A Cellular Deficiency of Gangliosides Causes Hypersensitivity to Clostridium perfringens Phospholipase C*

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Clostridium perfringens phospholipase C (Cp-PLC), also called α-toxin, is the major virulence factor in the pathogenesis of gas gangrene. Previously, a cellular UDP-Glc deficiency was related with a hypersensitivity to the cytotoxic effect of Cp-PLC. Because UDP-Glc is required in the synthesis of proteoglycans, N-linked glycoproteins, and glycosphingolipids, the role of these glycoconjugates in the cellular sensitivity to Cp-PLC was studied. The cellular sensitivity to Cp-PLC was significantly enhanced by glycosphingolipid synthesis inhibitors, and a mutant cell line deficient in gangliosides was found to be hypersensitive to Cp-PLC. Gangliosides protected hypersensitive cells from the cytotoxic effect of Cp-PLC and prevented its membrane-disrupting effect on artificial membranes. Removal of sialic acids by C. perfringens sialidase increases the sensitivity of cultured cells to Cp-PLC and intramuscular co-injection of C. perfringens sialidase, and Cp-PLC in mice potentiates the myotoxic effect of the latter. This work demonstrated that a reduction in gangliosides renders cells more susceptible to the membrane damage caused by Cp-PLC and revealed a previously unrecognized synergism between Cp-PLC and C. perfringens sialidase, providing new insights toward understanding the pathogenesis of clostridial myonecrosis.

Bacterial phospholipases C (PLCs)1 (EC 3.1.4.3) are secreted proteins that display a variable preference for different phospholipids (1). Bacterial PLCs that hydrolyze sphingomyelin (SM) and phosphatidylcholine (PC) are important virulence factors in the pathogenesis of diseases caused by Listeria monocytogenes, Pseudomonas aeruginosa, and several Clostridium sp. (2–4). Clostridium perfringens, the pathogenic bacterium most widely distributed in nature (5), produces one phospholipase C (referred to here as Cp-PLC), which is highly toxic; it decreases cardiac contractility, increases capillary permeability, and displays platelet-aggregating, hemolytic, cytotoxic, and myotoxic activities (6). Cp-PLC is also called α-toxin and has been associated with enteritis in domestic animals, Crohn disease, and gas gangrene in humans (6).

Gas gangrene is an acute and life-threatening infection most frequently caused by C. perfringens and characterized by massive local edema, severe myonecrosis, and the accumulation of gas at the site of infection (7). Gas gangrene without external injury is associated with diabetes mellitus, peripheral vascular disease, or an underlying gastrointestinal or hematologic malignancy (8). More often, gas gangrene is associated either to trauma or surgery, occurring after the introduction of bacterial spores in a deep lesion or in a surgical wound (7). Despite the use of antibiotics and intensive care regimes, in many cases of gas gangrene radical amputation is the treatment of choice to avoid shock, multiorgan failure, and death (7). The plc gene, encoded in the C. perfringens chromosome, shows minimal inter-strain sequence variations and is highly expressed in all strains associated with gas gangrene (6, 9). Homologous genes are found in other Clostridium species such as Clostridium sordellii, Clostridium absonum, and Clostridium novyi, which occasionally cause gas gangrene (10, 11).

Several lines of evidence indicate that Cp-PLC is the major virulence factor in C. perfringens-induced gas gangrene. First, when injected intramuscularly in mice, recombinant Cp-PLC causes myonecrosis and reproduces the histopathological features of gas gangrene (12). Second, immunization with the recombinant Cp-PLC C-terminal domain protects mice from the intramuscular challenge with a lethal dose of C. perfringens vegetative cells (13). Third, a C. perfringens mutant strain, in which the plc gene has been inactivated by homologous recombination, is unable to produce gas gangrene (14). Although

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considerable progress has been made in recent years in the knowledge of the structure of Cp-PLC, our understanding of the cellular and molecular basis of its toxic effects is still incomplete (15).

A mutant cell line hypersensitive to Cp-PLC, referred to here as Don Q, was isolated previously (16). This cell line has a permanent low UDP-glucose (UDP-Glc) level due to a recessive point mutation in the UDP-Glcyrophosphorylase (UDPG:PP) gene (17). We showed previously (18) that upon one allele reversion of the mutation or transfection with a wild type UDPG:PP, the mutant cell compensates the UDP-Glc deficiency and regains the same relative resistance to Cp-PLC as the one displayed by the parental cell. However, the molecular basis relating the cellular UDP-Glc deficiency to the sensitization to Cp-PLC has not been established.

