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Effect of the Hemolytic Lectin CEL-III from Holothuroidea *Cucumaria echinata* on the ANS Fluorescence Responses in Sensitive MDCK and Resistant CHO Cells

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The addition of CEL-III to sensitive MDCK cells preincubated with 8-anilino-1-naphthalenesulfonate (ANS) caused an increase in the fluorescence intensity of the probe. The increase in the ANS fluorescence caused by CEL-III was Ca^{2+}-dependent and strongly inhibited by 0.1 M lactose, indicating that Ca^{2+}-dependent binding of CEL-III to specific carbohydrate receptors on the plasma membrane is responsible for this phenomenon. In contrast, no significant effect of CEL-III on the ANS fluorescence was observed in CHO cells, which are highly resistant to CEL-III cytotoxicity. In MDCK cells, energy transfer from tryptophan residues to bound ANS molecules was observed in the presence of CEL-III, but not in CHO cells. Furthermore, the amount of ANS bound to MDCK cells increased as the concentration of CEL-III increased. Therefore, a simple interpretation is that the CEL-III-induced increase in ANS fluorescence is attributable to an increase of the hydrophobic region in the plasma membrane where ANS could bind. Immunoblotting analysis of proteins from cells treated with CEL-III indicated that CEL-III oligomers were irreversibly bound to the cells, and the amount of oligomer bound to MDCK cells was much greater than that bound to CHO cells under any conditions tested. The oligomerization may be accompanied by an enhancement of the hydrophobicity of CEL-III molecules, which in turn provides new ANS-binding sites. The difference in susceptibility of MDCK and CHO cells to CEL-III cytotoxicity may be due to a difference in oligomerization of bound CEL-III.

Key words: ANS-fluorescence, CEL-III, cytotoxicity, lectin, pore-forming protein.

A number of animal lectins have been isolated from various organisms and studied along with their physiological functions (*1, 2*). Most animal lectins are generally classified into Ca^{2+}-dependent (C-type) or independent (S-type or galectin) (*3, 4*). The C-type animal lectins have a characteristic carbohydrate-recognition domain consisting of 120-130 amino acids residues, which exhibits some degree of sequence homology between species. Several C-type lectins have been found in marine invertebrates (*5-8*). Recently, Hatakeyama *et al.* purified four galactose/GalNAc-specific Ca^{2+}-dependent lectins with different molecular masses (CEL-I to CEL-IV) from the marine invertebrate *Cucumaria echinata* (Holothuroidea) (*9, 10*). Interestingly, one of these lectins, CEL-III, caused hemolysis of rabbit and human erythrocytes but showed only weak hemagglutination of chicken and horse erythrocytes. The hemolytic activity of CEL-III was inhibited by galactose or GalNAc-containing carbohydrates and was dependent on the Ca^{2+}-concentration, suggesting that the hemolysis was caused by Ca^{2+}-dependent binding of CEL-III to specific carbohydrate on the erythrocyte membrane and the following partial destruction of the membrane. Although the details of the mechanism of hemolytic activity of CEL-III are still unclear, immunoblotting analysis of proteins from the erythrocyte membrane after treatment with CEL-III indicated that CEL-III oligomers were irreversibly bound to the membrane, suggesting that the hemolytic activity of CEL-III is mediated by the formation of ion-permeable pores in the erythrocyte membrane (*10*). From osmotic protection experiments using dextran with a molecular weight of over 4,000, it was suggested that the functional radii of the pores formed by CEL-III oligomers may be smaller than 18 Å (*10*). Some bacterial protein toxins have been reported to exhibit their toxic effect by pore-formation in the target cell membrane (*11-14*). Recently, we have found that CEL-III shows strong cytotoxicity to several cell lines, such as MDCK, HeLa, and Vero cells, while CHO cells were highly resistant to CEL-III cytotoxicity (*15*). It seems likely that the cytotoxicity, like hemolysis, is caused by the formation of ion-permeable pores by CEL-III oligomer. It might be expected that the interaction of CEL-III with specific receptors on the cell surface leads to dramatic structural changes in the plasma

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Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; BSA, bovine serum albumin; FBS, fetal bovine serum; F-CEL-III, FITC-labeled CEL-III; FITC, fluorescein isothiocyanate; MEM, minimal essential medium; PBS, phosphate-buffered saline, pH 7.4; TBS, Tris-buffered saline, pH 7.5.

membrane through the formation of CEL-III oligomers in the membrane.

In an attempt to assess changes in plasma membrane of cultured cells induced by the action of CEL-III, we used a fluorescence probe, 8-anilino-1-naphthalenesulfonate (ANS), which has been widely used to study changes of structure of proteins and membranes (*16, 17*). ANS has a low fluorescence yield in aqueous solution, but its fluorescence is enhanced when it is bound to hydrophobic sites on membranes or proteins.

Here, we report that CEL-III induces a marked increase in fluorescence intensity when it is added to MDCK cells preincubated with ANS, but only a small increase in ANS fluorescence in CHO cells. It appears that the increase in fluorescence intensity of bound ANS induced by CEL-III is correlated with its cytotoxic effect and the formation of CEL-III oligomers in the cell membrane as judged by SDS-PAGE and subsequent immunoblotting analysis.

