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Characterization of the Interaction of Hemolytic Lectin CEL-III from the Marine Invertebrate, *Cucumaria echinata*, with Artificial Lipid Membranes: Involvement of Neutral Sphingoglycolipids in the Pore-Forming Process[1]

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The hemolytic lectin, CEL-III, is a Ca^{2+}-dependent, galactose/*N*-acetylgalactosamine-specific lectin purified from the marine invertebrate, *Cucumaria echinata* (Holothuroidea). After binding to specific carbohydrates on the erythrocyte surface, CEL-III forms ion-permeable pores by oligomerizing in the membrane, which leads to colloid osmotic rupture of the cells. When incubated with liposomes composed of total lipids from the human erythrocyte membrane, CEL-III efficiently induced the leakage of carboxyfluorescein (CF) trapped in the vesicles, suggesting the presence of its receptor in the membrane lipids. The rate of CF-leakage increased with increasing temperature, although the hemolytic activity of CEL-III had been found to be much higher at lower temperatures (around 10°C). Identification of the receptor for CEL-III was performed by examining the ability of individual lipids from human erythrocytes to induce CF-leakage from DOPC-liposomes. As a result, the most effective receptor was found to be lactosyl ceramide (LacCer), while globoside (Gb_4Cer) also showed slight induction of CF-leakage. On the other hand, a binding assay involving CEL-III-horseradish peroxidase conjugate indicated that CEL-III exhibits similar affinity for LacCer and Gb_4Cer, suggesting that the structure or length of the carbohydrate portion of sphingoglycolipids is also relevant as to their ability to induce CF-leakage in addition to their affinity. Electron micrographs of CEL-III-treated liposomes revealed that CEL-III induced considerable morphological changes in the vesicles, while a clearly distinguishable oligomeric structure of the protein was not observed.

Key words: calcium, *Cucumaria echinata*, glycolipid, hemolysin, lectin.

CEL-III is a Ca^{2+}-dependent, galactose/*N*-acetylgalactosamine-specific lectin from a sea cucumber, *Cucumaria echinata* (*1*). This lectin shows strong hemolytic activity, especially toward human and rabbit erythrocytes. We recently found that CEL-III also exhibits strong toxicity toward several types of cultured cells, while some cell lines show remarkable resistance to its toxicity (*2*). These activities of CEL-III have been suggested to be mediated by the binding of the protein to specific carbohydrate chains on the cell surface, followed by its oligomerization and insertion into the lipid bilayer to form membrane pores (*3, 4*). Under some conditions, the binding of CEL-III to the carbohydrate chains on the cell surface can also occur without disruption of the cell membrane. For example, CEL-III shows only hemagglutinating activity, *i.e.* no hemolysis, at acidic pH (*3*). The succinylation of a few amino groups of CEL-III also leads to inactivation of its hemolytic activity but not its hemagglutinating activity (*5*). These data confirm that the hemolysis is not directly caused by the binding of CEL-III to the carbohydrate chains on the cell surface, and that additional interactions between CEL-III molecules or between CEL-III and membrane lipids are necessary.

The hemolytic activity is greatly enhanced by decreasing the temperature to 10°C and also by raising the pH to 10, which are remarkable features distinct from those of other pore-forming toxins, which generally act at higher temperatures and slightly acidic pHs. We have demonstrated that, at high pH and in the presence of a high concentration of salt, CEL-III forms an oligomer upon the binding of specific carbohydrates, such as lactose, even in an aqueous solution (*4*). The oligomer is the same size, as judged on SDS-PAGE (270 kDa, corresponding to a 6-7 mer), as that formed in erythrocyte membranes treated with CEL-III, suggesting that the two oligomers are similarly constituted. However, the actual size of the former in solution was recently found, by means of small angle X-ray scattering, to be much larger

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Abbreviations: CF, 4(5)-carboxyfluorescein; DOPC, dioleoyl phosphatidylcholine; Gb_3Cer, globotriaosyl ceramide; Gb_4Cer, globoside; HRP, horseradish peroxidase; LacCer, lactosyl ceramide; TBS, Tris-buffered saline.

(1,021 kDa, corresponding to a 21 mer) than that estimated on SDS-PAGE (*6*). Such promotion of oligomerization of CEL-III by carbohydrates was observed only with lactose, lactulose, and *N*-acetyllactosamine, all of which contain a β-1,4 galactosidic linkage. In contrast, *N*-acetylgalactosamine was not effective as to the formation of an oligomer in solution, although it exhibits the highest affinity for CEL-III among the carbohydrates so far tested. This suggests that, in addition to the affinity, the size or structure of specific carbohydrates is also important for induction of the conformational change in CEL-III. The oligomerization of CEL-III upon the binding of lactose in an aqueous solution is accompanied by a remarkable increase in the surface hydrophobicity of the protein (*4*). Therefore, it seems possible that the binding of CEL-III to the erythrocyte surface through specific carbohydrate chains as receptors also promotes the conformational change, which leads to an increase in the surface hydrophobicity of the protein. The importance of the conformational changes induced by the binding to the specific carbohydrates as receptors was suggested in the case of some bacterial toxins, such as pertussis toxin (*7*) and verotoxin (*8*). The binding to the receptors was thought to induce the conformational changes and enhance the surface hydrophobicity of the toxins, resulting in the insertion of the toxins into the lipid membrane. Therefore, it seems very important to investigate the structure of the receptor for CEL-III to elucidate the mechanism of its interaction with the lipid membrane.

