

Direct binding of gangliosides to *Helicobacter pylori* vacuolating cytotoxin (VacA) neutralizes its toxin activity

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Abbreviations: FCS, Fluorescence correlation spectroscopy; SL, Sialyllactose; DT,
diffusion time; CT, cholera toxin; CTB, cholera toxin B subunit

Summary

Gangliosides are target receptors for bacterial entry yet those present in human milk exhibit a protective role against bacterial infection. Here, we show that treatment with ganglioside mixture at a concentration of 100 µg/ml resulted in significant inhibition of the vacuole formation activity of *Helicobacter pylori* vacuolating cytotoxin (VacA) in gastric epithelial cancer AZ-521 cells. All gangliosides (GM1, GM2, GM3, GD1a, GD1b, GD3 and GT1b) examined showed good neutralizing capacity against VacA. A pull-down assay was performed using lyso-GM1 coupled to Sepharose as the tagged polysaccharide polymer to capture VacA from *H. pylori* culture supernatant. GM1-VacA complexes were successfully precipitated suggesting that GM1 binds directly to VacA. The hydrodynamic binding of lyso-GM1 and VacA measured by fluorescence correlation spectroscopy (FCS) had a K_d value of 190 nM. VacA also bound to lyso-GM1 at pH 2 corresponding to the physiological pH of human stomach. Collectively, these results showed that direct binding of *H. pylori* VacA to free gangliosides neutralizes the toxin activity of VacA. These findings offer an alternative insight into the role of gangliosides in VacA toxicity and the pathogenesis of *H. pylori*.

Introduction

Helicobacter pylori, a gram-negative microaerophilic bacterium is of growing concern today because of its crucial role in the pathogenesis of gastritis, peptic ulcer disease, gastric lymphoma and gastric adenocarcinoma (Warren and Marshall, 1984; Parsonnet *et al.*, 1991; Axon, 1999). More than half of the world population is chronically infected with this organism (Mann and Westblom, 1999). Cytotoxin-associated gene A (*cagA*), its related pathogenicity island (*cagPAI*), vacuolating toxin A (VacA) and BabA adhesin are among virulence factors important in the pathogenicity of *H. pylori*. VacA is a protein toxin of 95 kDa which induces multiple structural and functional alterations in eukaryotic cells. It forms voltage-dependent, anion-selective channels in planar lipid membranes and is essential for the transformation of late endosomal compartments into vacuoles (Szabò *et al.*, 1999; Tombola *et al.*, 1999). The vacuolar membrane contains an active vacuolar ATPase proton pump (V-ATPase) which plays a crucial role in cell vacuolization by favouring water uptake and swelling. These vacuoles contain membrane protein markers of the late endosomes and lysosomes (Papini *et al.*, 1994; Molinari *et al.*, 1997), and are capable of incorporating fluid phase markers of extracellular medium (Catrenich and Chestnut, 1992; Cover *et al.*, 1992; Papini *et al.*, 1994). Exposure of VacA to either acidic or alkaline pH results in structural changes of the protein and a marked enhancement of its cell-vacuolating activity (Cover *et al.*, 1992; Leunk *et al.*, 1998; Cover and Blanke, 2005). Such cell alterations facilitate the migration and the accumulation of membrane-permeable weak bases, including dyes such as neutral red (Cover *et al.*, 1992) into the acidic vacuolar lumen and is used as a measure of the changes in the internal volume of these compartments.

VacA and other bacterial toxins are internalized following the adhesion of the pathogens to target receptors on cell surfaces. Glycosphingolipids (GSLs) such as gangliosides (Schengrund, 2003) are targets for the adhesion of viruses, bacteria, or bacterial toxins. A ganglioside is a sialylated carbohydrate in glycosidic linkage to a ceramide lipid consisting of a sphingosine chain and a fatty acid amide (Fig. 1). For example, heat-labile enterotoxin (LT) from *Escherichia coli* and cholera toxin (CT) from *Vibrio cholerae*, interact with GM1 ganglioside on cell surfaces (Otnaess *et al.*, 1983). A recent study reported that VacA is one of the glycosphingolipid (GSLs)-binding proteins that interacts with short carbohydrate chain of GSLs but does not bind to acid glycosphingolipids such as GM1 and GM4 gangliosides (Roche *et al.*, 2007). Another report, however, showed that *H. pylori* cells can bind to GM3 ganglioside (Abul-Milh *et al.*, 2001). While the role of cell surface-associated gangliosides as target molecules for the binding of VacA remains unclear, some gangliosides are also known to have a protective role against enteric pathogens (Newberg *et al.*, 2005). Human milk gangliosides can neutralize *Escherichia coli* and *Vibrio cholerae* toxins (Otnaess *et al.*, 1983) but their effects on *H. pylori* toxin remain to be investigated. The present study, hence, aims to investigate whether VacA of *H. pylori* interacts with gangliosides and whether this interaction would have an effect on the toxicity of VacA.

Results

Cell surface gangliosides are targets for binding of various bacterial toxins. Cholera toxin (CT) for example, specifically binds to ganglioside subtype GM1 on cell surfaces. They are also found in human milk and it was shown that these free gangliosides can

neutralize bacterial toxins (Newberg *et al.*, 2005). In the present study, we examined the neutralization capacity and binding affinity of free gangliosides to *Helicobacter pylori* vacuolation cytotoxin (VacA type s1m1).

The neutralization capacity of gangliosides was examined by incubating acid-activated VacA with various concentrations of ganglioside mixture in AZ-521 gastric epithelial cancer cells (Fig. 2A). Induction of vacuolation in AZ-521 cells by VacA was significantly inhibited in the presence of 100 µg/ml ganglioside mixture, compared to cells cultured with VacA only ($p < 0.01$, ANOVA with Dunnett's post-hoc test; Fig. 2A). Gangliosides are amphipathic compounds and form micelles at critical micelles concentrations (CMC) at 10^{-9} to 10^{-10} M in an aqueous medium (Yohe *et al.*, 1976). Hence, this finding suggested that gangliosides at concentrations beyond CMC can neutralize the vacuolation activity of VacA. Various gangliosides, including GM1, GM2, GM3, GD1a, GD1b, GD3 and GT1b were next individually examined to identify specifically which ganglioside can neutralize the vacuolation activity of VacA. Results showed that all gangliosides neutralized the vacuolation activity of VacA ($p < 0.01$, T-test; Fig. 2B).

Gangliosides consist of side chains such as sphingosine and fatty acids (Fig. 1). To determine whether the fatty acid moiety of ganglioside neutralizes the vacuolation activity of VacA, we used Lyso-gangliosides (Lyso-GM1, Lyso-GM2, and Lyso-GM3), which are devoid of fatty acid chains for subsequent examination of the neutralization effects on the vacuolation activity of VacA. All three Lyso-gangliosides neutralized the vacuolation activity of VacA in a dose-dependent manner (Fig. 3). This finding suggested that the neutralization effects on the vacuolation activity of VacA by gangliosides were not attributed to the fatty acid moiety of gangliosides.

As gangliosides are important for signals transduction, it is possible that the neutralization of the vacuolation activity of VacA by gangliosides can be mediated through specific intracellular pathways. Therefore, we examined whether internalization of VacA by AZ-521 cells can occur in the presence of Lyso-GM1 by immunofluorescence staining. Fig. 4 showed that VacA was not internalized in the presence of Lyso-GM1 and this observation suggested that the neutralization effect of ganglioside could be due to the extracellular binding of VacA to ganglioside.