MATERIALS AND METHODS

Materials—Specimens of *C. echinata* were collected in the Sea of Genkai (Fukuoka). The samples were stored at −30°C until use. CEL-III was purified from the body fluid of *C. echinata* by column chromatography on lactosyl-Cellulofine, GalNAc-Cellulofine, and Sephacryl S-200 as previously described (*9*). The molecular mass of purified CEL-III is determined to be 47.5 kDa (*18*). The purified protein was dialyzed against 10 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl and 10 mM $CaCl_2$ (TBS), and stored frozen at −83°C until use. Concanavalin A and wheat germ agglutinin were obtained from Sigma Chemical (St. Luis, MO). Castor bean hemagglutinin and ricin were isolated from small castor beans as described by Mise *et al.* (*19*). Diphtheria toxin and *Pseudomonas* toxin were purchased from the Swiss Serum and Vaccine Institute (Berne, Switzerland). Modeccin was obtained from Inland Laboratories (Austin, TX). FITC isomer I was from Dojin Chemical Laboratories, Kumamoto.

FITC Labeling of CEL-III—Fluorescein isothiocyanate (FITC)-labeled CEL-III (F-CEL-III) was prepared essentially as described previously (*20*). In brief, 2 mg of FITC was added to 1 ml of 0.5 M sodium bicarbonate buffer, pH 8.3, containing 10 mg of CEL-III and 0.1 M lactose. After stirring for 4 h at 4°C, the reaction mixture was applied to a column (1.5×10 cm) of Sephadex G-25 previously equilibrated with phosphate-buffered saline, pH 7.4 (PBS), followed by dialysis against PBS. F-CEL-III retained the original hemolytic activity toward human erythrocytes.

Cell Culture—MDCK (Madin-Darby canine kidney) and CHO (Chinese hamster ovary) cells were cultured in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 10 μg each of adenosine, guanosine, cytidine, and thymidine per ml of medium, penicillin (100 μg/ml), and streptomycin (100 μg/ml) as described (*21*).

Fluorescence Measurements—Adherent MDCK and CHO cells were detached by treatment with 0.1% trypsin and 0.05% EDTA in PBS and harvested by centrifugation, washed twice with PBS, and resuspended in suitable buffer. Unless otherwise specified, the buffer was 10 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, 10 mM $CaCl_2$, and 11 mM glucose. A small volume of ANS aqueous solution (final concentration of 100 μM) was added to 1 ml of cell suspension (1×10^6 cell/ml) and allowed to stand for 5 min at 37°C, then the indicated concentration of CEL-III was added, and fluorescence was measured with a fluorescence spectrophotometer (Hitachi Model 650-40). ANS was excited at 380 nm and its emission was monitored between 400 and 610 nm. For kinetic measurements, the fluorescence intensity was measured at 480 nm with excitation at 380 nm as a function of time. For the determination of energy transfer between tryptophan and ANS, the excitation wavelength was 285 nm and the emission fluorescence spectra were recorded in the 290–550 nm range.

Measurement of CEL-III Cytotoxicity—Cell viability was estimated by trypan blue exclusion, using 0.5% trypan blue. At least 300 cells were counted on a hemocytometer for each determination.

Measurement of ANS Binding—Cell suspension (1×10^6 cells/ml) was preincubated with ANS in 10 mM Tris-HCl pH 7.5 containing 0.15 M NaCl, 10 mM $CaCl_2$, and 11 mM glucose at 37°C for 5 min, then CEL-III was added in varying concentrations. After 15 min, cells were centrifuged and washed twice with PBS, and ANS was extracted with dioxane/methanol (4:1) (*22*). The amount of bound ANS was determined from the fluorescence intensity of the extract at 470 nm with excitation at 370 nm, using a standard curve.

Immunoblotting—Monolayers of confluent cells (1×10^6 cell/dish) in 35-mm dishes were incubated with CEL-III (10 μg/ml) in 10 mM Tris-HCl buffer, pH 7.5, or 10 mM sodium borate buffer, pH 10, containing 10 mM $CaCl_2$, 0.15 M NaCl, 11 mM glucose for 1 h at 4 or 37°C. Cells were washed twice with PBS containing 0.1 M lactose to remove cell surface-bound CEL-III, then scraped off and pelleted by centrifugation. The resulting pellet was solubilized with the sample buffer containing 0.1% SDS, then subjected to 5% SDS-PAGE. After transfer of separated proteins in the polyacrylamide gel to nitrocellulose membrane, immunoblotting was performed using mouse anti-CEL-III antiserum as described previously (*10*).

Fluorescence Microscopy—Cells were grown on glass coverslips to subconfluence, then incubated with CEL-III (40 μg/ml) in serum-free α-MEM at 37°C. After 30 min, the cells were washed twice with PBS, then fixed with 10% formaldehyde in PBS for 15 min at room temperature. After washing with PBS, the coverslips with cells were mounted on a glass slide with 50% glycerol in PBS containing 2.6% 1,4-diazabicyclo[2,2,2]-octane and viewed with a fluorescence microscope (Carl Zeiss Axiophot).