As previously reported (*3*), CEL-III caused carboxyfluorescein (CF)-leakage from liposomes containing *N*-acetylgalactosamine-containing glycolipid (*3*), which was competitively inhibited by lactose. In addition, our preliminary experiment indicated that CEL-III forms oligomers in liposome membranes, which are the same size as those observed in erythrocyte membranes treated with CEL-III (data not shown). These results support that the CF-leakage from the liposomes was mediated by pores formed by the CEL-III oligomer in the lipid membrane, and that the assay can be utilized to investigate the interaction between CEL-III and the erythrocyte membrane. In this paper, we report the characterization of the interaction of CEL-III with lipid membranes mainly by means of the CF-leakage assay involving liposomes containing human erythrocyte lipids. As a result, the involvement of neutral sphingoglycolipids in the pore-forming process of CEL-III was revealed.

MATERIALS AND METHODS

Materials—Specimens of *Cucumaria echinata* were collected in the Sea of Genkai (Fukuoka). The samples were stored at −30°C until use. The rabbit blood was obtained from Nippon Bio-Test Laboratories (Tokyo). Dioleoyl phosphatidylcholine (DOPC), lactosyl ceramide (LacCer), globotriaosyl ceramide (Gb_3Cer), and globoside (Gb_4Cer) were purchased from Sigma. 4 (5)-Carboxyfluorescein (CF) was from Aldrich. Horseradish peroxidase was from Wako (Tokyo).

Purification of CEL-III—CEL-III was purified from the extract of a homogenate of *C. echinata* by column chromatography on lactosyl-Cellulofine, GalNAc-Cellulofine, and Sephacryl S-200 as previously described (*2*). We recently found that the yield of the Ca^{2+}-dependent lectins in *C. echinata* could be considerably improved by using an extraction buffer (10 mM Tris-HCl, pH 7.5) containing 20 mM EDTA. This suggests that the lectins were partly bound to the carbohydrate portions of insoluble matter in the homogenate and that EDTA dissociated them by abolishing the carbohydrate binding ability. The extracted proteins were dialyzed against 10 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl (TBS) to remove EDTA, and then applied to a lactosyl-Cellulofine column after the addition of $CaCl_2$ to 10 mM. Chromatography was performed at 4°C. The purified protein was stored in TBS at −30°C until use.

Protein Determination—Protein concentrations were calculated based on the absorbance value of 1.4 at 280 nm for a 0.1% (w/v) protein solution in TBS, which had been determined with bicinchoninic acid by the method reported by Smith *et al.* (*9*) with bovine serum albumin as a standard.

Separation of Lipids by Thin-Layer Chromatography (TLC)—The separation of lipids was performed by thin-layer chromatography on a silica gel plate (Silica gel 60, Merck) with a solvent system of chloroform/methanol/2.5 M NH_4OH (60:35:8, v/v). Detection was performed with Coomassie Brilliant Blue R-250 (*10*) or orcinol (for glycolipid).

Determination of Lipids—Phospholipids were determined based on the phosphoric acid content using a phospholipid assay kit from Wako. Glycolipids were determined by densitometry of the TLC spots stained with Coomassie Brilliant Blue R-250.

Separation of Membrane Lipids from Human Erythrocytes—Total membrane lipids were extracted from human erythrocytes. Erythrocyte ghosts from outdated human blood (blood group O) were prepared by treating the cells with distilled water, and the total lipid was extracted with chloroform/2-propanol (7:11, v/v) for 2 days essentially according to the method described by Nakamura *et al.* (*11*). The lipids were separated on a silica gel column (Merck Silica gel 60, 1.8×5 cm). Elution was performed with a solvent system of chloroform, acetone, and methanol. The partially purified glycolipids were further separated by HPLC on a column of Iatrobeads 6RS 8010 (4× 150 mm; Iatron Laboratories). Elution was performed with 2-propanol/*n*-hexane/water (55:44:1 to 55:30:15, v/v) (*12*). The eluate was monitored as to the absorbance at 205 nm. Identification of the separated lipids was performed by comparison with standard lipids on TLC.

The acidic lipid fraction including gangliosides was separately prepared from the total lipids essentially as described by Nakamura *et al.* (*11*). The total lipids were applied to a silica gel column equilibrated with chloroform/methanol (8:2, v/v), and after washing with the same solvent the adsorbed lipids were eluted with chloroform/methanol (1:2). The eluted lipids were then separated on a DEAE-Cellulofine column (1.6×2.5 cm; Seikagaku Kogyo, Tokyo). The column was washed with chloroform/methanol (1:2, v/v), methanol, and then 0.2 M sodium acetate in methanol. The acidic lipid fraction was then treated with 0.5 M NaOH to degrade the remaining phospholipids. The solution was neutralized by the addition of 1 M HCl, dried using N_2 gas, and then desalted by passage through a Sep-Pak C18 cartridge (Waters).