To determine whether GM1 ganglioside binds directly to VacA, we further examined its binding capacity by using Lyso-GM1 Sepharose prepared by covalent binding of Sepharose to Lyso-GM1 in another pull-down assay. VacA was substantially pulled down together with Lyso-GM1 Sepharose (~95 kDa), although a residual amount of VacA also bound non-specifically to Sepharose in a control experiment (Fig. 5A). VacA and Lyso-GM1 interacted under neutral (pH 7.5) as well as acidic (pH 2.0) conditions (Fig. 5A). These results showed that VacA binds to Lyso-GM1. Since cholera toxin B subunit (CTB) also binds to GM1, both VacA and CTB were incubated together in another pull-down assay to determine whether these toxins bind to the same site on Lyso-GM1. Both VacA and Lyso-GM1 were pulled down, regardless of the presence or absence of CTB, suggesting that VacA and CTB bound to different sites on Lyso-GM1 (Lane 7; Fig. 5B). The difference in the binding sites between VacA and CTB was further analyzed in the presence of avidin-Sepharose incubated with or without biotinylated CTB, Lyso-GM1 and VacA. Avidin-Sepharose was used to rule out the formation of biotinylated CTB-GM1-VacA complex. VacA was again pulled down in the presence of avidin-Sepharose, biotinylated CTB and Lyso-GM1 (Fig. 5C). Taken together, these findings suggested that VacA and CTB

bind to different sites on Lyso-GM1. A pull-down assay was performed on ammonium sulfate dialyzed *H. pylori* (ATCC49503) culture supernatant using Lyso-GM1 resin to identify potential GM1-binding factor in the culture supernatant of *H. pylori*. Two dense protein bands (~95 kDa and >200 kDa) bound to Lyso-GM1 but not to the control Sepharose were detected by SDS-PAGE analysis (Fig. 6). The protein bands were trypsin-digested and analyzed by mass spectrometry. The MASCOT program matched the 95 kDa MS/MS ions sequence to Vacuolating cytotoxin precursor (accession number Q48245, Supplementary Table 1B). Whereas, the peptide mass fingerprint and MS/MS ions sequence of the 200 kDa protein band were matched to both Apolipoprotein B (accession number XP_583270) and Apolipoprotein A-1 (Accession number P15497) (Supplementary Table 1A and B). ApoB is a low density lipoprotein found in serum and it is a typical human serum protein that interacts with gangliosides (25). Hence, this finding established that VacA is the primary GM1 binding protein of *H. pylori*.

In order to investigate whether VacA binds to the sugar moiety of a ganglioside, a commercially available carbohydrate moiety of GM3 (3'-SL; sialyllactose) was used instead of GM1. Incubation of the acid-activated VacA with various amounts of 3'-SL in the culture media of AZ-521 gastric epithelial cancer cells failed to inhibit VacA-induced vacuolation of these cells ($p > 0.01$, ANOVA with Dunnett's post-hoc test; Fig. 7A). In addition, induction of vacuoles formation on cells by VacA could not be inhibited by BSA conjugated to 13 oligomers of 3'-SLs ($p > 0.01$, ANOVA with Dunnett's post-hoc test; Fig. 7B). These findings suggest that the GM3 carbohydrate moiety alone might not be sufficient to bind or neutralize the vacuolating activity of VacA.

To determine if the sphingosine chain contributes to the neutralization of VacA toxicity, sphingosine Sepharose was used. Cellular vacuolation inhibition assay was not performed with sphingosine as it could induce cell apoptosis (data not shown). Binding of VacA to sphingosine Sepharose was instead examined using the pull-down assay. Similar amount of VacA was pulled down by both Sepharose control and sphingosine-Sepharose indicating that VacA did not bind specifically to sphingosine (Fig. 8A). Instead, sphingosine bound to both albumin and apolipoprotein A-1 in fetal calf serum (Fig. 8B). This finding suggested that the interaction of the sphingosine chain on its own is not sufficient for the binding of VacA to gangliosides. Taken together, these results showed that the carbohydrate, fatty acid and sphingosine moieties of ganglioside in isolation neither bind nor neutralize VacA activity.

Since Lyso-gangliosides can bind and neutralize the vacuolation activity of VacA, their relative binding affinities to VacA were further characterized using fluorescence correlation spectroscopy (FCS) (23, 24). This method measures the diffusion time (DT) of fluorescent-labeled molecules without fixation on a sensor device. Fluorescent labeling of Lyso-gangliosides was performed by a reaction with succinimidyl esters of Alexa Fluor 488 and 514. After reverse-phase HPLC isolation, their structures were confirmed by MS/MS measurements. As a representative example, the MS/MS spectrum of Alexa488-labeled Lyso-GM1 (precursor ion $[M+3Na+2H]^{2+} = 931.78$) is shown in supplementary Fig. 1, in which the mass differences between each of the fragment ions are consistent with residual mass values predicted from the Lyso-GM1 sugar chain structure. The MS analysis of the Lyso-gangliosides gave similar results and was consistent with their sugar chain structure (data not shown). Fluorescence spectra of the labeled Lyso-gangliosides were in agreement with expected values of the

emission wavelengths, 516-518 nm and 536-540 nm of Alexa Fluor 488 and 514, respectively.

Fig. 9A shows the optical setup of a FCS instrument. FCS is based on the analysis of time-dependent intensity fluctuations that are the result of translational motion of a fluorophore, typically diffusion into and out of a small observed volume defined by a focused laser beam and a confocal aperture. An average duration of a pass through the observed volume by the fluorophore, which is called diffusion time (DT) can be determined by correlation analysis of fluctuations. When the fluorophore diffuses into a focused light beam, there is a burst of emitted photons due to multiple excitation-emission cycles from the same fluorophore. If the fluorophore is a small molecule and diffuses rapidly out of volume, the photon burst is short-lived, whereas, a larger molecule would result in a longer duration of photon burst. Thus, DT values obtained from FCS measurements can provide information on molecular weight changes of the fluorophore.

In the absence of binding protein, each fluorescent-labeled Lyso-ganglioside typically gave a baseline DT of 50 μ sec (Fig. 9B-D) and increased to a larger value when the fluorescent-labeled Lyso-ganglioside bound to VacA. In order to offset variations due to fluctuations in temperature and fluid viscosity during each experimental condition, the differences of DTs of each Lyso-ganglioside were used. In the mixture containing the same concentration (10 nM) of Alexa 488-labeled Lyso-GM1 and Alexa 514-labeled Lyso-GM2, DT values of Lyso-GM1 were larger than those of Lyso-GM2 with increasing concentrations of VacA (Fig. 9B), thus indicating that Lyso-GM1 has a higher binding affinity than Lyso-GM2. The difference of DTs between Lyso-GM1 and Lyso-GM2 was 33 ± 18 μ sec at 1.0 μ M of

VacA. In the case of Alexa 488-labeled Lyso-GM1 and Alexa 514-labeled Lyso-GM3, DT values of Lyso-GM1 were larger than those of Lyso-GM3 with increasing concentrations of VacA (Fig. 9C), indicating that Lyso-GM1 had a higher binding affinity than Lyso-GM3. The difference of DTs between Lyso-GM1 and Lyso-GM3 was 49 ± 11 μ sec at 1.0 μ M of VacA. Furthermore, the combination of Alexa 488-labeled Lyso-GM2 and Alexa 514-labeled Lyso-GM3 did not show differences in their DT values, indicating that Lyso-GM2 and Lyso-GM3 have similar magnitude in binding affinity (Fig. 9D). These results strongly suggested that the binding affinity of Lyso-GM1 is higher than that of Lyso-GM2 and Lyso-GM3, which correlated with the cell-based inhibition of VacA toxicity as shown in Fig. 3.

The K_d values of VacA bound to Alexa 488-labeled Lyso-GM1, Alexa 514-labeled Lyso-GM2 and Alexa 514-labeled Lyso-GM3 are summarized in Table 1. To estimate Bound/Free (B/F) ratio of fluorescent-labeled gangliosides, composite function curves of the two components (VacA-bound and unbound gangliosides) autocorrelation function were fitted to the FCS data. The DT value deduced from the molecular mass of VacA hexamer (570 kDa) is 330 μ sec, because VacA has 300 times larger molecular mass than ganglioside, thus, DT of VacA would be 6.7 times (third root of 300) of the ganglioside DT value (50 μ sec). Under this consideration, curve-fitting procedure gave good fitting result. The serial B/F ratios in various VacA concentrations (Supplementary Fig. 2A-C) were used to depict Scatchard plots (Supplementary Fig. 2D-F). The obtained K_d values were 190 nM, 400 nM and 830 nM to Lyso-GM1, -GM2 and -GM3, respectively. For comparison, the K_d value of CTB bound to Alexa 488-labeled Lyso-GM1 as determined by FCS measurements was 24 nM (Table 1 and Supplementary Fig. 3). Alexa 514-labeled Lyso-GM2 showed no binding response to

CTB.