RESULTS

Effect of CEL-III on the Fluorescence Intensity and the Emission Spectrum of Cell-Bound ANS—Addition of CEL-III to sensitive MDCK cells preincubated with ANS resulted in a marked increase in fluorescence intensity of the probe (Fig. 1). On increasing the concentration of CEL-III, a parallel enhancement of fluorescence was observed (Fig. 2). The CEL-III-induced increase in ANS fluorescence intensity was inhibited by 0.1 M lactose. Furthermore, in the absence of Ca^{2+}, CEL-III even at 10,000 ng/ml caused little increase in ANS fluorescence (Fig. 2). Thus, the fluorescence increase can be interpreted in terms of the Ca^{2+}-dependent binding of CEL-III to

specific carbohydrate receptors on the cell surface. In CHO cells, which are highly resistant to CEL-III cytotoxicity, only a little change in ANS fluorescence was induced by CEL-III (Fig. 1). The increase in fluorescence of ANS bound to MDCK cells caused by CEL-III appeared to be mostly due to an increase in the probe binding (Fig. 3), since the amount of ANS bound to MDCK cells increased depending

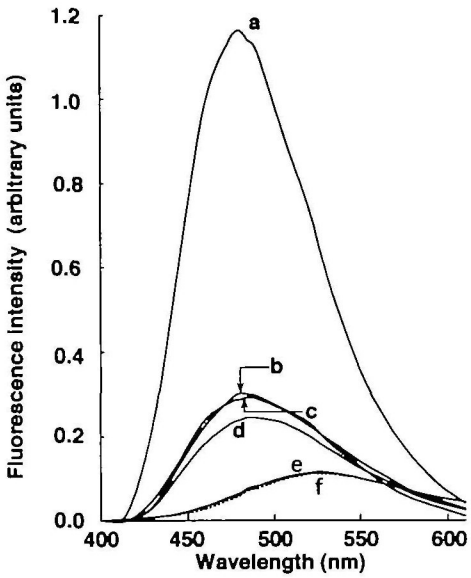

Fig. 1. **Fluorescence emission spectra of ANS in buffer solution (e, f) or cell suspensions of MDCK (a, b) and CHO cells (c, d) in the presence (a, c, e) or absence (b, d, f) of CEL-III.** The excitation wavelength used was 380 nm, and spectra were scanned 15 min after the addition of CEL-III (10,000 ng/ml) at 37°C. The medium for these samples was 10 mM Tris-HCl, pH 7.5, buffer containing 0.15 M NaCl, 10 mM $CaCl_2$, and 11 mM glucose.

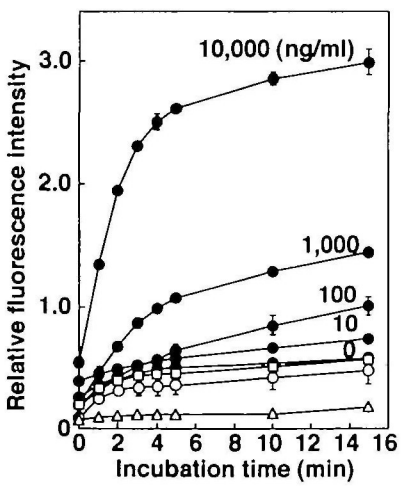

Fig. 2. **Effects of CEL-III concentration, Ca^{2+}, and lactose on the increase in ANS fluorescence in a suspension of MDCK cells.** After the addition of various concentrations (10-10,000 ng/ml) of CEL-III, fluorescence intensity at 480 nm was recorded with excitation at 380 nm (●) under the conditions described in the legend to Fig. 1. After the addition of 10,000 ng/ml of CEL-III, ANS fluorescence was measured under the same conditions but without $CaCl_2$ (△) or in the presence of 0.1 M lactose (○). ANS fluorescence in the presence of CEL-III (10,000 ng/ml) alone (without cells) (□).

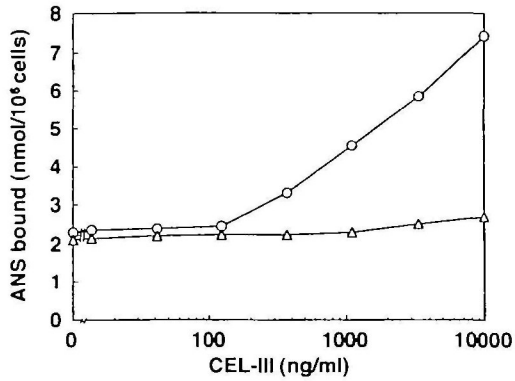

Fig. 3. **Effect of CEL-III on the ANS binding to MDCK (○) and CHO cells (△).** Cell suspensions preincubated with ANS were incubated with various concentrations of CEL-III at 37°C. After 15 min, the amounts of cell-bound ANS were determined as described under "MATERIALS AND METHODS."