Preparation of Liposomes Containing Carboxyfluorescein—Liposomes were prepared with either total lipids

from human erythrocyte membranes (1 mg), or DOPC (1 mg) and separated membrane lipids from human erythrocytes. The lipids were dissolved in 0.5 ml of chloroform/methanol (2:1, v/v) and then dried under a stream of N_2 gas in a conical glass tube. After the addition of 0.5 ml of 20 mM Tris/HCl buffer-150 mM NaCl, pH 8.5, containing 0.1 M carboxyfluorescein, the lipids were hydrated by vortex-mixing at room temperature. The suspension was then sonicated for 1 min at room temperature using a Taitec Ultrasonic Processor VP-5T at 10 W intensity. The resulting liposomes were separated from free CF by spun-column chromatography using a 1-ml disposable syringe filled with Sephadex G-75 in 20 mM Tris/HCl buffer-150 mM NaCl, pH 8.5.

Measurement of CF-Leakage from Liposomes—The liposome solution (50 μl) was diluted with 950 μl of 10 mM Tris/HCl buffer-150 mM NaCl, pH 8.5, containing 10 mM $CaCl_2$ in a quartz cuvette kept at constant temperature. A CEL-III solution (5 μl) in the same buffer was then added to this solution and, after mixing, the fluorescence intensity at 518 nm on excitation at 490 nm was immediately recorded using a Hitachi F-3010 Fluorescence Spectrophotometer. For 100% leakage of CF, Triton X-100 was added to a final concentration of 0.1%.

Preparation of CEL-III-Horseradish Peroxidase (HRP) Conjugate—CEL-III-HRP conjugate was prepared essentially according to the method of Tijssen and Kurstak (*13*). To a HRP (1 mg) solution in 0.1 ml of 0.1 M $NaHCO_3$, 0.1 ml of 12 mM $NaIO_4$ was added to oxidize the carbohydrate chains. After 4 h at 20°C, 1 mg of CEL-III in 0.2 ml of 0.1 M sodium carbonate buffer, pH 9.2, and 66 mg of dry Sephadex G-25 were added. After 3 h at room temperature, the Sephadex G-25 gel was removed by centrifugation, and then a 1/20 volume of $NaBH_4$ (5 mg/ml) and a 1/10 volume of the same solution were added at an interval of 30 min. The resultant CEL-III-HRP conjugate was separated from the unreacted proteins on a Sephadex G-75 column. The hemagglutinating and peroxidase activities of the conjugate were confirmed by means of the hemagglutination assay involving rabbit erythrocytes and the coloring reaction using 3,3′-diaminobenzidine. The conjugate thus prepared was found to be a mixture of different sizes of over 83 kDa, indicating cross-linking between multiple molecules of CEL-III and HRP, and was used without further purification.

Measurement of Affinity of CEL-III for Immobilized Glycolipids—The affinity of CEL-III for glycolipids was measured using a microtiter plate coated with glycolipids. Coating of the wells of a polystyrene microtiter plate (96-well; Sanko Jun-yaku, Tokyo) with glycolipids was performed by adding 50 μl of a phospholipid solution (30 μg/ml) in chloroform/methanol (1:9, v/v) to the wells and heating at 55°C to allow the solution to evaporate. To avoid nonspecific binding, the wells were then coated with 400 μl of 1% bovine serum albumin in TBS for 1 h. After the solution had been removed, 100 μl of various concentrations of CEL-III-HRP conjugate in TBS containing 10 mM $CaCl_2$ (TBS-$CaCl_2$) was added to the wells. After incubation for 30 min, the wells were washed with the same buffer three times, and then the bound conjugate was detected as HRP activity using 100 μl of a substrate solution (0.4 mg/ml *o*-phenylenediamine-0.01% H_2O_2 in citrate-phosphate buffer). The reaction was stopped after 30 min by the addition of 0.1 ml of 2 M H_2SO_4. The absorbance at 490 nm was measured with a microplate reader (Immuno Mini NJ-2300, Intermed, Tokyo). The affinities of individual lipids for CEL-III-HRP conjugate were compared using the values which gave the half maximum increase in the absorbance. They were calculated by nonlinear least-squares fitting using the equation: $A_{490} = A_{490max} \cdot [\text{conjugate}]/([\text{conjugate}] + K)$; where A_{490max} and K represent the maximum and half-maximum of the absorbance at 490 nm, respectively.

Electron Microscopy—The effect of CEL-III on lipid vesicles containing LacCer was observed by electron microscopy. Five microliters of a DOPC/LacCer (9:1, w/w)-liposome solution in 20 mM Tris-HCl, pH 8.5, containing 150 mM NaCl and 10 mM $CaCl_2$ was put on a carbon-coated copper grid, and then the liposomes were allowed to adsorb on the grid. After removing the solution by blotting with filter paper, 5 μl of CEL-III (1.2 mg/ml) in the same buffer was added, followed by incubation for 5 min at room temperature. The grid was then washed with the same buffer, stained with 1% (w/v) uranyl acetate, and examined under a JEOL JEM-100S electron microscope (100 kV).