UDP-Glc is required for the synthesis of the carbohydrate moiety of cell surface glycoconjugates, such as proteoglycans, N-glycosylated glycoproteins, and glycosphingolipids (GSLs) (17). Proteoglycans consist of variable polysaccharide chains, the glycosaminoglycans, linked to a core polypeptide. The mature glycosaminoglycans contain glucosamine or galactosamine alternating in glycosidic linkages with glucuronic acid, iduronic acid, or galactose and are sulfated or acetylated to varying degrees (19). The glycosaminoglycans are assembled in the lumen of the Golgi apparatus by sequential addition of monosaccharides to the linker tetrasaccharide GlcUA/β1,3Gal/β1,3Gal/β1,4Xyl/β, which is bound to a Ser or a Thr residue of the core polypeptide (20). UDP-Glc is essential for the synthesis of UDP-glucuronic acid and UDP-galactose, two monosaccharide donors used in glycosaminoglycans synthesis.

N-Glycosylated glycoproteins have covalently bound to an Asn residue, oligosaccharides of variable composition, all of which arise from the common core precursor GlcManβ1,3GlcNAcβ1,4PP-dolichol (21). The oligosaccharide moiety of this precursor is synthesized by the stepwise addition of monosaccharide units onto dolichol pyrophosphate on the endoplasmic reticulum (ER) membrane and is then transferred en bloc onto the nascent polypeptide chains (21). UDP-Glc is actively transported to the lumen of the ER, where it serves as donor of the three terminal glucose of the core precursor (21). Within the ER, UDP-Glc is also used by the UDP-Glc-glycoprotein glucosyltransferase, which recognizes and glycosylates misfolded glycoproteins (21). This glycosylation prolongs the contact of N-linked glycoproteins with the lectin-type chaperones calnexin and calreticulin and thus leads to their retention within the ER until they are correctly folded (21).

GSLs are composed of a variable carbohydrate moiety linked to ceramide and derive either from glucosylceramide (GlcCer) or galactosylceramide (GalCer) (22). GalCer is synthesized from ceramide and UDP-galactose on the luminal face of the Golgi apparatus (23). Ceramide, a common precursor of GSLs and SM, is either synthesized from sphinganine/sphingosine (23) or by a salvage pathway from sphingomyelinase, 1-deoxynojirimycin, castanospermine, and the ganglioside GM3 were from Calbiochem. Both asparagine II (B. aspar MT II) was purified as described (25). 1,4-Amino-3-isoxazolidinone (L-cycloserine) and DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) were from Biomol. The ganglioside-specific monoclonal antibodies (mAbs) DH2, KM871, 101, and 102 were produced and purified as described (26–28). The ganglioside-specific monoclonal antibodies (mAbs) DH2, KM871, 101, and 102 were produced and purified as described (26–28). The anti-glucosylceramide rabbit antibody anti-glucosylceramide rabbit antibody DH2, KM871, 101, and 102 were produced and purified as described (26–28). The inhibitors of the pathway used in this study are indicated in italic.

FIG. 1. Metabolic pathway of glycosphingolipid synthesis and sites susceptible to pharmacologic inhibition. The inhibitors of the pathway used in this study are indicated in italic.

the 3-position of the inner galactose moiety or may lack SiaAc at this position (0 series) (23). Complex gangliosides have another SiaAc moiety linked to the 3-position of the terminal galactose (23).

Because a UDP-Glc deficiency leads to a cellular hypersensitivity to Cp-PLC (18) and this metabolite is essential in the synthesis of proteoglycans, glycoproteins, and GSLs, the aim of this work was to clarify the role of these glycoconjugates in the cellular sensitivity to this toxin.

EXPERIMENTAL PROCEDURES

Toxins, Antibodies, and Chemicals—Wild type recombinant Cp-PLC from the strain NCTC 8237 or its C-terminal fragment (C-PLC) were expressed in Escherichia coli and purified as described (24). Bacillus cereus PLC, 1-deoxynojirimycin, castanospermine, and the ganglioside GM3 were from Calbiochem. Both asparagine II (B. aspar MT II) was purified as described (25). 1,4-Amino-3-isoxazolidinone (L-cycloserine) and DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) were from Biomol. The ganglioside-specific monoclonal antibodies (mAbs) DH2, KM871, 101, and 102 were produced and purified as described (26–28). The inhibitors of the pathway used in this study are indicated in italic.