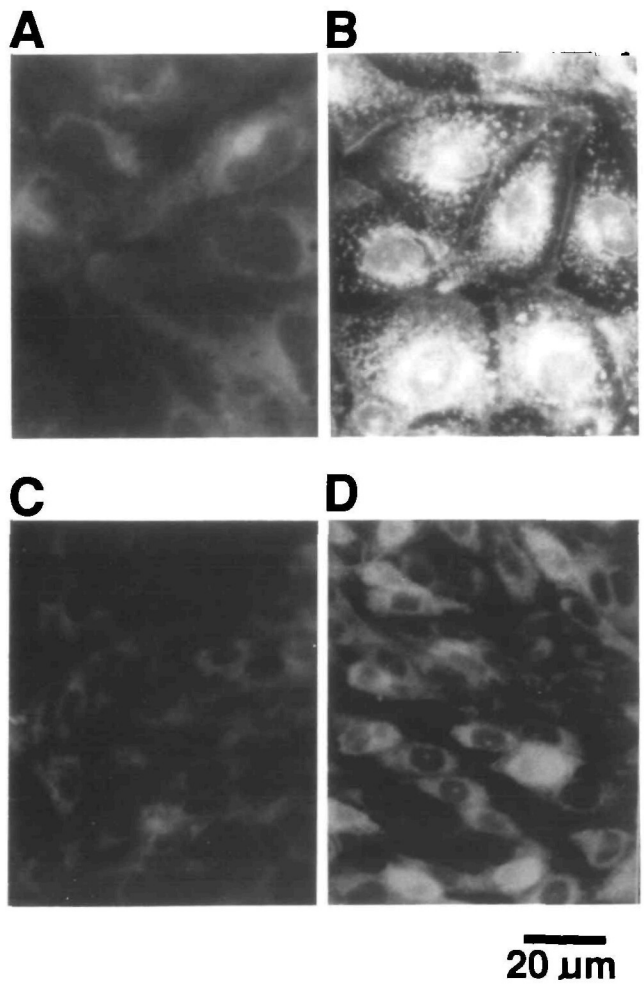

Fig. 4. **Fluorescence micrographs of MDCK (A, B) and CHO (C, D) cells preincubated with ANS before (A, C) or after (B, D) the incubation with CEL-III.** Cells grown on glass coverslips were preincubated with 100 µM ANS for 5 min at 37°C, then 10,000 ng/ml of CEL-III was added. After 15 min at 37°C, cells were immediately taken for microscopic observation.

on the concentration of CEL-III added (Fig. 3). In contrast, no such increase in ANS binding was observed in CHO cells, even though ANS bound to these cells at similar level in the absence of CEL-III. Fluorescence microscopic studies confirmed that CEL-III caused a dramatic increase in ANS fluorescence in MDCK cells. In these cells, ANS fluorescence was found in numerous punctuate dots distributed throughout the cell, while only slightly enhanced ANS fluorescence in a dispersed pattern was observed in CHO cells (Fig. 4).

CEL-III-Induced Energy Transfer between Tryptophan and ANS—Figure 5 shows the emission spectra of MDCK and CHO cell suspensions excited at 285 nm, where membrane protein tryptophan residues absorb. In the absence of ANS, fluorescence was emitted by tryptophan residues only, with a band maximum at 330 nm. On addition of ANS, the tryptophan-derived fluorescence band at 330 nm was markedly quenched to a similar extent in both cell lines. In MDCK cells, a second fluorescence band appeared with a maximum at 470 nm, which, since free ANS does not fluoresce under these conditions, represents tryptophan-excited ANS fluorescence (energy transfer). This second fluorescence band was almost negligible in CHO cells. In MDCK cells, CEL-III caused further increase in ANS emission at 470 nm and a concomitant decrease in tryptophan emission at 330 nm. No such effects of CEL-III were observed in CHO cells (Fig. 5).

Effect of Temperature and pH on CEL-III-Induced Changes in ANS Fluorescence Intensity and CEL-III Cytotoxicity—The hemolytic activity and cytotoxicity of CEL-III are known to be influenced by pH and temperature (*10, 15*). To clarify the relationship between the biological activities of CEL-III and its effect on ANS fluorescence, we examined the effect of CEL-III on ANS fluorescence under different conditions in MDCK and CHO cells. As shown in Fig. 6, the effect of CEL-III on ANS fluorescence was enhanced at pH 10 in both cell lines. Even in CHO cells, CEL-III induced a clear increase in fluorescence intensity at pH 10 and at 37°C. The increase in ANS fluorescence response at higher pH may relate to the fact that the cytotoxicity and hemolytic activity of CEL-III increase with increase in pH (*10, 15*). In MDCK cells, the effect of CEL-III decreased at 4°C at both pHs. This may be due to