Discussion

Gangliosides are found in milk and blood apart from mammalian cell surfaces. Earlier studies reported that gangliosides from human milk exhibit the ability to attenuate the binding of cholera toxin to GM1, and those obtained from goat milk inhibit the binding of botulinum type A neurotoxin to GT1b (Iwamori *et al.*, 2008). In view of these findings, the present study examines the neutralization capacity and binding affinity of gangliosides to *Helicobacter pylori* vacuolation cytotoxin (VacA). GM3 is the major ganglioside found in milk (Pan *et al.*, 2000) and human serum (Senn *et al.*, 1989) while GM1 is found in low concentration in human milk (Pan *et al.*, 2000). Results from the present study showed that GM1 and other gangliosides neutralize the toxin activity of VacA on human gastric cancer cells by preventing internalization of VacA into the cells. Secreted VacA from the culture supernatant of *H. pylori* was successfully captured by GM1-Sepharose. This suggests that GM1 ganglioside prevents the internalization of VacA by binding directly to the cytotoxin and neutralizes the toxin activity of VacA on human gastric cancer cells. In addition, GM1-VacA interaction can also occur under an acidic condition similar to that of the stomach environment. We further demonstrated using FCS that Lyso-GM1 has a higher binding affinity for VacA as compared to Lyso-GM3 and other gangliosides examined.

Initial studies of potential receptors for *H. pylori* suggested that acid glycosphingolipids, such as GM3 ganglioside and sulfatide can function as receptors for the bacterium (Kamisago *et al.*, 1996). Using bacterial cells, Abul-Milh *et al.* (Abul-Milh *et al.*, 2001) reported variable binding abilities in selected *H. pylori* strains

to GM3 but not to other acid glycosphingolipids. More recently, Roche *et al.* reported that *H. pylori* binds to N-acetyllactosamine-based gangliosides with terminal $\alpha 3$ -linked NeuAc but does not recognize gangliosides with terminal NeuGc $\alpha 3$, NeuAc $\alpha 6$, nor NeuAc $\alpha 8$ NeuAc $\alpha 3$ on thin layer chromatography surface (Roche *et al.*, 2004). In contrast to surface binding analysis, FCS measures real-time binding that needs neither ligand immobilization nor bound/free separation. Moreover, measuring freely diffusing proteins represent a more physiologically-relevant state. The measurement of the hydrodynamic binding between monomeric Lyso-ganglioside and hexameric VacA in a homogenous solution perhaps contributed to the differences observed in our study as compared with earlier studies. On the other hand, it is plausible that VacA binds to all gangliosides as they are known to be the “unintended” target receptors for the adhesion of various pathogens (Rueda, 2007). It is also plausible that on cell surfaces, binding to other glycosphingolipids with higher affinities may be preferred over acid sphingoglycolipids such as gangliosides. We showed in the present study that cell internalization and intoxication by VacA were inhibited in the presence of gangliosides and this further supports the notion that gangliosides can act as decoy receptors that interfere with the binding of pathogens (Rueda, 2007).

In the present study, the strength of VacA-Lyso-GM1 interaction ($K_d = 190$ nM) and CTB-Lyso-GM1 (24 nM) measured under hydrodynamic condition differs from interactions measured using surface binding analysis such as that observed for CT-GM1, with K_d values of 730 pM (MacKenzie *et al.*, 1997), 53 nM (Masserini *et al.*, 1992) and *E. coli* heat-stable enterotoxin (STa)-guanylyl cyclase receptor (GC-C), with K_d values of ~ 1.0 nM in human (de Sauvage *et al.*, 1991) and 7.9 nM in pig (Wada *et al.*, 1994). The differences may not necessarily reflect unimportant interactions but

more likely due to the fact that FCS is hydrodynamic analysis, while other surface binding analysis measures molecular distribution average between a solution and a solid surface. Hence, FCS has an advantage for measuring rapid equilibrium changes that could not be estimated by other surface binding analysis such as those often observed in the interaction of sugar moieties and proteins.

We also investigated the importance of each component of ganglioside in the interaction with VacA. 3'-Sialyllactose (3'-SL), is a carbohydrate moiety present on GM3 and is naturally secreted in human milk together with other sialylated oligosaccharides. It is also a common constituent of cell surface glycoproteins and glycolipids. Several studies have shown that 3'-SL inhibits the adhesion of *H. pylori* to epithelial cells (Simon *et al.*, 1997) and colonization of *H. pylori* to gastric epithelial cells *in vitro*, in monkeys infected by *H. pylori* (Mysore *et al.*, 1999). As *H. pylori* adherence blocker, multivalent 3'-SL (~20 oligosaccharides attached with human serum albumin) is 2 to 3 orders higher in magnitude than monovalent 3'-SL (Simon *et al.*, 1997). Although these studies showed multivalent 3'-SL inhibits *H. pylori* adhesion, VacA-induced vacuolation of epithelial cells was not inhibited by multivalent 3'-SL in the present study. Similarly, we found that sphingosine and carbohydrate alone do not exhibit any binding or neutralizing activity for VacA but structural differences between GM1 and GM3 sialyllactose and saccharide contributing to this observation could not be ruled out in the present study. Nevertheless, chemical structure resulting from the linking of these side chains to the carbohydrate moiety is essential for the binding of VacA to ganglioside.

Although results from the present study only showed that gangliosides in aqueous medium bind to VacA, the importance of cell surface-associated gangliosides

in the binding of VacA cannot be undermined as gangliosides together with sphingomyelin (SM) exist in lipid rafts on the gastric cell surface. Lipid rafts have been shown to play an important role for VacA interaction with bacterial cells as disruption of lipid rafts inhibits the toxin activity of VacA (Schraw *et al.*, 2002; Kuo *et al.*, 2003; Nakayama *et al.*, 2006). Moreover, it is reported that SM is the VacA target receptor on cell surface (Gupta *et al.*, 2008) and both SM and gangliosides share the same chemical structure that consists of sphingosine and fatty acid, suggesting that these common structures may play an important role in the binding to VacA.

In conclusion, results from the present study showed that gangliosides can directly bind to *H. pylori* VacA. Even though their roles as target receptors for the adhesion and entry of *H. pylori* remain to be investigated, it is clear that the binding of gangliosides to VacA inhibits internalization of VacA and cell intoxication. This finding offers new insight into the role of gangliosides in the prevention of *H. pylori* infection.

Materials and methods

Materials

VacA (s1m1) of *H. pylori* strain ATCC49503 was purified as previously described (Yahiro *et al.*, 2004). The toxin was activated by incubating in HCl-PBS buffer, pH 2.0, at room temperature (RT) for 10 min before it was added to the cells. Bovine brain gangliosides mixture was obtained from IsoSep AB (Sweden). Lyso-GM1, sphingosine and cholera toxin B subunit (CTB) were purchased from Sigma-Aldrich (USA). Protein marker was obtained from Bio-Rad (USA). Alexa Fluor dyes were purchased from Invitrogen (USA) and Alexa Fluor 488-cojugated antibody was purchased from

Invitrogen (USA). Anti-VacA antibody was prepared as previously described (Yahiro et al, 2004).

Cellular assays for vacuolation activity

AZ-521 cells were purchased from the Culture Collection of Health Science Resources Bank, Japan Health Science Foundation and maintained in complete Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO₂ atmosphere environment. Cells were seeded in 96-well tissue culture plates at the density of 2.0×10^4 cells in 100 µl/well and grown for 24 h. Purified VacA was added to a final concentration of 240 nM and cells were incubated in EMEM containing 5 mM NH₄Cl for an additional 3 h at 37°C. Quantification of cell vacuolation by VacA was performed using Neutral Red Uptake (NRU) assay as previously described (Cover *et al.*, 1992).

Confocal analysis of VacA localization

Cells were seeded at a density of 1.0×10^4 cells in 200 µl/well in 8-well chamber microscope slide (Nalgen Nunc, Denmark) and were grown overnight. Cells were incubated with VacA (240 nM) with or without 20 µM Lyso-GM1 for 3 h at 37°C. Following treatment, the cells were washed with Phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde in PBS for 30 minutes. The cells were then permeabilized for 5 min at RT with 0.1% Triton X-100 in PBS, washed three times with PBS and blocked with blocking solution (1.5% Block Ace, Snow-Brand, Tokyo, Japan) in PBS for 1 h. The cells were then incubated with anti-VacA antibody diluted 1:5000 in blocking buffer (0.4% Block Ace in PBS) for 1 h at RT, followed by an incubation

with anti-rabbit Alexa Fluor 488-conjugated secondary antibody diluted 1:1000 in blocking buffer for 1 h at room temperature in the dark. Images were captured by a confocal laser microscope (TSC SP2; Leica, Heidelberg, Germany) using Ar/Kr laser at the excitation wavelength of 488 nm.