Cells and Cell Culture—Chinese hamster lung fibroblasts, referred to here as Don wt, mouse melanoma cells (B16), Chinese hamster ovary cells (CHO-K1), and human larynx carcinoma cells (HEp-2) were from ATCC. The UDP-Glc-deficient mutant of Don wt cells, Don Q, which has a defective UDPG:PP, the spontaneous revertant of this mutant, Don QR, and the UDPG:PP transfectant clone. B9 was described previously (18). A proteoglycan-deficient mutant of CHO cells, referred to here as pgsG-110, which has a defective UDP-GlcUA:Galβ1,3Galβ1,4GlcNAcβ1,4PP-dolichol glycosyltransferase I (GlcUA-T1), and the GlcUA-T1 transfectant clone pgs-T
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were described previously (20). A ganglioside-deficient mutant of B16 cells, referred to here as GM5, which has a defective UDP-Glc-ceramide glucosyltransferase (UDPG:CGT, EC 2.4.1.80), and the UDPG:CGT transfected CG-G1 were described in previous studies (20). Don wt, Q, QR, B9, B16, GM5, and CG-1 cells were cultivated at 37 °C in Eagle’s minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 5 mM l-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humid atmosphere containing 5% CO₂, 98% (g/g), pgs-110, and pgs-T cells were grown in Ham’s F-12 medium (Invitrogen), supplemented as above.

Cytotoxicity Assays—Cells in 96-well plates grown to 80–90% confluence were exposed to Cp-PLC or to wheat germ lectin in 200 μl of medium/well, and cell viability was assessed 24 h after using a neutral red assay (18). Briefly, the cells were incubated for 2 h with 200 μl/well of neutral red (50 μg/ml) dissolved in supplemented culture medium, and the incorporated dye was extracted with 100 μl of acetone/ethanol/water (90:900:1). After recording the absorbance at 540 nm (Invitrogen), neutral red was expressed as a percentage, considering as 100% the value of parallel cultures incubated with only medium or with medium and drugs at the indicated concentrations. Assays were performed with 3–6 replicate samples. When not shown, the standard deviation was <10%.

Pharmacological or Enzymatic Treatments and Evaluation of Their Effects—Cells were preincubated for 18–24 h with 1-deoxyxojirimycin (1–90 μM), auranofin (1–90 μM), chlorpromazine (1–90 μg/ml), brefeldin A (0.1–9 μg/ml), monensin (1–90 μg/ml), tunicamycin (0.1–9 μg/ml), β-chloroalanoline (1–90 μg/ml), β-cyclohexidyne (3–300 μg/ml), myriocin (3–300 μg/ml), fumonisin B1 (0.3–30 μM), PMK (0.3–30 μg/ml), C. perfringens large sialidase (0.3–100 units/ml) or V. cholerae small sialidase (0.3–100 units/ml) or for 1 h to 1% Triton X-100 at 37 °C or to borate saline buffer, pH 7.6, respectively.

Proteins in lysates of untreated and treated cells were submitted to SDS-PAGE and electrophorotated onto 0.45-μm nitrocellulose membranes as described (17). Carbohydrate moieties in glycoproteins were oxidized with periodate and detected using biotin-hydrazide and streptavidin-peroxidase as described (17). Carbohydrate moieties in glycoproteins were oxidized with periodate and detected using biotin-hydrazide and streptavidin-peroxidase as described (17). Ganglioside GT1b was dissolved in acetone/ethanol/acetic acid (90:900:1) and gangliosides (90–900 μg/ml) in supplemented medium containing 10% FBS, or for 18–24 h in Opti-MEM (Invitrogen) with 2% serum as described (27). When not shown, the standard deviation was <10%.

To evaluate ganglioside incorporation into the membrane, cells were trypsinized and pelleted by centrifugation at 4 °C at 300 × g for 10 min. Pellets were lyophilized and extracted twice with chloroform/methanol 1:1 (v/v). The combined extracts were reduced to 25% of the original volume and kept at 4 °C overnight, and then the insoluble material was removed by centrifugation at 10,000 × g for 10 min. The supernatant was washed successively with nitrocellulose membranes (in 500 μl of distilled water). Salts were removed by gel filtration in Sephadex G-50.

Membrane-disrupting Effect in Liposomes—Unilamellar liposomes composed of cholesterol and either PC or SM (in 1:1 molar ratio) encapsulating carboxyfluorescein were prepared as described (23). In some cases purified gangliosides were incorporated into the liposomes at concentrations of 100, 33, and 11 μg/ml (which would correspond to PC/ganglioside or SM/ganglioside molar ratios of about 30:1; 90:1, and 270:1). After a 20-min exposure to Cp-PLC (20 μg/ml), fluorescence at 520 nm (excitation at 485 nm) was measured using a Fluoroscan Ascent fluorimeter (PerkinElmer Life Sciences). Samples were assayed in triplicate. Carboxyfluorescein release was expressed as a percentage, considering as 100% the value of the same liposomes exposed for 1 h to 1% Triton X-100 at 37 °C or to borate saline buffer, pH 7.6, respectively.