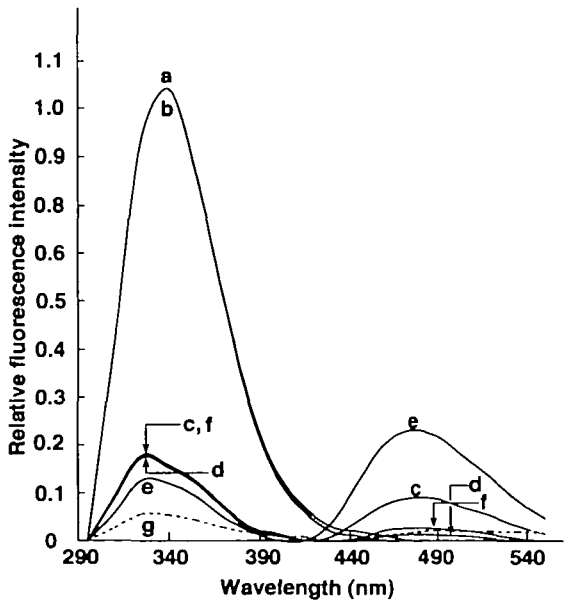

Fig. 5. **Energy transfer from membrane tryptophan to ANS in suspensions of MDCK (a, c, e) and CHO cells (b, d, f).** The excitation wavelength used was 285 nm, and spectra of the cell suspensions preincubated with ANS (c, d, e, f) were scanned 15 min before (c, d) and after (e, f) the addition of CEL-III (10,000 ng/ml) at 37°C. (a, b), cell suspensions alone. The emission spectrum of ANS plus CEL-III (10,000 ng/ml), without cells, is shown in curve g.

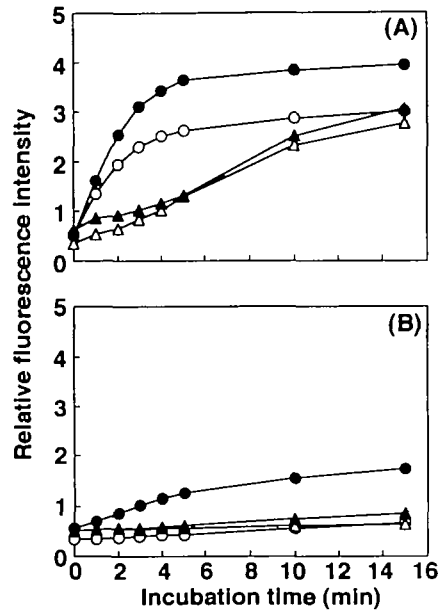

Fig. 6. **Effects of pH and temperature on the CEL-III-induced change in ANS fluorescence in MDCK (A) and CHO (B) cell suspensions.** After the addition of CEL-III (10,000 ng/ml) to cell suspensions in 10 mM Tris-HCl (pH 7.5) (○, △) or 10 mM sodium borate (pH 10) (●, ▲) buffer containing 0.15 M NaCl, 10 mM $CaCl_2$, and 11 mM glucose, fluorescence intensity at 480 nm was recorded with excitation at 380 nm at 4°C (△, ▲) or 37°C (○, ●).

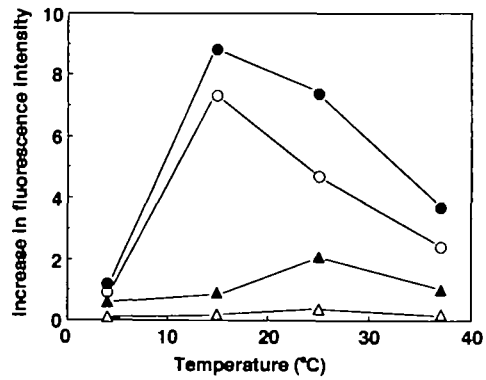

Fig. 7. **CEL-III-induced change in ANS fluorescence at various temperatures in MDCK (○, ●) and CHO (△, ▲) cell suspensions.** After the addition of CEL-III (10,000 ng/ml) to cell suspensions in 10 mM Tris-HCl (pH 7.5) (○, △) or 10 mM sodium borate (pH 10) (●, ▲) buffer containing 0.15 M NaCl, 10 mM $CaCl_2$, and 11 mM glucose, cells were incubated at the indicated temperatures for 5 min. The fluorescence intensity of each sample at 480 nm was measured with excitation at 380 nm.

the decrease in membrane fluidity at lower temperature. When the CEL-III-induced changes in ANS fluorescence were measured at various temperatures, the highest increase in ANS fluorescence was observed at around 15°C in MDCK cells and 25°C in CHO cells (Fig. 7). These results suggest that each cell line may have an optimal temperature for CEL-III-induced ANS fluorescence response. To obtain the further information on the relationship between ANS fluorescence response and CEL-III actions, we examined the cytotoxicity of CEL-III by trypan blue exclusion test under the conditions used in the ANS fluorescence study. Reflecting ANS fluorescence responses, CEL-III showed the strongest cytotoxicity at 37°C at pH 10 in both cell lines among the conditions tested (Fig. 8). These results suggest that ANS fluorescence response may be partly correlated to the CEL-III-induced cytotoxicity or at least membrane damage. Regarding the effect of temperature on CEL-III cytotoxicity, however, our previous results indicated that CEL-III showed stronger cytotoxicity at 4°C than at 37°C even at pH 10. The reason for the discrepancy is uncertain, but one possibility is that it might be due to different experimental conditions. In our previous study, we examined CEL-III cytotoxicity by colony formation assay using adherent cells, which required incubation periods of 5–7 days to obtain the results; whereas in this study suspended cells were used, and cell viability was determined by trypan blue exclusion test immediately after the treatment.