Preparation of Lyso-GM1 (GM1 analog) Sepharose or sphingosine Sepharose

Lyso-GM1 or sphingosine was coupled to Sepharose using the NHS-activated Sepharose 4 Fast Flow setup (GE Healthcare, England) according to the manufacturer's procedure. Briefly, Lyso-GM1 or sphingosine was coupled using NHS-activated Sepharose 4 Fast Flow in coupling solution containing 0.2 M NaHCO₃ and 0.5 M NaCl (pH 8.3) at RT for 4 h. After the completion of the coupling processes, any non-reacted groups on the Sepharose were blocked by 0.5 M ethanolamine in the coupling solution. Lyso-GM1 or sphingosine Sepharose was then washed 0.1 M Tris-HCl, 0.1 M acetate and 0.5 M NaCl and resuspended with phosphate buffered saline (PBS) in a 1:1 (volume/volume) ratio.

Analysis of the binding of VacA to Lyso-GM1 or sphingosine Sepharose

VacA was incubated with Lyso-GM1 or sphingosine Sepharose in PBS for 1 h at 4°C. After incubation, Sepharose was sedimented by centrifugation at 12000 x g. The supernatant was discarded and the remaining Sepharose was washed twice with PBS. The bound protein complexes were then solubilized with sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.001% bromophenol blue, and 5% mercaptoethanol) and boiled for 10 min at 100°C. The sample was analyzed by SDS-PAGE and visualized by EzStain Aqua (ATTO, Japan). In a separate experiment,

the binding of VacA to Lyso-GM1 Sepharose was also examined in the presence of cholera toxin B (CTB). VacA and CTB were incubated with Lyso-GM1 in PBS for 1 h at 4°C and subsequently washed and solubilized as above.

Protein identification by mass spectrometry (MS)

Protein bands of interest from a Coomassie brilliant blue (CBB)-stained SDS-PAGE gel were excised and rinsed with 50 mM NaHCO₃/50% acetonitrile. Gel particles were dried in a vacuum concentrator and rehydrated in 50 µl of 50 mM NaHCO₃ buffer containing 10 ng/µl sequencing grade trypsin (Sigma-Aldrich, USA). After the removal of excess trypsin solution, gel particles were overlaid with 30 µl of 50 mM NaHCO₃ and were incubated for 16 hrs at 37°C. Supernatant containing peptides were subsequently used for MS analysis.

Each sample was loaded onto a nano-LC equipped with PicoFrit column (New Objective Inc., MA). A Prominence Nano binary pump system (Shimadzu Co., Kyoto, Japan) was programmed to elute the peptides at a gradual ramp of a linear gradient of 5 to 90% acetonitrile with 0.1% formic acid in 60 min. The flow rate in the column was 200 nl/min, and the column was directly coupled to a nanospray tip of LCMS-IT-TOF System (Shimadzu Co.). The mass spectrometer was set to a cycle of one full mass scan, followed by three tandem mass scans of the three most intense ions. All tandem mass spectra obtained were subjected for database blast against *Helicobacter pylori* ORF database of Swiss-Prot using the MASCOT program (Matrix Science Inc., MA).

Fluorescence correlation spectroscopy (FCS) measurement

One hundred nmol of Lyso-GM1, Lyso-GM2 or Lyso-GM3 (Takara-Bio Co., Shiga,

Japan) was mixed with an equi-molar Alexa Fluor 488 or Alexa Fluor 514 carboxylic acid succinimidyl ester (Invitrogen Co., USA) in dimethylformamide with 0.1% triethylamine. After 24 hrs incubation at RT in the dark, fluorescent-labeled Lyso-gangliosides were purified with HPLC equipped Cosmosil AR-II 4.6 x 150 mm column (Nacalai tesque Inc., Kyoto, Japan). Their structures were confirmed by LC-MS analysis using the LCMS-IT-TOF System (Shimadzu Co., Japan) (Supplementary Figure 1).

In a FCS measurement, the fluorescence intensity fluctuations of a single molecule, which passed across a confocally defined volume element in a fluid were recorded as photon counts and correlated in time (Eigen and Rigler, 1994; Maiti *et al*, 1997). A commercial FCS setup (Hamamatsu Photonics, Shizuoka, Japan), which is equipped with a water immersion objective (UPAPLO 40 x /1.2 W, Olympus) was used (Fig. 9D). The observed light cavity was reduced to less than a femto-liter, which was made by confocal illumination with a tightly focused laser beam and image formation on a small aperture in front of a photo multiplier. In this configuration, the 473 nm line of a semiconductor laser (output power 1 mW) was applied and attenuated adequately with light filters. The laser beam was focused at about 0.2 mm above the bottom of Nunc cuvettes (Nalge Nunc International, Naperville, IL) in a typical sample droplet volume 20 μ l. Fluorescence intensity data through each band-path filter, 500-530 nm or 540-570 nm, located adjacent to the photo multiplier tube were collected. Autocorrelation analysis of the fluorescence intensity fluctuations were performed by the control software (Hamamatsu Photonics, Japan).

Statistical analysis

Statistical analysis was performed using commercially available statistical software (StatView version 5.0). Data were expressed as the mean \pm standard error of mean (SEM) from at least three independent experiments. Statistical differences were analyzed using ANOVA or *T*-test where applicable. A *p*-value < 0.01 was considered as significantly different.

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Figure Legends

Fig. 1. Wada A. et al.

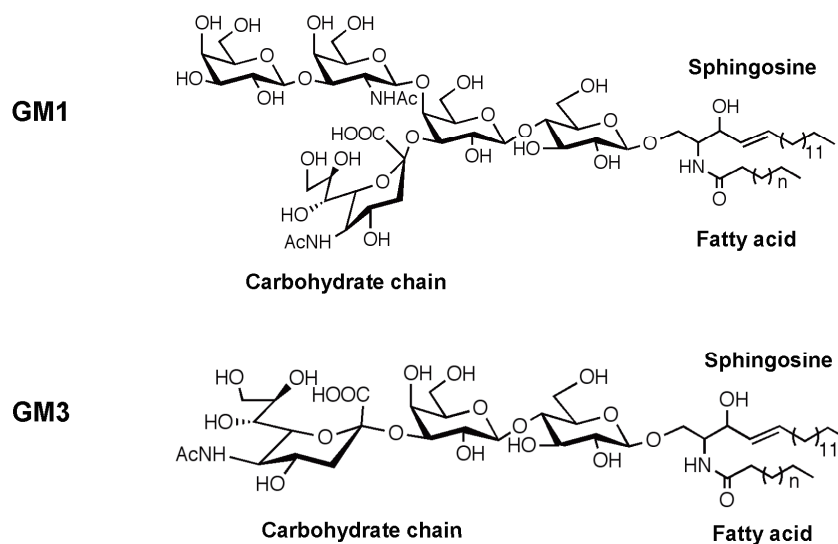


Fig. 1. Schematic representation of the chemical structure of gangliosides, GM1 and GM3. Ganglioside consists of a common carbohydrate moiety attached to sphingosine and fatty acid chains.

Fig. 2A. Wada A. et al.

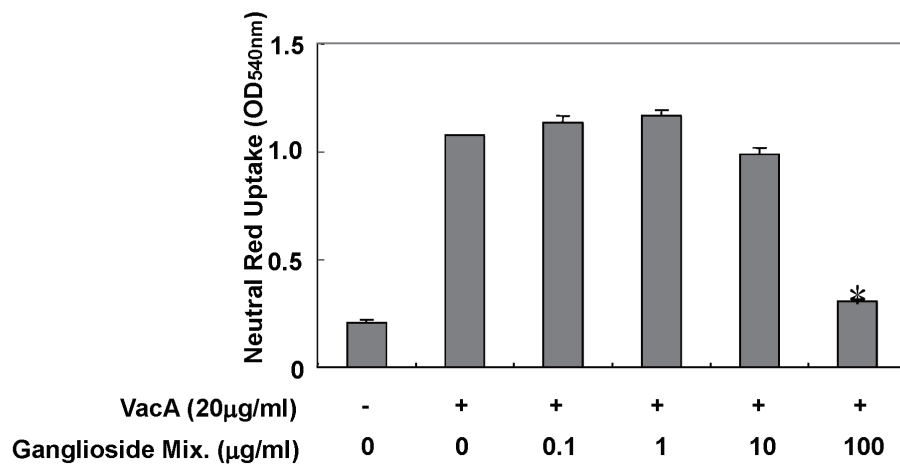


Fig. 2B. Wada A. et al.