Biosensor Studies—Analysis was performed using a BLAcore 2000 biosensor and the carboxymethyl-dextran-coated sensor chip, CM5, or the lipophilic pioneer L1 sensor chip (Pharmacia Biosensor, Uppsala, Sweden). GT1b ganglioside was immobilized onto a CM5 surface as described previously (27). Briefly, ganglioside GT1b was dissolved in ethanol and grafted at a density of 120 RU (which correspond to PC/ganglioside or SM/ganglioside molar ratios of about 30:1; 90:1, and 270:1). After a 20-min exposure to Cp-PLC (20 μg/ml), fluorescence at 520 nm (excitation at 485 nm) was measured using a Fluoroscan Ascent fluorimeter (PerkinElmer Life Sciences). Samples were assayed in triplicate. Carboxyfluorescein release was expressed as a percentage, considering as 100% the value of the same liposomes exposed for 1 h to 1% Triton X-100 at 37 °C or to borate saline buffer, pH 7.6, respectively.

RESULTS

Inhibition of Proteoglycan Synthesis Does Not Affect Cellular Sensitivity to Cp-PLC—To evaluate the role of proteoglycans in the cell sensitivity to Cp-PLC, the following two cell lines having extreme differences in their proteoglycan content due to a genetic reason were used: the proteoglycan-deficient mutant cell line, pgs-G110, which has an impaired activity of the enzyme GlcUAT-1, that catalyzes the first step in the synthesis of the tetrasaccharide primer of glycosaminoglycans (20), and the clone pgs-T, which is a stable transfectant having a wild-type GlcUAT-1 gene. Remarkably, these two cell lines exhibited the same sensitivity to Cp-PLC as the wild-type CHO cell (Fig. 2), which conclusively demonstrates that a deficiency in proteoglycans does not sensitize cells to Cp-PLC.
**Inhibition of N-Linked Glycoprotein Processing Does Not Increase the Cellular Sensitivity to Cp-PLC**—We assessed the role of N-linked glycoproteins in the cellular sensitivity to Cp-PLC by treating the cells prior to and during the toxin exposure with substances that interfere with their processing or transport to the surface. These substances lead to the production of glycoproteins with under-processed or incomplete oligosaccharide chains and thus alter the cellular glycoprotein pattern (33, 34). Deoxynojirimycin, an inhibitor of glucosidase I, and castanospermine, an inhibitor of glucosidases I and II, prevent removal of the glucose residues from the oligosaccharide core thereby inhibiting further processing of the oligosaccharide chain (33). Swainsonine affects a later step of glycoprotein processing by inhibiting the Golgi α-mannosidase II and thus preventing further glycoprotein processing at the Golgi apparatus (33). Brefeldin A and monensin interfere with the organization of the Golgi apparatus and thereby interrupt the trafficking of glycoproteins through this compartment (34). As reported for other cell lines, growth of Don wt cells in the presence of deoxynojirimycin, castanospermine, swainsonine, brefeldin A, or monensin altered the glycoprotein pattern (not shown). However, none of these treatments increase the sensitivity of Don wt cells to Cp-PLC (Fig. 3A, bars 1–5), in comparison with untreated cells. Consistently, the sensitivity of Don wt cells to the cytotoxic effect of Cp-PLC was not affected when the cells were pretreated neither with endoglycosidase H, which cleaves high mannose and hybrid N-linked oligosaccharides from glycoproteins, nor with endoglycosidase F, which cleaves high mannose, hybrid, and biantennary complex N-linked oligosaccharides (Fig. 3A, bars 6 and 7). In contrast, the exposure to the same inhibitors of glycoprotein processing/transport or endoglycosidases changed significantly the cellular sensitivity to the cytotoxic effect of wheat germ lectin (Fig. 3B), which requires binding to cell surface glycoproteins in order to cause cytotoxicity (35). These results corroborate that the treatments with the inhibitors or the enzymes had the predicted effect and indicate that a reduction in cell surface N-linked glycoproteins does not increase the cellular sensitivity to Cp-PLC. However, pretreatment of Don wt with tunicamycin, an inhibitor of the N-acetylglucosamine phosphotransferase, which blocks the synthesis of all N-linked glycoproteins (21), was found to increase the cellular sensitivity to both Cp-PLC and wheat germ lectin, in comparison with control cells (Fig. 3, A and B, bars 8). Because tunicamycin also blocks the synthesis of GSLs (36–38), we were prompted to study the effects of specific inhibitors of GSLs synthesis in the cellular sensitivity to Cp-PLC.