RESULTS

CF-Leakage from Liposomes Prepared with Human Erythrocyte Lipids—As reported in the previous paper, CEL-III induced CF-leakage from egg phosphatidylcholine (PC)-liposomes containing Gb_4Cer (*3*). In the present study, we examined the interaction of CEL-III with liposomes containing human erythrocyte lipids. Figure 1, A and B, shows the time courses of CF-leakage from liposomes composed of the total lipids of human erythrocytes, and the leakage after 10 min with different concentrations of CEL-III, respectively. The fluorescence at 518 nm arising from CF released from the liposomes increased immediately after the addition of CEL-III, while the rate of leakage rapidly decreased, as can be seen in these curves. This might be related to the fact that the membrane pores are formed through oligomerization of multiple molecules of CEL-III, probably through a cooperative interaction. Therefore, a relatively small amount of CEL-III could not induce the entire CF-leakage, leaving some of the liposomes intact. The CF-leakage was almost completely inhibited when the assay was performed in the presence of 0.1 M lactose (data not shown), suggesting that the reaction was induced through specific binding of CEL-III to the carbohydrate chains of some glycolipids. As shown in Fig. 2, when the measurements were performed at different temperatures, the rate of CF-leakage was greater at higher temperatures. This is in contrast to the fact that the hemolytic activity of CEL-III was highest at around 10°C and then decreased drastically with increasing temperature (*3*). The reason for such a discrepancy is unclear; yet the higher efficiency of the interaction between the protein and the lipid bilayer could be due to the higher membrane fluidity at higher temperatures, which may facilitate the interaction between CEL-III molecules in the membrane.

Separation of Lipids from Human Erythrocytes and Examination of Their Ability to Function as CEL-III Receptors—Since the lipid fraction of human erythrocytes was suggested to contain CEL-III receptors, as described above, we examined the individual lipids separated from

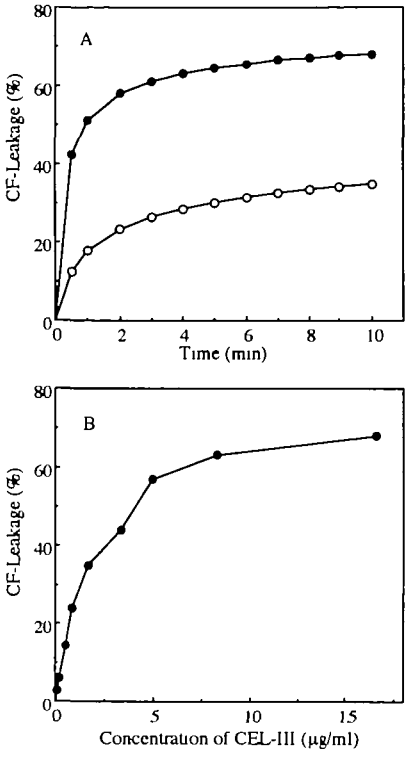

Fig. 1. **Pore-formation by CEL-III in artificial lipid vesicles composed of total membrane lipids from human erythrocytes.** A, time-course of CF-leakage from liposomes in 20 mM Tris-HCl, pH 8.5, containing 0.15 M NaCl and 10 mM $CaCl_2$. CEL-III (○, 1.6 μg/ml; ●, 16 μg/ml) was added to the liposome solution in a quartz cuvette at 20°C, and then the fluorescence intensity at 518 nm on excitation at 490 nm was measured. The fluorescence intensity of the solution after the addition of Triton X-100 (final concentration, 0.1%) was taken as 100%. B, CF-leakage from total-lipid liposomes after 10 min induced by various concentrations of CEL-III.

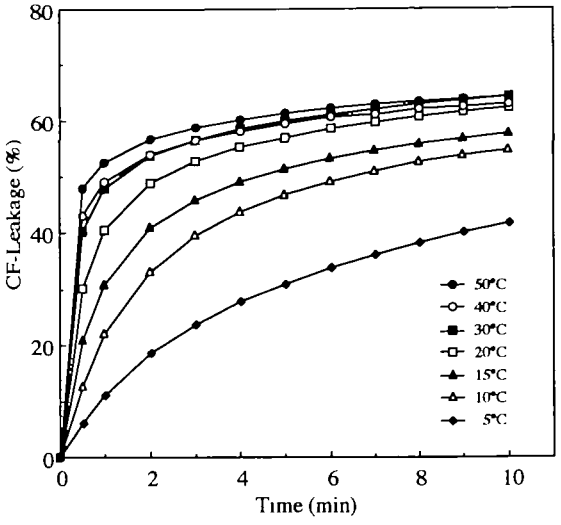

Fig. 2. **Temperature-dependence of the CF-leakage from total-lipid liposomes.** CEL-III (final concentration, 1.6 μg/ml) was added to 1 ml of the liposome solution in 20 mM Tris-HCl, pH 8.5, containing 0.15 M NaCl and 10 mM $CaCl_2$ in a quartz cuvette, which had been previously incubated at a given temperature, and after rapid mixing, the fluorescence at 518 nm on excitation at 490 nm was recorded.