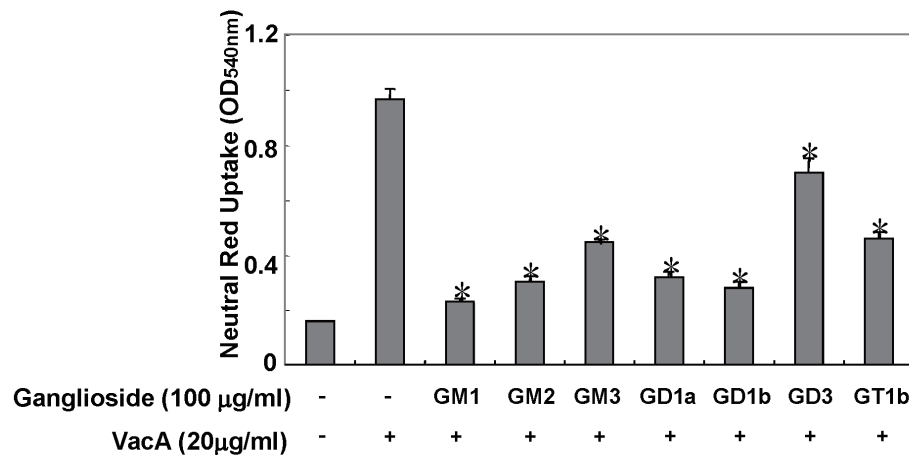


Fig. 2. Inhibition of VacA-induced vacuolation in AZ-521 cells by gangliosides.

The effects of various concentrations of bovine brain gangliosides mixture (A) and specific ganglioside on VacA-induced cellular vacuolation (B) are shown. AZ-521 cells were incubated for 3 h with or without 240 nM acid-activated VacA in the presence or absence of ganglioside in EMEM containing 5 mM NH₄Cl. Vacuoles formation was examined using Neutral Red uptake assay. * $p < 0.01$ (ANOVA with Dunett's post-hoc test (A) and *T*-test (B)) indicates a significant difference in comparison to cells incubated with only VacA.

Fig. 3. Wada A. et al.

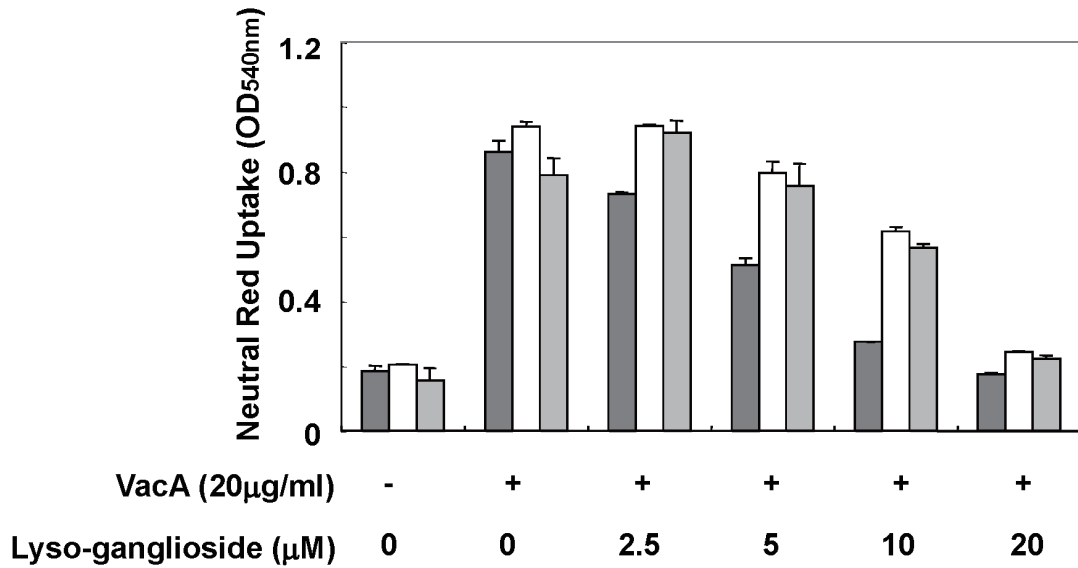
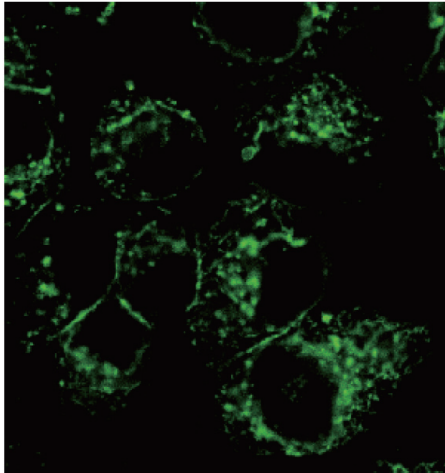


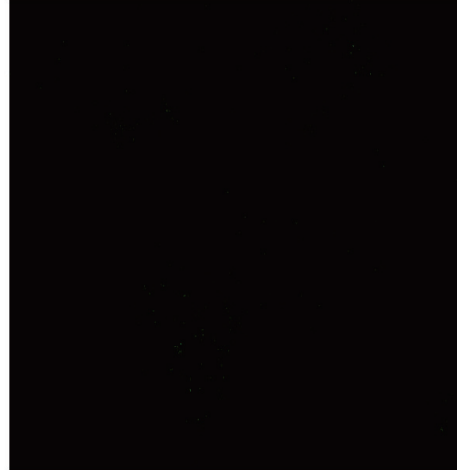
Fig. 3. Inhibition of VacA-induced vacuolation in AZ-521 cells by Lyso-GM1, Lyso-GM2 or Lyso-GM3 ganglioside. AZ-521 cells were grown overnight and incubated for 3 h with Lyso-ganglioside and 240 nM acid-activated VacA in EMEM containing 5 mM NH₄Cl. Vacuoles formation was examined using Neutral Red uptake assay. Black, white and gray bars represent Lyso-GM1, Lyso-GM2 and Lyso-GM3, respectively.

Fig. 4. Wada A. et al.

(A) VacA



(B) VacA + Lyso-GM1



IF: anti-VacA

Fig. 4. Internalization of VacA by AZ-521 cells in the presence or absence of Lyso-GM1.

AZ-521 cells were incubated with 240 nM acid-activated VacA for 3 h in EMEM supplemented with 5 mM NH₄Cl with or without 20 μM Lyso-GM1. Cells were stained with anti-VacA antibody and viewed under a confocal microscope.

Fig. 5. Wada A. et al.

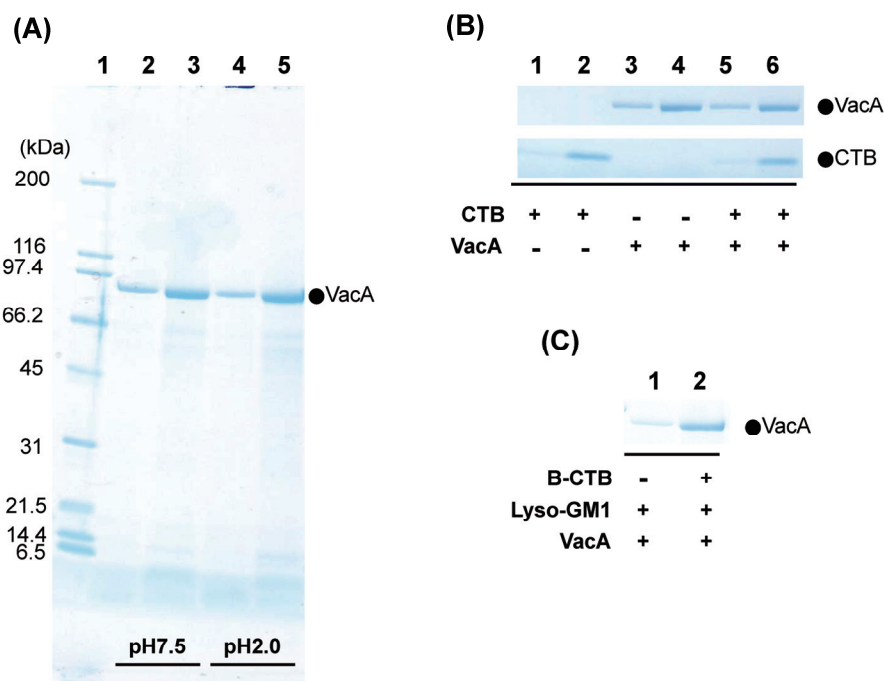


Fig. 5. Binding analyses of VacA or cholera toxin B (CTB) to Lyso-GM1 Sepharose.