**A Specific Reduction in Cellular Gangliosides Increases the Sensitivity to the Cytotoxic Effect of Cp-PLC**—We initially assessed the role of GSLs in the cellular sensitivity to Cp-PLC by treating the cells prior to and during the toxin exposure with various inhibitors of the enzymes involved in GSLs synthesis (see Fig. 1). Myriocin, L-cycloserine, and β-chloroalanilin inhibit the serine palmitoyltransferase, blocking the pathway of ceramide synthesis (22). Fumonisin B1 inhibits the sphinganine/sphingosine fatty-acyl-CoA N-acyltransferase, blocking both ceramide synthesis de novo (22), whereas PPMP inhibits UTP-GC7, the enzyme that catalyzes glucosylceramide synthesis (39). The treatment of Don cells with these substances reduces the synthesis of glucosylceramide and GM3 to 20–35% in comparison with untreated cells (not shown) and all increased significantly their sensitivity to Cp-PLC (Fig. 4A). In contrast, none of these GSLs synthesis inhibitors affected the cellular sensitivity to the cytotoxic effect of the B. cereus PLC (not shown). Similar results were obtained with the same treatments in the cell lines CHO and HEp-2 (not shown). These results suggest that the reduction of glucosylceramide-derived GSLs specifically sensitizes different cell lines to Cp-PLC. To substantiate this conclusion further, we compared the sensitivity to Cp-PLC of two cell lines having extreme differences in their GSLs content due to a genetic reason. We used the ganglioside-deficient mouse melanoma cell line GM95, which has an impaired activity of UDPG:GC7 (29), and the clone CG-1, which is a stable transfected having a wild type UDPG:GC7 and has the same ganglioside content as the parental cell (29). Remarkably, the ganglioside-deficient cell GM95 was found to be 10^2 times more sensitive to Cp-PLC than the transfected clone CG-1, which is resistant to the cytotoxic effect of the Cp-PLC as the parental B16 cell (Fig. 4B). In contrast, GM95, CG-1, and B16 exhibited the same relative resistance to the B. cereus PLC (not shown). These results conclusively demonstrated that a cellular deficiency of glucosylceramide-derived GSLs specifically causes hypersensitivity to the cytotoxic effect of Cp-PLC. Indeed, the content of the ganglioside GM3, evaluated by HPTLC and immunostaining with mAb DH2, was found to be similar in Don Q and GM95 cells (2.4 ± 0.3 and 2.0 ± 0.4 nmol/10⁶ cells respectively) and is significantly reduced (about 4 and 19 times, respectively) in
Inhibition of glycosphingolipid synthesis sensitzes cells to Cp-PLC. A, Don wt cells were incubated without any pretreatment (bar 1), pretreated with 60 mg/ml β-chloroalanine (bar 2), 60 mg/ml L-cycloserine (bar 3), 60 mg/ml myriocin (bar 4), 0.01 mg/ml fumonisin B1 (bar 5), and 6 mg/ml FFMP (bar 6), as described under “Experimental Procedures,” before exposure to Cp-PLC (214 ng/ml). Cell viability was determined 24 h later using the neutral red assay. The results are expressed as the percentages of neutral red uptake in controls incubated with the same concentration of the inhibitors but without Cp-PLC. B, a ganglioside deficiency due to a genetic defect sensitizes cells to Cp-PLC. The mouse melanoma ganglioside-deficient mutant cell line GM95, which has a deficient UDPG:CGT (filled circles), the UDPG:CGT transfectant clone CG-1 (×), and the wild type B16 Cells (open circles) were exposed to serial 10-fold dilutions of Cp-PLC (214 μg/ml). Cell viability was determined 24 h later using the neutral red assay. The results are expressed as the percentages of neutral red uptake in controls incubated without Cp-PLC.

Gangliosides Protect Hypersensitive Cells from the Cytotoxic Effect of Cp-PLC—It has been shown that gangliosides added to culture media are incorporated in the plasma membrane by a wide range of cells (23). Most interestingly, Don Q or GM95 cells preincubated with a mixture of gangliosides extracted from bovine brain were protected in a dose-dependent manner from the cytotoxic effect of Cp-PLC (not shown). In contrast, pretreatment of Don Q or GM95 cells with galactocerebrosides extracted from bovine brain did not have any effect on their sensitivity to Cp-PLC (not shown). Because exogenously added gangliosides could attach to cells as micelles removable by proteases (40), control experiments were performed in which cells were treated with trypsin after ganglioside addition. However, trypsinization did not affect the protective effect of the gangliosides (not shown). In addition, the incorporation of gangliosides was verified by flow cytometry using the mAbs 101 and 102 in cells exposed to the ganglioside mixture and then treated with trypsin (not shown). Taken together, these results indicated that the protection from the cytotoxic effect of Cp-PLC was exerted by ganglioside molecules inserted into the plasma membrane.