Fluorescence Microscopy—Figure 9 shows fluorescence micrographs of MDCK and CHO cells treated with FITC-labeled CEL-III (F-CEL-III). Adherent cells were incubated with F-CEL-III in TBS at 37°C for 30 min, then washed with PBS, and fixed. In both cell lines, but especially in MDCK cells, F-CEL-III was seen as small punctuate dots distributed throughout the cells. These dots may be clusters of bound CEL-III on the cell membranes. Evidently stronger fluorescence was observed in MDCK cells than CHO cells, indicating that a greater amount of F-CEL-III is associated with MDCK cells.

Immunoblotting Analysis—Several lines of evidence indicate that hemolysis is caused by the formation of ion-permeable pores by CEL-III oligomer after it has irreversibly bound to the membrane (*10*). Thus, the interaction between CEL-III and the cultured cells was further examined by immunoblotting analysis of the proteins solubilized from MDCK and CHO cells treated with CEL-III under various conditions. After the incubation with CEL-III, cells were washed with 0.1 M lactose to remove CEL-III bound on the cell surface. The proteins solubilized from the cells were then subjected to SDS-PAGE and subsequent immunoblotting analysis. As shown in Fig. 10, the immunoblotting of MDCK cells treated with

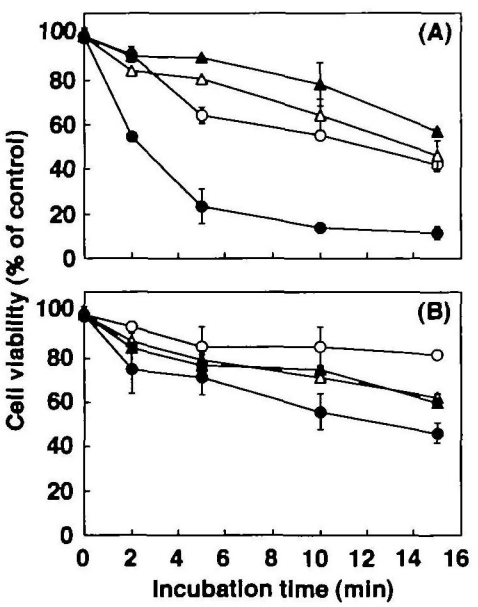

Fig. 8. **Effects of pH and temperature on the CEL-III cytotoxicity in MDCK (A) and CHO (B) cells.** Cell suspensions in 10 mM Tris-HCl (pH 7.5) (○, △) or 10 mM sodium borate (pH 10) (●, ▲) buffer containing 0.15 M NaCl, 10 mM $CaCl_2$, and 11 mM glucose were treated with CEL-III (10,000 ng/ml) at 4°C (△, ▲) or 37°C (○, ●). After 15 min, an aliquot of each cell suspension was withdrawn for the determination of cell viability by trypan blue exclusion. Each value represents an average of triplicate measurements.

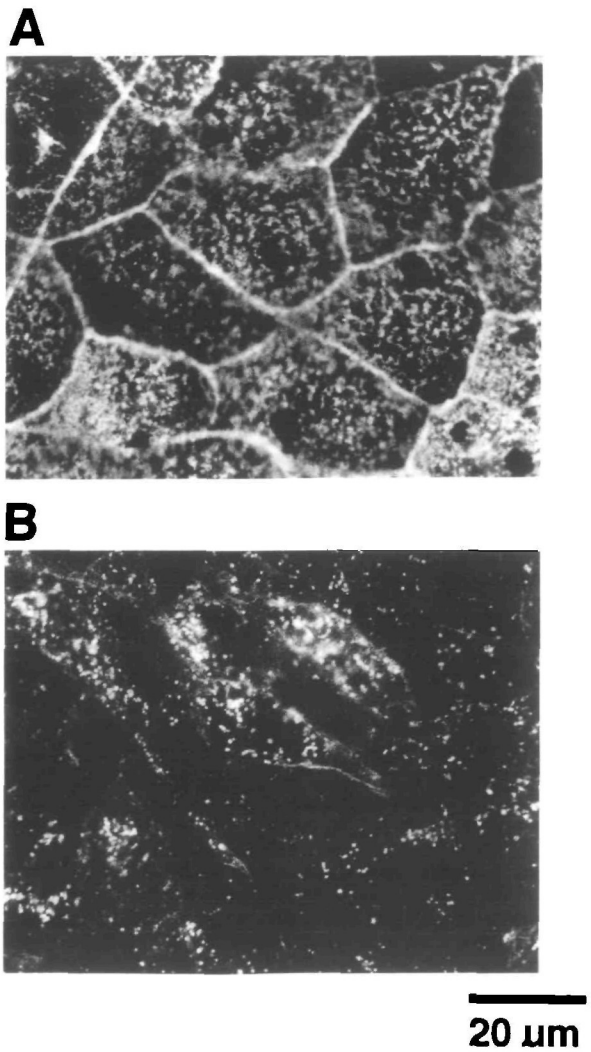

Fig. 9. **Fluorescence micrographs of MDCK (A) and CHO (B) cells incubated with FITC-labeled CEL-III.** Cells grown on glass coverslips were incubated with 40 μg/ml of F-CEL-III for 30 min at 37°C, washed three times with PBS, and fixed.