(A) Electrophoresis separation of VacA and Lyso-GM1 Sepharose following binding under neutral (pH 7.5) or acidic (pH 2.0) conditions. Lane 1, molecular mass protein markers (kDa); lane 2, 4, control Sepharose; lane 3, 5, Lyso-GM1 Sepharose. Lane 2, 3, PBS in neutral condition (pH 7.5); lane 4, 5, PBS containing HCl (pH 2.0). (B) Electrophoresis separation of VacA and Lyso-GM1 Sepharose following binding in the presence or absence of CTB. Lane 1, 3, 5, control Sepharose; lane 2, 4, 6, Lyso-GM1 Sepharose. (C) Electrophoresis separation of VacA and Lyso-GM1-biotinylated CTB complex. Biotinylated CTB was captured by avidin-Sepharose together with VacA-Lyso-GM1 complex. Lane 1, without biotinylated CTB; lane 2, with biotinylated CTB.

Fig. 6. Wada A. et al.

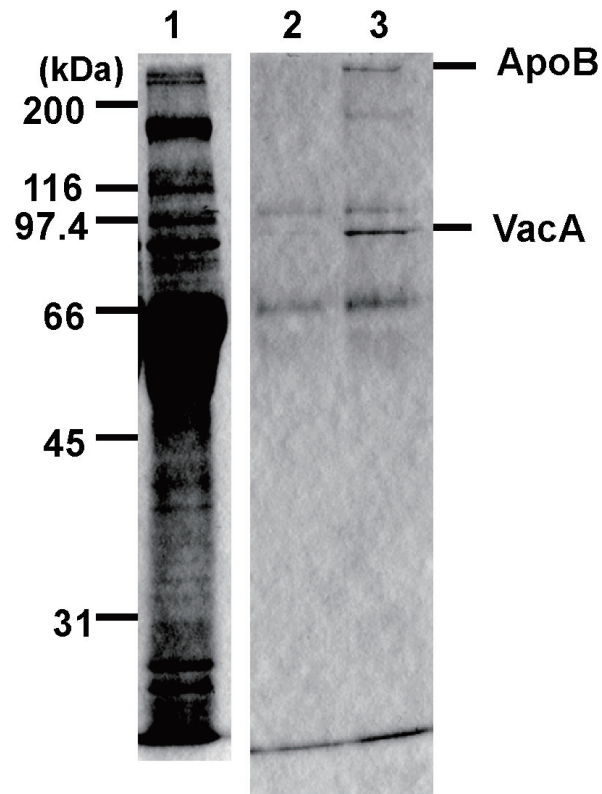


Fig. 6. Identification of the major binding protein of Lyso-GM1 in *H. pylori* culture supernatant. *H. pylori* proteins bound to Lyso-GM1 Sepharose were captured and proteins bound to these complexes were separated by 5-20 % gradient SDS-PAGE gel. Protein bands were excised, trypsin digested and analyzed by mass spectrometry. ApoB and VacA were identified as proteins that bind to Lyso-GM1 Sepharose. Lane 1, *H. pylori* culture supernatant; lane 2, control Sepharose; lane 3, Lyso-GM1 Sepharose.

Fig. 7A. Wada A. et al.

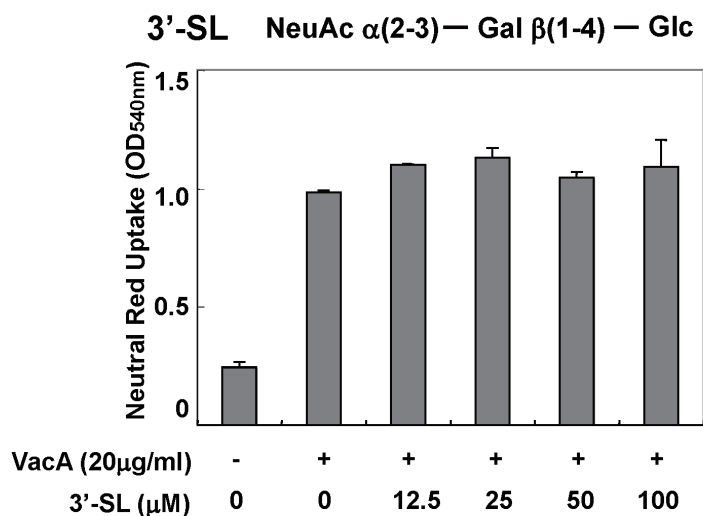


Fig. 7B. Wada A. et al.

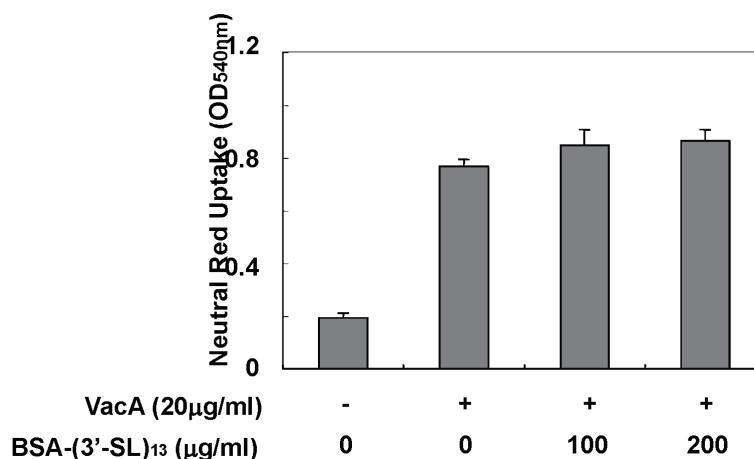


Fig. 7. Examination of the neutralization capacity of the carbohydrate moiety of GM3 (3'-Sialyllactose; SL) on vacuoles formation in AZ-521 cells. AZ-521 cells were incubated for 3 h with or without 240 nM acid-activated VacA in EMEM containing 5 mM NH₄Cl and subsequently treated with 3'-SL (A); BSA-(3'-SL)₁₃ (B). Neutral red uptake assay was then performed to measure the vacuolation activity of VacA. 200 mg/ml BSA-(3'-SL)₁₃ corresponds to 33 µM 3'-SL. No significant differences were observed between cells incubated with only VacA and with various concentrations of 3'-SL or BSA-(3'-SL)₁₃, ($p > 0.01$, ANOVA with Dunett's post-hoc test).

Fig. 8. Wada A. et al.