human erythrocyte membranes as to their ability to induce CF-leakage due to CEL-III. Figure 3A shows the TLC spots of neutral lipids separated from human erythrocyte membranes on silica gel column chromatography. Simple lipids, glycolipids, and phospholipids were successively eluted with the indicated solvents. Orcinol staining was also performed to detect glycolipids (Fig. 3B). Aliquots of the fractions were subjected to the CF-leakage assay. Figure 3C shows the CF-leakage from liposomes containing these fractions induced by CEL-III. DOPC was used for the preparation of the liposomes, since we have observed that DOPC-liposomes are relatively stable and also sensitive enough for this assay (data not shown). Neutral sphingoglycolipids eluted in fractions 23-26 effectively induced CF-leakage from the liposomes. In contrast, the liposomes containing other fractions showed only slight or no leakage at all. These results indicate that neutral sphingoglycolipids could be candidate CEL-III receptors. Acidic sphingoglycolipids, such as gangliosides, were not recovered in the fractions obtained on silica gel chromatography, probably because of their higher hydrophilicity; thus we separately obtained acidic sphingoglycolipids by anion-exchange chromatography as described under "MATERIALS AND METHODS." However, DOPC-liposomes containing this fraction exhibited no CF-leakage, indicating that the acidic sphingoglycolipids were unable to function as CEL-III receptors. Neutral sphingoglycolipids obtained by silica gel chromatography (fractions 23-26 in Fig. 3) were combined and further separated by HPLC on an Iatrobeads column, and then each fraction was examined by TLC. As shown in Fig. 4, LacCer, Gb_3Cer, and Gb_4Cer were separated by elution with 2-propanol/*n*-hexane/water (*12*), and aliquots of the purified lipids were subjected to the assay. Figure 5 shows a comparison of the CF-leakage from DOPC-liposomes containing these lipids. Effective leakage was observed for LacCer, whereas Gb_4Cer induced appreciable leakage due to CEL-III only above 10 μg/ml. On the other hand, Gb_3Cer caused almost no leakage. These results clearly indicate that LacCer is the most effective as a receptor for inducing membrane pore formation by CEL-III.

Binding of CEL-III to Glycolipids from Erythrocyte Membranes—To compare the ability of the sphingoglycolipids to induce CF-leakage due to CEL-III and their affinity for the protein, a binding assay involving a glycolipid-coated microtiter plate and CEL-III-HRP conjugate was performed (Fig. 6). The conjugate was prepared by cross-linking the amino groups of CEL-III and the $NaIO_4$-oxidized carbohydrate chains of HRP (*13*). Since the apparent molecular size of the conjugate was found to be more than 83 kDa on SDS-PAGE, it was inferred that multiple molecules of both CEL-III and HRP were present in a CEL-III-HRP conjugate molecule. Such incorporation of multiple molecules into one conjugate is expected to lead to rather higher affinity for the ligand than that of a monomer lectin without a change in its binding specificity, thereby improving the sensitivity of the measurement. The binding was detected as to the absorbance at 490 nm resulting from the enzymatic reaction of HRP with *o*-phenylenediamine as the substrate. As shown in Fig. 6, the absorbance increased with increasing concentration of added CEL-III-HRP conjugate. The concentrations of the conjugate which gave the