To evaluate whether the sensitization to Cp-PLC caused by tunicamycin was preventable by gangliosides, cells were treated with tunicamycin in the presence of different concentrations of bovine brain gangliosides. This mixture fully protected Don wt cells (Fig. 5A) or CHO cells (not shown) from the sensitization caused by tunicamycin, thus demonstrating that the sensitization caused by tunicamycin was indeed due to the inhibition of ganglioside synthesis rather than to the inhibition of N-linked glycoprotein synthesis.

To determine whether protection from the cytotoxic effect of Cp-PLC is conferred by specific type(s) of gangliosides, experiments were performed adding different gangliosides to the cells. Pretreatment of Don Q cells with the ganglioside asialo-GM1a did not protect from the cytotoxic effect of Cp-PLC (Fig. 5B). However, pretreatment with gangliosides of the a series (GM3, GM1a, or GD1a) or the b series (GD1b or GT1b) (Fig. 1) increased cell resistance to Cp-PLC in a dose-dependent manner (Fig. 5B). The protective effect increased with the content of SiaAc of the ganglioside oligosaccharide chain, indicating that multiple SiaAc units of complex gangliosides play a protective role. Remarkably, the protective effect of GM3 and GT1b could be reduced or even abolished if they are added in the presence of the “C. perfringens large sialidase” (Fig. 5C), which catalyzes the hydrolysis of α2,3 SiaAc residues in gangliosides (41).
These results further substantiate the conclusion that the SiaAc of these GSLs play a role in their cytoprotective effect from Cp-PLC.

**Exposure to Sialidases Potentiates the Toxicity of Cp-PLC—**
The effect of a pretreatment of the target cells with sialidases from Cp-PLC was evaluated. The exposure of Don wt or CG-1 cells to the *C. perfringens* large sialidase did not cause cell death by itself but increased significantly their sensitivity to the cytotoxic effect of Cp-PLC in a dose-dependent manner (Fig. 6A). In contrast, the exposure of Don wt cells to *C. perfringens* large sialidase alone, 0.7 μg of Cp-PLC alone, or with a combination of Cp-PLC and 17.5 or 35 milliunits of the sialidase, and 3 h later plasma CK activity was measured as described under “Experimental Procedures.”

To evaluate whether a potentiation of Cp-PLC toxicity by the *C. perfringens* large sialidase also would occur *in vivo*, both proteins were injected separately and together intramuscularly in mice, and myotoxicity was evaluated as the increase in plasma CK activity. The sialidase was not myotoxic by itself at the doses used, but increased the muscle damage caused by Cp-PLC in a dose-dependent manner (Fig. 6C).

*C. perfringens* Causes Disruption of the Cellular Membrane in Ganglioside-deficient Cells—We have shown previously that there are no significant differences in the incorporation of [3H]choline among cells with different UDP-Glc levels (18). We now compared the degradation of choline-containing phospholipids and the disruption of the cellular membrane caused by Cp-PLC in the cell lines Don wt, Q, and QR, which differ in their cellular UDP-Glc concentration (17). The degradation of choline-containing phospholipids induced by Cp-PLC, measured as the release of radioactive choline from prelabeled cells, occurred in a dose- and time-dependent manner (Fig. 6, A and B). Remarkably, Cp-PLC induced a higher release of labeled choline from the membrane of Don Q than from Don wt or Don QR cells, which have partially compensated the UDP-Glc deficiency (17) (Fig. 7, A and B). Furthermore, it also induced a higher release of LDH from Don Q than from the UDPG:PP transfectant clone B9 (Fig. 8A). Similarly, the release of LDH induced by Cp-PLC from GM95 cells was higher than that induced from the UDPG:CGT transfectant clone CG-1 (Fig. 8A). In contrast, *B. asper* myotoxin II, a membrane-disrupting toxin with broad cytolytic specificity (35), induced a similar LDH release from Don Q and GM95 as from their transfectant counterparts (Fig. 8B). Therefore, these data show that Cp-PLC causes extensive membrane damage in Don Q and GM95 cells and suggest that their hypersensitivity to this toxin depends, at least in part, on an increased disruption of the cellular membrane.