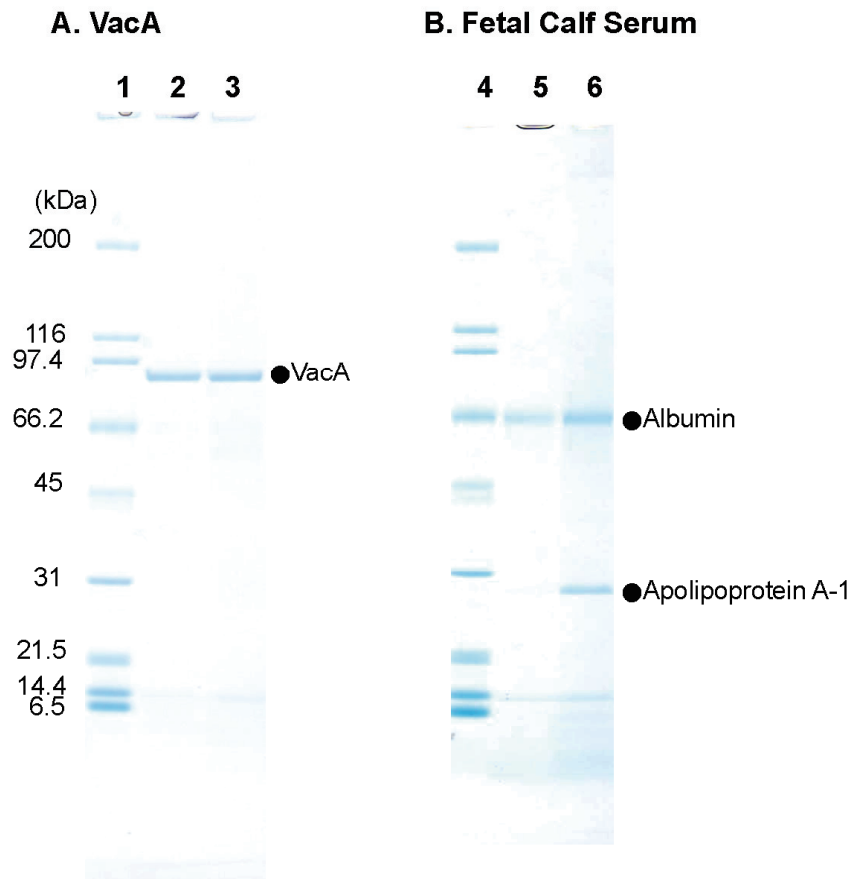


Fig. 8. Identification of sphingosine-binding protein. Pull-down assay was performed using sphingosine Sepharose. (A) VacA binds non-specifically to both control Sepharose and sphingosine Sepharose indicating that VacA does not bind to sphingosine. (B) Albumin and apolipoprotein A-1 from FCS instead bind to sphingosine Sepharose. Lane 1 and 4, molecular mass protein marker (kDa); lane 2 and 5, control Sepharose; lane 3 and 6, sphingosine Sepharose.

Fig. 9A. Wada A. et al.

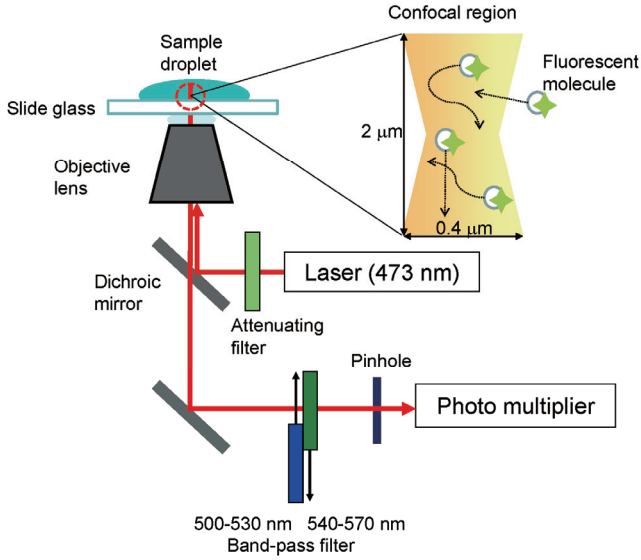


Fig. 9B. Wada A. et al.

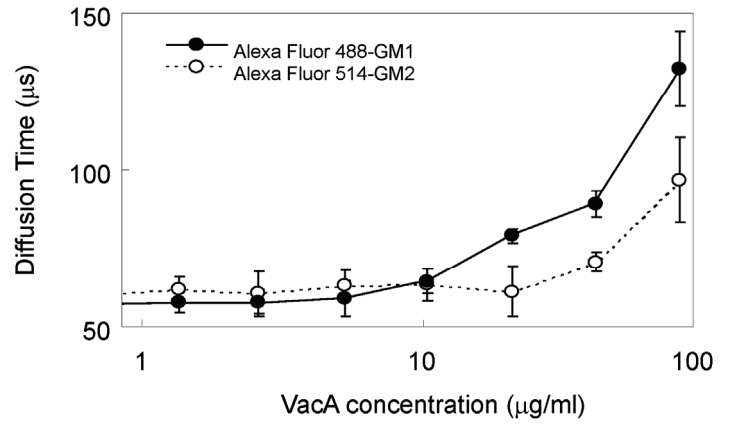


Fig. 9C. Wada A. et al.

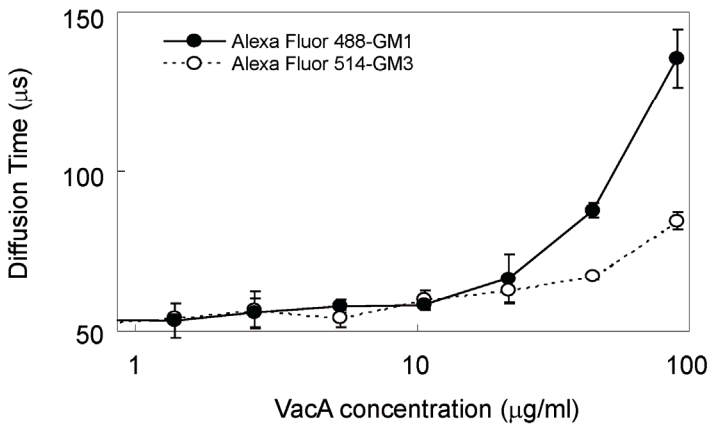


Fig. 9D. Wada A. et al.

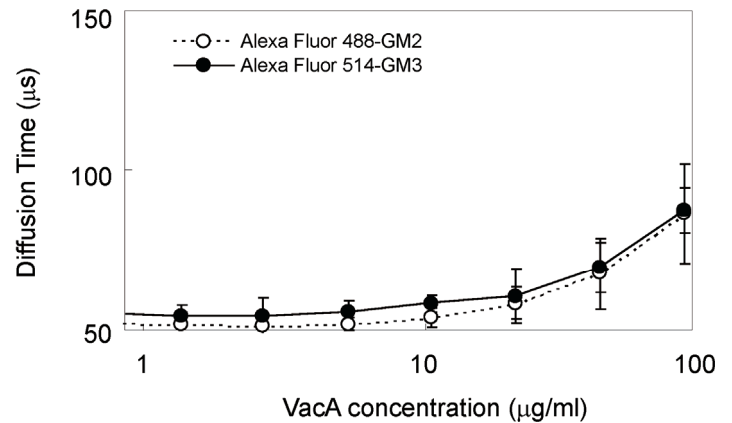


Fig. 9. Estimation of the relative binding affinity of Lyso-GM1, Lyso-GM2, and Lyso-GM3 to VacA using fluorescence correlation spectroscopy (FCS). (A) Schematic representation of the instrumentation setup for FCS. Fluorescence intensity is collected from the molecules in a small well defined volume in the confocal region within a sample droplet. The fluorescence intensity fluctuates according to diffusion velocity of a fluorescent molecule, and its autocorrelation yields the diffusion time. Each fluorescence intensity of Alexa Fluor 488-labeled and Alexa Fluor 514-labeled

molecules is observed through 500-530 nm and 540-570 nm band-pass filters switching, respectively. (B) Diffusion time (DT) values of Alexa Fluor 488-labeled Lyso-GM1 (closed circle) and Alexa Fluor 514-labeled Lyso-GM2 (open circle) in the presence of various concentrations (0-100 $\mu\text{g/ml}$) of VacA in PBS. (C). DT values of Alexa Fluor 488-labeled Lyso-GM1 (closed circle) and Alexa Fluor 514-labeled Lyso-GM3 (open circle). (D) DT values of Alexa Fluor 488-labeled Lyso-GM2 (closed circle) and Alexa Fluor 514-labeled Lyso-GM3 (open circle).

Table 1. Wada A. et al.

	LysoGM1	LysoGM2	LysoGM3
DT (μsec) in the presence of 180 nM VacA (% bound ratio)	124.7 \pm 8.1 (52%)	64.7 \pm 7.8 (20%)	66.5 \pm 6.6 (18%)
K_d (nM) for VacA	190	400	830
DT (μsec) in the presence of 280 nM CTB (% bound ratio)	100.3 \pm 7.3 (100%)	Not Detected	—
K_d (nM) for CTB	24	Not Determined	—

Table 1. K_d values of VacA-Lyso-gangliosides and CTB-Lyso-gangliosides

Supplementary Table 1. Wada A. et al.