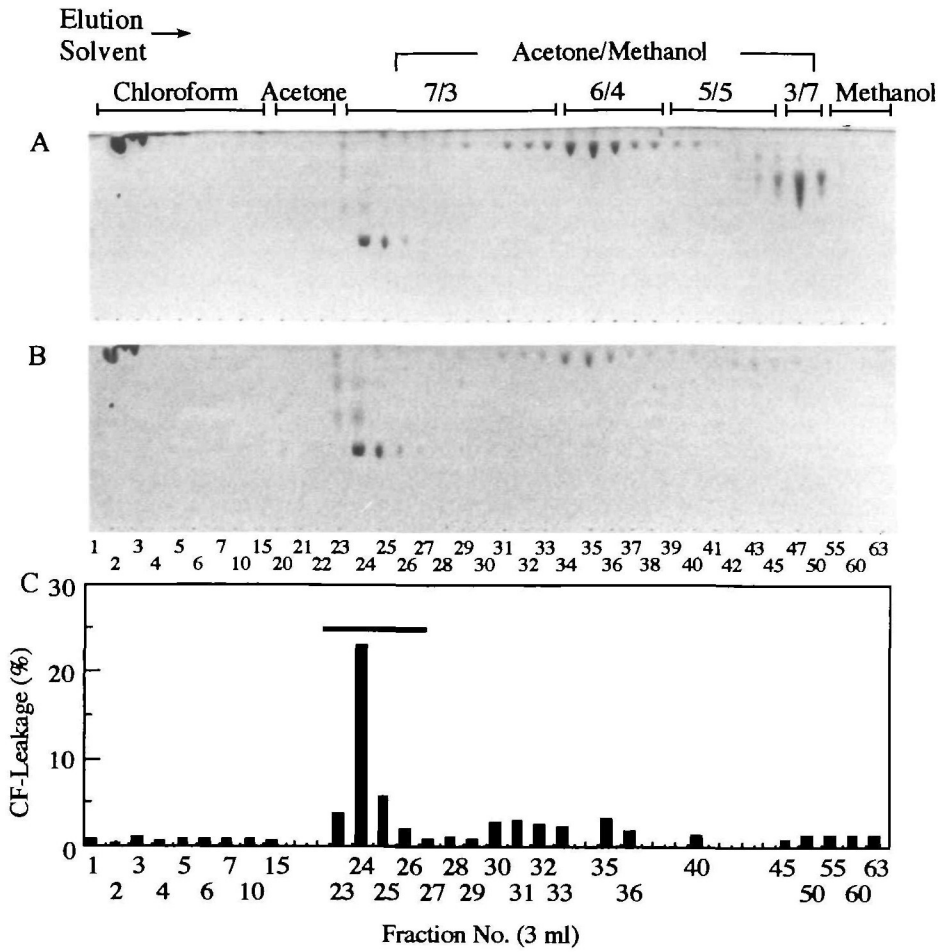

Fig. 3. **Separation of lipids from human erythrocyte membranes and their ability to induce CF-leakage from liposomes.** Total lipids (83 mg) from human erythrocyte membranes were separated on a silica gel column (1.8×5 cm) by elution with the indicated solvents. The separated lipids were identified by TLC with staining with orcinol (B) and then Coomassie Brilliant Blue R-250 (A). One-fifth of each fraction was subjected to the CF-leakage assay (C). The fractions indicated by the bar were pooled, concentrated with N_2 gas, and further purified by HPLC.

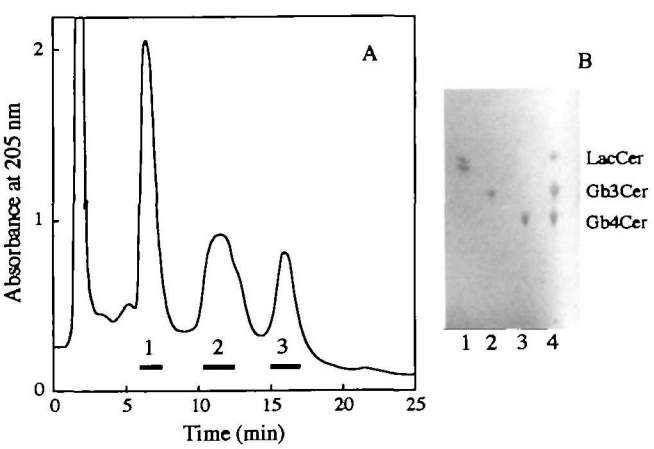

Fig. 4. **HPLC of the glycolipid fractions from human erythrocyte membranes.** The glycolipid fractions indicated by the bar in Fig. 3 were separated by HPLC on a column of Iatrobeads 6RS 8010 (4×150 mm). Lipids were eluted with a linear gradient of 2-propanol/*n*-hexane/water (55:44:1 to 55:30:15, v/v). The eluate was monitored as to the absorbance at 205 nm (A), and the numbered fractions (lanes 1-3) were identified by TLC in comparison with standard sphingoglycolipids (lane 4) (B). LacCer in fraction 1 appeared as two spots, the materials differing only in the length of the ceramide portion (*14*).

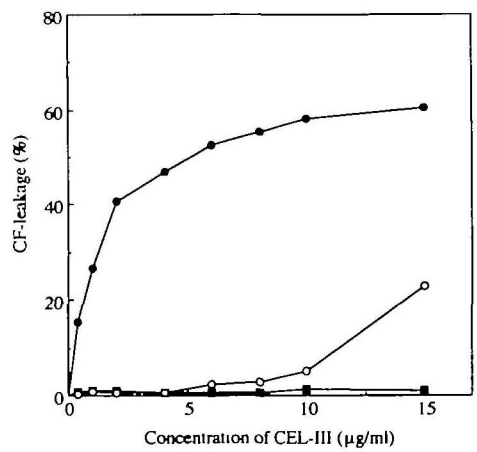

Fig. 5. **CF-leakage from DOPC-liposomes containing LacCer, Gb_3Cer, or Gb_4Cer.** LacCer (●), Gb_4Cer (○), and Gb_3Cer (■) (100 μg each) purified from human erythrocyte membranes were incorporated into DOPC-liposomes (1 mg), followed by assaying of CF-leakage ability. Measurements were performed at 20°C in 20 mM Tris-HCl, pH 8.5, containing 0.15 M NaCl and 10 mM $CaCl_2$.

half-maximum binding were calculated to be 0.09, 0.14, and 1.2 μg/ml for LacCer, Gb_4Cer, and Gb_3Cer, respectively. This order of binding affinity is consistent with the CF-leakage from the liposomes containing these glyco-

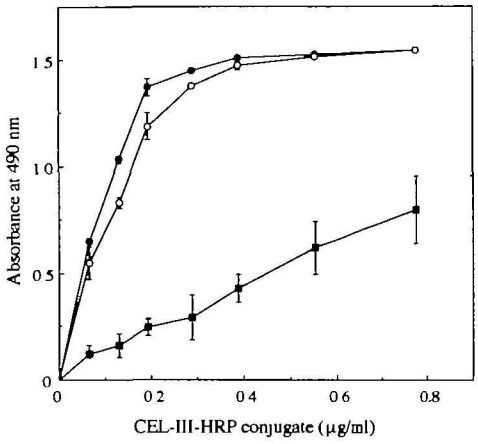

Fig. 6. **Binding of CEL-III-HRP conjugate to sphingoglycolipids coated on microtiter plate wells.** Microtiter plate wells were coated with LacCer (●), Gb_4Cer (○), and Gb_3Cer (■) in chloroform/methanol (1:9, v/v), and then the binding of CEL-III-HRP conjugate was measured using *o*-phenylenediamine as the substrate for HRP. Error bars represent the means ± SD of three measurements.

lipids, suggesting that the affinity of the glycolipids for CEL-III is related to their ability to induce CF-leakage. However, as can be seen in Fig. 5, since a much larger difference in CF-leakage was observed between LacCer and Gb_4Cer than expected from their affinities for CEL-III, it is likely that there is an additional factor(s) in the structures of these glycolipids affecting the interaction of CEL-III with the lipid bilayer.