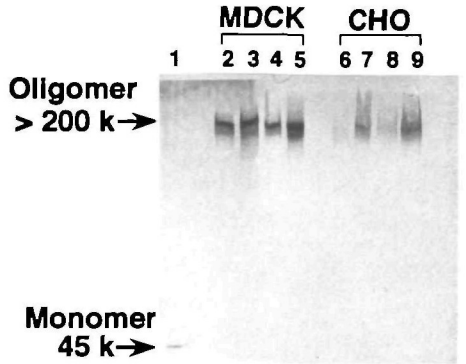

Fig. 10. **Immunoblotting of MDCK (lanes 2-5) and CHO (lanes 6-9) cells treated with CEL-III under various conditions.** Cells were treated with CEL-III (10,000 ng/ml) for 1 h at 4°C (lanes 2, 3, 6, 7) or 37°C (lanes 4, 5, 8, 9) in 10 mM Tris-HCl (pH 7.5) (lanes 2, 4, 6, 8) or 10 mM sodium borate (pH 10) (lanes 3, 5, 7, 9) buffer containing 0.15 M NaCl, 10 mM $CaCl_2$, and 11 mM glucose. After washing with 0.1 M lactose, cells were solubilized with the sample buffer containing 0.1% SDS and subjected to SDS-PAGE (5% gel) and immunoblotting. Detection was done as described in "MATERIALS AND METHODS."

CEL-III gave clear bands of CEL-III oligomer with a molecular mass of more than 200 kDa, which is similar in size to the CEL-III oligomer bands detected in the erythrocyte membrane (*10*), and stronger bands were seen at pH 10 than at pH 7.4. CHO cells gave much weaker bands than MDCK cells under all conditions tested, but the bands corresponding to CEL-III oligomer were obvious at pH 10.

Effect of Various Lectins and Protein Toxins on the Fluorescence Intensity of Cell-Bound ANS—To ascertain whether the effect of CEL-III on ANS fluorescence is specific to CEL-III or a common feature of membrane-binding proteins, we examined the effects of ricin, castor bean hemagglutinin, concanavalin A, wheat germ agglutinin, diphtheria toxin, and *Pseudomonas* toxin on ANS fluorescence in MDCK cells. As shown in Table I, none of these proteins showed any significant effect on ANS fluorescence, even though ricin and castor bean hemagglutinin are known to recognize galactose residues, as does CEL-III. Furthermore, strong cytotoxicity of ricin, diphtheria toxin, and *Pseudomonas* toxin had been observed in MDCK cells (*21*). Thus, these results suggest that the binding of certain proteins to the specific receptors on the plasma membrane is insufficient for induction of ANS fluorescence response. Further CEL-III-specific action on the plasma membrane, such as oligomer formation, may be responsible for the increase in ANS fluorescence.

TABLE I. **Effects of various lectins and protein toxins on the ANS fluorescence in MDCK cells.**

Treatment	ANS fluorescence (relative fluorescence intensity after 15 min)
None	0.403±0.001
+CEL-III[a]	2.990±0.010
+Ricin[b]	0.300±0.001
+Castor bean hemagglutinin[c]	0.340±0.006
+Modeccin[d]	0.382±0.001
+Concanavalin A[e]	0.367±0.002
+Wheat germ agglutinin[f]	0.424±0.007
+Diphtheria toxin[g]	0.369±0.010
+*Pseudomonas* toxin[h]	0.398±0.008

Cell suspensions preincubated with ANS were treated with each protein (10,000 ng/ml). After incubation for 15 min at 37°C, the fluorescence intensity of cell suspension was measured with excitation wavelength of 380 nm and emission wavelength of 480 nm. Sugar specificity or recognition sites: (a, b, c, d), *N*-acetylgalactosamine, galactose; (e), mannose, glucose; (f), $(N\text{-acetylglucosamine})_2$, *N*-acetylneuraminic acid; (g, h), toxin-specific receptors.