Supplementary Table 1. Identification of VacA and Apolipoprotein by mass spectrometry

A. Peptide mass fingerprint blast result using ProteinProspector MS-Fit

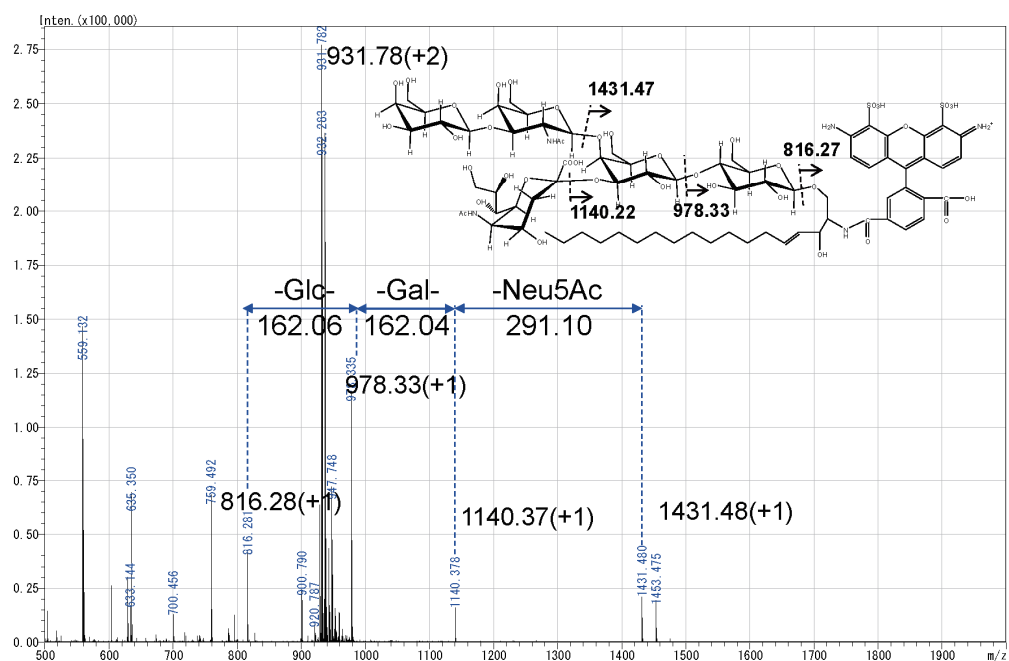
Identified protein	Accession number	Theoretical molecular mass	Mowse score	Sequence coverage (%)
Apolipoprotein B	XP_583270	211685	2.17e+013	31

B. MS/MS peptide sequence blast results using MASCOT

Identified protein	Accession number	Theoretical molecular mass	Mascot score	Expect	Sequence coverage
Vacuolating cytotoxin precursor	Q48245 (VACA2_HELPY)	139070	1650	5.1E-160	41
Albumin	P02769 (ALBU_BOVIN)	71279	624	2E-57	35
Apolipoprotein A-1	P15497 (APOA1_BOVIN)	30258	925	1.6E-87	63

Supplementary Table 1. Identification of VacA and Apolipoprotein by mass spectrometry

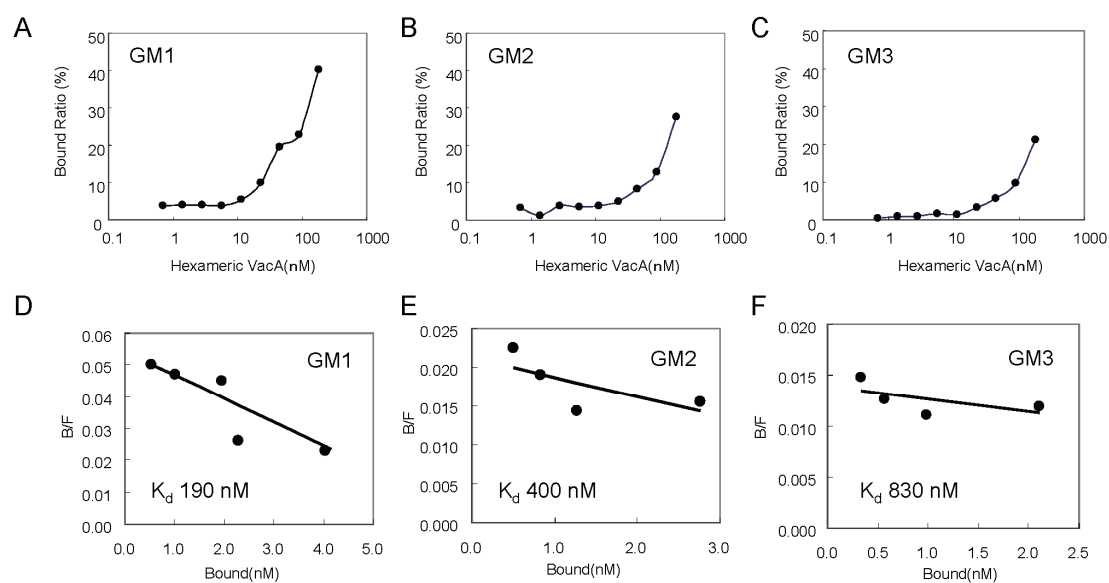
Supplement. Figure 1. Wada A. et al.



Supplementary Fig. 1. MS/MS spectrum of Alexa Fluor 488-labeled Lyso-GM1.

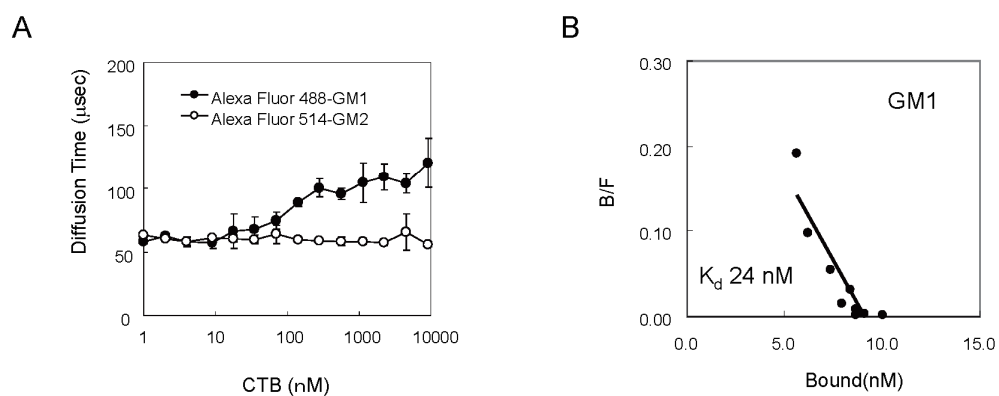
Lyso-GM1 was labeled with Alexa Fluor 488 and its structure was confirmed by LC-MS analysis using the LCMS-IT-TOF System (Shimadzu Co., Japan). The precursor ion m/z 931.78 ($[M+3Na+2H]^{2+}$) and fragment ion peaks are shown. The mass differences between each of the fragment ion peaks were consistent with residual mass values predicted from the Lyso-GM1 sugar chain structure.

Supplementary. Figure 2. Wada A. et al.



Supplementary Fig. 2. Bound ratio of the fluorescent-labeled Lyso-GM1 (A), Lyso-GM2 (B), or Lyso-GM3 (C) at various concentrations of VacA. Each ratio was estimated from composite curves fitting of two components (VacA-bound and unbound gangliosides) autocorrelation function of FCS data. The bound ratio data were applied to Scatchard plot analysis, and each K_d value of Alexa Fluor488-labeled Lyso-GM1 (D), Alexa Fluor514-labeled Lyso-GM2 (E), and Alexa Fluor514-labeled Lyso-GM3 (F) was estimated.

Supplement. Figure 3. Wada A. et al.



Supplementary Fig. 3. FCS measurements of the fluorescent-labeled Lyso-GM1 and CTB. (A) Diffusion time (DT) values of Alexa Fluor 488-labeled Lyso-GM1 (closed circle) and Alexa Fluor 514-labeled Lyso-GM2 (open circle) in the presence of various concentrations (0-10 μ M) of CTB in PBS. (B) Scatchard plot analysis for estimation of the K_d value of Alexa Fluor488-labeled Lyso-GM1 bound to CTB.