Electron Microscopy of Liposomes Treated with CEL-III—The effect of CEL-III treatment on DOPC/LacCer-liposomes was examined by electron microscopy as shown in Fig. 7. CEL-III was incubated with liposomes adsorbed on a carbon-coated copper grid for 5 min at pH 8.5 in the presence of 10 mM $CaCl_2$, followed by observation after negative-staining with uranyl acetate. CEL-III induced remarkable changes in the shapes of the liposomes (Fig. 7, B and C); in addition to agglutination of the vesicles caused by cross-linking due to the lectin activity of CEL-III, many vesicles with a conspicuous ring- or C-like appearance were observed. Interestingly, the membranes of some vesicles appeared to be caved in, as indicated by arrowheads in Fig. 7C, suggesting considerable structural changes in the lipid bilayer, which were probably caused by the CEL-III oligomer irreversibly bound to the membrane. However, a distinct oligomeric structure of CEL-III was not observed. Similar morphological changes were also observed for total lipid liposomes, when incubated with CEL-III (data not shown).

DISCUSSION

CEL-III effectively induced CF-leakage from liposomes composed of total lipids from human erythrocytes. This indicated that specific carbohydrate ligands, which trigger the formation of membrane pores by CEL-III, were contained in the membrane lipid fraction. Since such CF-leakage induced by CEL-III is almost completely inhibited by specific carbohydrates, such as lactose, it seems reasonable to consider that the action of CEL-III on liposomes reflects its pore-formation process in the erythrocyte

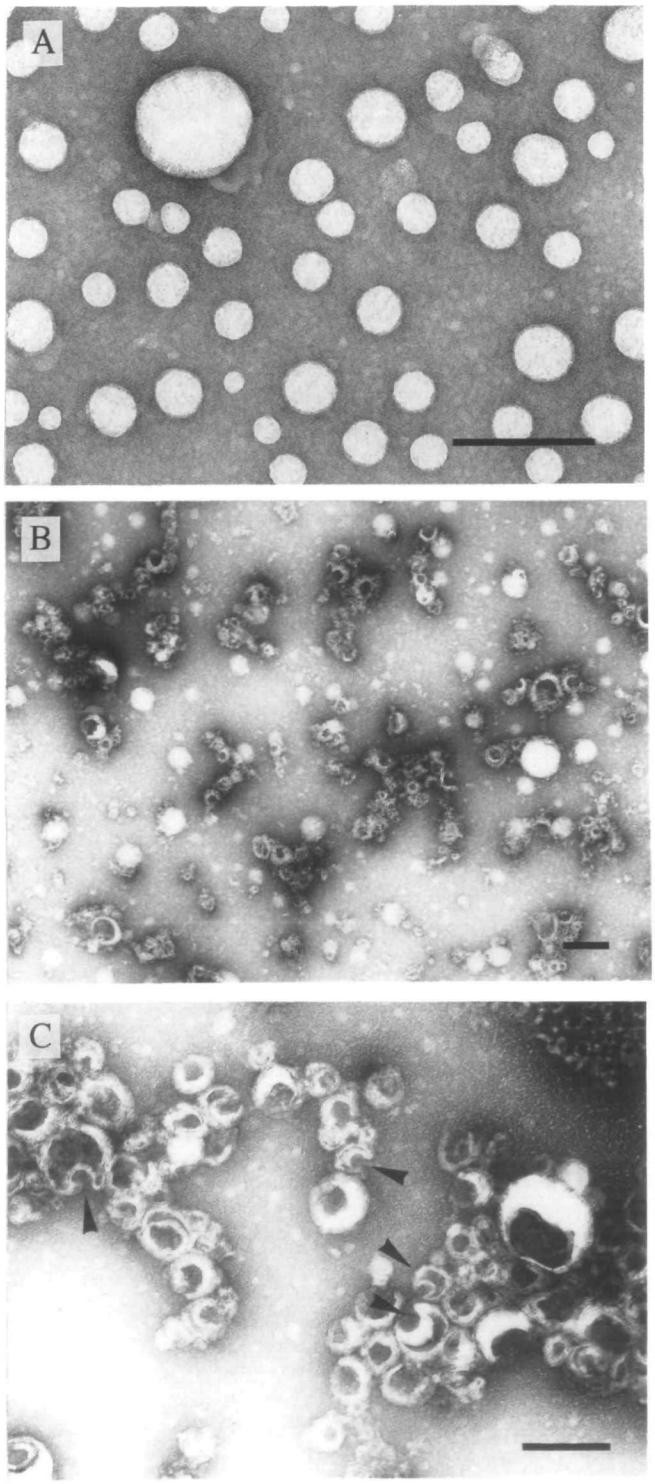

Fig. 7. **Effect of CEL-III-treatment on DOPC/LacCer-liposomes.** DOPC/LacCer (9:1, w/w)-liposomes in 20 mM Tris-HCl buffer, pH 8.5, containing 150 mM NaCl and 10 mM $CaCl_2$ were adsorbed on a carbon-coated grid and, after removing the solution, incubated with CEL-III for 5 min at room temperature. Negative staining was performed with a 1% uranyl acetate solution. A, control liposomes; B and C, liposomes treated with CEL-III. Bars represent 200 nm.