Gangliosides Protect PC- or SM-containing Liposomes from the Membrane-disrupting Effect of Cp-PLC—Although Cp-PLC has a broad spectrum of substrates (1), it only disrupts artificial membranes made of cholesterol and PC or SM, but not those made of cholesterol and phosphatidylinerine, phosphati-
onto the CM5 surface (140 and 260 RU, respectively, Fig. 9 specifically to immobilized GT1b via hydrophobic interaction surfaces. The anti-GT1b mAbs 101 and 102 were shown to bind with GT1b was evaluated by biosensor analysis. GT1b was immobilized onto a carboxymethyl-dextran-coated CM5 sensor chip or onto a lipophilic L1 sensor chip. To evaluate the specificity of the GT1b-immobilized surfaces, anti-GT1b ganglioside mAb 101, mAb 102, or anti-GD3 ganglioside mAb KM871 (165 nM) were injected on both the GT1b-coated and the control surfaces. The anti-GT1b mAbs 101 and 102 were shown to bind specifically to immobilized GT1b via hydrophobic interaction onto the CM5 surface (140 and 260 RU, respectively, Fig. 9A) compared with the CM5 control surface. The mAb KM871 (Fig. 10A) and bovine serum albumin (not shown) did not bind to immobilized GT1b. Similar results were obtained by using the L1 chip. The anti-GT1b mAbs 101 and 102 were shown to bind specifically to immobilized GT1b/PC vesicles (170 and 130 RU, respectively, see Fig. 10B) compared with a PC control surface, whereas the mAb KM871 (Fig. 10B) or bovine serum albumin (not shown) did not bind to the GT1b/PC vesicles. No binding was observed upon injection of Cp-PLC or its C-terminal domain (C-PLC) (2.35 and 3.5 mM, respectively) onto GT1b-coated surfaces (Fig. 10A and B). Similar results were obtained when Cp-PLC or C-PLC was injected at concentrations of 7 and 10 mM, respectively.

**DISCUSSION**

Cp-PLC is the major virulence factor in the pathogenesis of gas gangrene, a devastating disease associated with traumatic injuries or surgical wounds, which has increasing significance in diabetes. Despite the recent identification of the residues critical for toxicity in Cp-PLC (24, 43, 44), the molecular mechanism of action of this toxin is still not completely understood. Cp-PLC is hemolytic, necrotizing, lethal, and highly toxic to various cell types, particularly those that are deficient in UDP-Glc (18). However, the reason why a UDP-Glc deficiency induces cellular hypersensitivity to Cp-PLC was unknown. UDP-Glc is an important precursor in the synthesis of cell surface glycoconjugates such as proteoglycans, glycoproteins, and GSLs. This work demonstrates that a reduction of cellular gangliosides increases the cellular sensitivity to Cp-PLC. Several lines of evidence support this conclusion. (i) Pharmacological inhibition of ganglioside synthesis sensitizes different cell lines to the cytotoxic effect of Cp-PLC. (ii) Mutant cell lines with a reduced content of gangliosides, due to a mutation either in the UDPG:CGT or in the UDPG:FP, are 10^5 times more sensitive to Cp-PLC than the corresponding clones transfected with the wild type enzymes. (iii) The addition of exogenous gangliosides protects hypersensitive cells from the cytotoxic effect of Cp-PLC.

Regarding the type of gangliosides involved in this phenomenon, it was found that gangliosides protected artificial and cellular membranes from the disruption caused by Cp-PLC, whereas asialo-GM1a did not protect them. The fact that GM3 and GM1a have a protective effect, whereas asialo-GM1a does not, demonstrates that the SiaAc attached to the terminal galactose plays a role in protection. Furthermore, because GM1a and GD1b were less protective than GD1a and GT1b, respectively, the results indicate that the SiaAc attached to the terminal galactose also contributes to the protective effect of complex gangliosides. Two lines of evidence support the importance of the SiaAc units in cytoprotection. (i) C. perfringens sialidase abolishes the capacity of GM3 and GT1b to protect GM95 cells from the cytotoxic effect of Cp-PLC. (ii) Pretreatment of cultured cells with C. perfringens sialidase increases significantly their sensitivity to Cp-PLC.