DISCUSSION

Recent studies suggest that the hemolytic activity of CEL-III is caused by the formation of ion-permeable pores by CEL-III oligomer after its binding to carbohydrate receptors on the cell surface. Similar mechanisms have been postulated for other pore-forming proteins, such as staphylococcal α-toxin, streptolysin O, *Tethya* hemolysin from marine sponge, and complement C5b-9 (*14, 23-25*). Previously, we found that CEL-III exhibits strong cytotoxicity against cultured cell lines such as MDCK, HeLa, and Vero cells, whereas CHO, XC, and PtK_1 cells were highly resistant to CEL-III cytotoxicity (*15*). The LD_{50} value of CEL-III in the most sensitive cells, MDCK, was 53 ng/ml, while no significant toxic effect was observed in CHO cells up to 10,000 ng/ml. Binding studies using FITC-labeled CEL-III indicated that the amounts of CEL-III bound to sensitive MDCK, HeLa, and Vero cells were 1.5-2-fold greater than those bound to relatively resistant CHO, PtK_1, and XC cells (*15*). Thus, the binding efficiency of CEL-III partly accounts for the differences in susceptibility among these cell lines. However, the differences in binding efficiency of CEL-III seem to be too small to explain the difference in LD_{50} values between MDCK and CHO cells (>180-fold). It seems likely that the interaction between CEL-III and the cell membrane leading to pore-formation is more important in determining the susceptibility to CEL-III cytotoxicity. In fact, it has been reported that the affinity of CEL-III for lactose increased with decreasing pH, even though no hemolytic activity was observed in the acidic pH region (*10, 26*). Therefore, it is apparent that effective binding of CEL-III to the carbohydrate receptors is a prerequisite, but binding alone is insufficient for its biological activities; the subsequent interaction between CEL-III and the lipid bilayer may be necessary. It is conceivable that the variations in susceptibility to CEL-III cytotoxicity are attributable to complex factors related to the differences in composition and organization of membrane constituents. In this study, we used a fluorescence probe of protein and membrane structure, 8-anilino-1-naphthalenesulfonate (ANS), to investigate this point. When ANS binds to biological membranes, it gives information about alterations in their structure, since the fluorescence intensity of this probe is influenced by polarity changes in its microenvironment. Thus ANS has been widely used to study the dynamic state of membrane systems, such as mitochondria, microsomes, bacterial cytoplasmic membrane, and erythrocyte ghosts, through changes of fluorescence intensity and emission spectra of ANS bound to these membranes (*27*). Accordingly, an increase in fluorescence intensity of ANS concomitant with slight blue shift in its emission maximum from 526 to 484 nm was observed in both MDCK and CHO cell suspensions (Fig. 1). However, the addition of CEL-III to cell suspensions of CEL-III-sensitive MDCK cells, but not to those of resistant CHO cells, caused pronounced increase in ANS

fluorescence (Fig. 1), suggesting that CEL-III-induced probe response is correlated to the different susceptibilities of these cell lines to CEL-III cytotoxicity. The increase in ANS fluorescence response at pH 10 may also support this notion (Fig. 6), since stronger cytotoxicity of CEL-III was observed in MDCK and CHO cells at alkaline pH (*15*). In addition to the effect of pH, CEL-III-induced ANS responses were also markedly affected by temperature, and the highest response was observed around 15 and 25°C in MDCK and CHO cells, respectively (Fig. 7). This may be partly related to the fact that the hemolytic activity of CEL-III is highest around 10°C and decreases with increasing temperature (*10*). Interestingly, a similar temperature-dependent hemolytic activity is shown by Staphylococcal α-toxin, which is maximal at below 25°C and decreases with increasing temperature (*28*). Further study is required to clarify the mechanism of temperature-dependency of these hemolytic proteins.

On increasing the concentration of CEL-III, a parallel increase in probe binding was observed in MDCK cells (Fig. 3). Therefore, the increase in ANS fluorescence caused by CEL-III is probably due to the creation of additional probe-binding sites. Fluorescence microscopic observation showed that a part of the bound ANS was distributed as numerous punctuate dots that seemed to be localized on the plasma membrane, while strong ANS fluorescence was observed in the cytosolic region around nucleus (Fig. 4). Although the detailed mechanism of CEL-III-induced increase in ANS fluorescence and the effect on the distribution of bound ANS is still unclear, a dot-like distribution of bound FITC-labeled CEL-III was also observed (Fig. 9). Thus one can speculate that the clusters of bound CEL-III formed in the plasma membrane may provide new ANS binding sites. This hypothesis is supported by the recent finding that the surface hydrophobicity of CEL-III in aqueous solution, as measured by ANS binding, increased markedly with the binding of specific carbohydrates such as lactose, lactulose, and *N*-acetyllactosamine under certain conditions, and that this change was accompanied by the formation of a CEL-III oligomer of similar size to that formed in the erythrocyte membrane treated with CEL-III (*29*). Therefore, it is probable that CEL-III monomers bound to cell-surface carbohydrates spontaneously oligomerize in the membrane (*10*). Since oligomer formation of CEL-III was also observed when CEL-III was incubated with detergents such as SDS, Triton X-100, and Tween 20 (*10*), hydrophobic interaction with the membrane lipid-bilayer may trigger the formation of CEL-III oligomers.

Interaction between ANS and CEL-III bound to cells is also suggested by the results of energy transfer from tryptophan to ANS (Fig. 5). Since the efficiency of energy transfer varies inversely with the distance between the donor and acceptor fluorophores (*30*), the occurrence of CEL-III-enhanced energy transfer in MDCK cells may indicate that at least part of the ANS bound to the membranes is in close proximity to bound CEL-III.

Immunoblotting analysis of proteins from cells treated with CEL-III revealed that CEL-III forms oligomers irreversibly bound to the membrane (Fig. 10). Stronger bands of CEL-III oligomer were detected in sensitive MDCK cells than resistant CHO cells (Fig. 10) under all conditions tested. At pH 10, an increase in CEL-III oligomer formation was observed even in CHO cells. Therefore, these results suggest that the cytotoxicity of CEL-III is closely related to its capability to form an oligomer in the membrane, and this process may be reflected in the ANS fluorescence response.

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