membrane. However, there is a discrepancy as to their temperature dependence; we have observed that the hemolytic activity as well as cytotoxicity toward cultured cells of CEL-III were higher at relatively low temperatures (5-10°C) (*2, 3*), while CF-leakage from the liposomes increased with increasing temperatures in this study. This implies that, in the hemolytic process, there is a still unknown interaction between CEL-III and cell membrane components which makes the cells unusually susceptible at lower temperatures.

Although specific binding of CEL-III to the carbohydrate chains on the erythrocyte surface is important for the subsequent oligomer formation in the erythrocyte membrane, the affinity of specific carbohydrates for CEL-III is not always correlated with their ability to induce a conformational change in CEL-III. For example, *N*-acetylgalactosamine exhibits the highest affinity for CEL-III among several simple carbohydrates we have tested (*15*), whereas β-1,4-linked galactosides, such as lactose, lactulose, and *N*-acetyllactosamine, are much more effective in inducing the oligomerization of CEL-III in an aqueous solution (*4*). This suggests that not only the affinity but also the appropriate structure of the carbohydrate ligand is important for induction of a conformational change in CEL-III. Therefore, it appeared necessary to examine the ability of individual membrane lipids to induce CF-leakage due to CEL-III for identification of specific receptors in the erythrocyte membrane.

Fractionation of the membrane lipids on a silica gel column and the following CF-leakage assay indicated that most of the CEL-III receptor was confined in the neutral sphingoglycolipid fractions, including LacCer, Gb_3Cer, and Gb_4Cer, while the acidic lipid fraction induced no CF-leakage due to CEL-III. After further separation by HPLC, LacCer was identified as the most effective CEL-III receptor. This is consistent with the finding that β-1,4-linked galactosides were effective for inducing oligomerization and enhancement of the hydrophobicity of CEL-III in solution (*4*). However, since Gb_4Cer is the most abundant glycolipid in the human erythrocyte membrane (LacCer:Gb_3Cer:Gb_4Cer=0.77:0.60:3.55 in molar ratio) (*16*), Gb_4Cer may also actually provide a some contribution as a CEL-III receptor in the intact erythrocyte membrane. The results of the binding assay involving immobilized glycolipids and CEL-III-HRP conjugate (Fig. 6) indicated that the ability of the glycolipids to induce CF-leakage is related to their affinity for CEL-III. However, the difference in the degree of CF-leakage with LacCer and Gb_4Cer is much larger than that in their binding affinities. This strongly suggests the importance of the structure or length of the carbohydrate chains of the glycolipids for induction of the interaction between CEL-III and the lipid bilayer.

Electron micrographs of CEL-III-treated liposomes demonstrated that CEL-III induced considerable morphological changes in the vesicles, although clearly distinguishable oligomers of CEL-III were not observed. Many of the liposomes had ring- or C-like forms and some parts of the vesicle membranes appeared to be caved in. This appearance of the vesicles suggests that CEL-III oligomers were strongly associated in the membrane, thus changing the membrane structure. This is one of the conspicuous differences from bacterial pore-forming toxins, which often show oligomeric structures protruding from the surface of the vesicles, the overall shape of the vesicles being maintained (*17-19*). The far-UV CD spectra of CEL-III and its oligomer suggest that a relatively large secondary structural change occurs during the oligomerization process. In contrast, bacterial pore-forming toxins such as aerolysin (*20*), staphylococcal α-toxin (*21*), and anthrax toxin protective antigen (*22*) have been shown to undergo little change in secondary structure during oligomerization. Although it is apparent that, at least in the early stages, CEL-III induces hemolysis by means of colloid-osmotic rupture through the formation of membrane pores (*3*), the mechanism as well as the structure of the oligomer inserted into the membrane might be considerably different from those in the cases of bacterial toxins.

A sphingoglycolipid having a relatively short carbohydrate chain such as LacCer may be more suitable than membrane glycoproteins to bring bound CEL-III close to the membrane surface so as to promote its interaction with the lipid bilayer. Since the interaction of CEL-III and the erythrocyte membrane largely depends on the conformational change in the protein after the specific-binding to the carbohydrate chains, a partially hydrophobic environment on the membrane surface is expected to provide appropriate conditions for induction of the conformational change in the protein. It has been shown that the receptors for several bacterial toxins which interact with the target cell membrane are glycolipids (*23-26*). They interact with the lipid bilayer to facilitate translocation of the effector subunit into the cytosol (*27-29*). CEL-III exerts a selective toxic action on some types of cultured cells (*2*); therefore, it would be particularly interesting to determine their receptors in these cells. This may provide further insight into the mechanism of the interaction of CEL-III and the cell membrane.

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