Our observations shed light on general issues of muscle pathology during clostridial myonecrosis. When injected intramuscularly, Cp-PLC causes rapid and extensive damage to...
muscle fibers, inducing a significant release of CK to the plasma (24). However, the reason for the high sensitivity of muscle cells to this toxin is not clear. Muscle is known to have the lowest concentration of complex gangliosides among all mammalian tissues studied so far (45, 46). Therefore, our findings offer a possible explanation for the high susceptibility of muscle fibers to the cytotoxic effect of Cp-PLC. A UDP-Glc deficiency has been reported to occur in the muscular tissue during diabetes (47), probably as a consequence of the defective glucose transport and/or phosphorylation (48). The effect of this metabolic deficiency on ganglioside synthesis could be a predisposing factor to the severity of the tissue damage caused by C. perfringens infections in these patients.

Sequence analysis of the C. perfringens chromosome reveals the presence of two genes encoding secreted sialidases that are able to hydrolyze the SiaAc residues from glycoconjugates of the mammalian cell membranes (5). The nanI gene (Locus CPE 0725) encodes a 694-amino acid protein known as the “large sialidase enzyme” (49). The nanJ gene (Locus CPE 0553) encodes a 1173-amino acid protein not yet characterized, which displays a 51% sequence similarity to the 111-kDa sialidase from Clostridium septicum (50). Sialidase activity is detectable in culture supernatants during exponential growth of C. perfringens, and in vivo it is detectable in the bloodstream of experimental animals or patients with gas gangrene in early stages of the disease (51, 52). In patients suffering clostridial myonecrosis sialidase in amounts as high as 200 units/liter and 110 milliunits/g can be found in exudates and tissue homogenates, respectively (51, 52). The results of the myotoxicity studies performed in this work clearly support the hypothesis that C. perfringens sialidase has a synergistic effect with Cp-PLC in the pathology of clostridial myonecrosis.

The damage inflicted by Cp-PLC to the muscle tissue during C. perfringens infections could be also potentiated by its effects on diverse signal transduction pathways in endothelial cells, platelets, and neutrophils (7). In these cells, Cp-PLC leads to the uncontrolled production of several intercellular mediators and adhesion molecules, which alters the traffic of neutrophils to the infected tissue and promotes thrombotic events leading to further ischemia (7). Because UDPG:CGT expression decreases rapidly in cells exposed to low oxygen tension (53), the perfusion deficit caused by Cp-PLC in the infected tissue might lead to a reduction in cellular gangliosides, further increasing the susceptibility of muscle cells to its membrane damaging effect.

The capacity of Cp-PLC to disrupt artificial membranes depends on its ability to bind and enzymatically degrade their constituent phospholipids (54). We showed that the disruption of PC- or SM-containing liposomes by Cp-PLC is prevented by the incorporation of gangliosides into the liposomes membrane. It has been reported that the incorporation of gangliosides into artificial membranes does not affect the binding of Cp-PLC (55). Therefore, the inhibition of the membrane-disrupting effect of Cp-PLC by gangliosides could result from a direct interaction of the Cp-PLC with the SiaAc units, which would prevent it from reaching the acyl-ester bonds to be cleaved. However, the results of the biosensor studies excluded that possibility. Because gangliosides reduce membrane fluidity and shift the cut-off pressure to lower values (56), their inhibitory effect on the Cp-PLC membrane-disrupting activity could result from a tighter packing of the phospholipids in the target membrane. Alternatively, because the bulky head groups of gangliosides protrude from the interface and create a polar and hydrated microenvironment (56), the inhibition could be due either to an alteration of the availability of substrate due to a steric effect or to the electrostatic changes induced at the interface. The latter possibility is supported by the fact that GD1a and GT1b, which carry higher number of charges, exert the highest inhibition.

The three-dimensional structure of Cp-PLC shows two domains joined by a short flexible linker region (57). The N-terminal domain contains the active site, whereas the C-terminal domain, which is needed to interact with aggregated phospholipids, is analogous to C2 domains of intracellular eukaryotic proteins involved in signal transduction (57). Recent crystallographic studies have revealed that Cp-PLC exists in two conformations as follows: an open active form and a closed inactive form. In the open form three Zn2+ ions are bound, and the active site is accessible (58). In the closed form there are only two Zn2+ ions bound, and the active site is hindered by the loop encompassing residues 132–149 (58). It is suggested that the binding of the Cp-PLC C-terminal domain to the target membrane induces a conformational change in the N-terminal domain, which uncovers the active site, allowing hydrolysis of the aggregated phospholipid substrates (58). The changes induced by the reduction of the gangliosides content of the target membrane could favor that Cp-PLC acquires and/or maintains its active conformation. Regardless of the mechanism, our results demonstrate that the reduction of gangliosides in the target membrane increases the sensitivity to the Cp-PLC-induced disruption.
Ganglioside Reduction Causes α-Toxin Hypersensitivity

References


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