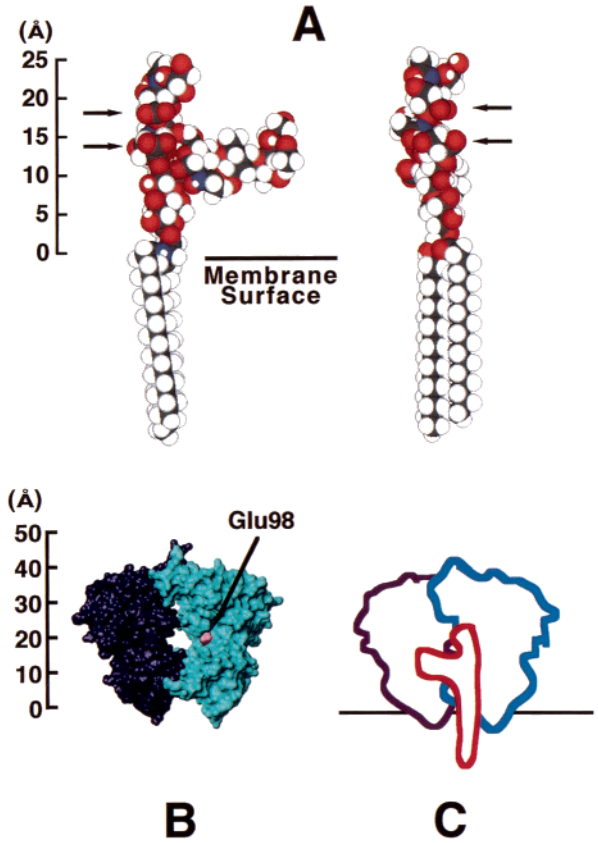

FIGURE 8: Space-filling models of G_{T1b} (A) and the CD38 homologue ADP-ribosyl cyclase (B) and a proposed model of the interaction (C). (A) Space-filling models of G_{T1b} (drawn by Chem 3D Plus) from different views are shown. The two carboxyl groups are indicated by arrows. Hydrogen, carbon, nitrogen, and oxygen atoms are colored white, black, blue, and red, respectively. (B) The structure of ADP-ribosyl cyclase was taken from the Protein Data Bank (accession code 1LBE). (C) Proposed interaction between CD38 and G_{T1b} on the membrane surface is indicated. The membrane surface is indicated by horizontal lines.

Investigation of complex ganglioside-lacking mice revealed the attenuation of interleukin 2 signaling in spleen T cells (*10*). The impaired response is due to the loss of G_{M1a}, asialo-G_{M1a}, and G_{D1b} gangliosides, which are present in wild-type spleen T cells. However, the underlying mechanism has not been understood. In other results, however, T cell receptor activation leads to Ca^{2+} signaling caused by cADPR in Jurkat T-lymphocytes (*48*), while activation was modulated by cell surface ADP-ribosyltransferase in mouse T cell lymphoma (*14*). Our study suggests the possibility that complex gangliosides contribute to the regulation of T cell function through interaction with cell surface NAD^+-metabolizing enzymes. The effect of complex gangliosides on other CD38-related NAD^+-metabolizing ectoenzymes, BST-1 (*49*), Rt6.1 (*15*), Rt6.2 (*16*), or lymphocyte GPI-anchored ADP-ribosyltransferase (*12*−*14*) should be investigated in future studies.

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