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Temperature- and pH-Dependent Cytotoxic Effect of the Hemolytic Lectin CEL-III from the Marine Invertebrate *Cucumaria echinata* on Various Cell Lines

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We investigated the cytotoxicity of CEL-III, one of four Ca^{2+}-dependent galactose/*N*-acetylgalactosamine (GalNAc)-binding lectins from the marine invertebrate *Cucumaria echinata*. Among six cell lines tested, MDCK cells showed the highest susceptibility to CEL-III cytotoxicity and its LD_{50} was estimated to be 53 ng/ml, while no significant cytotoxicity of CEL-III was observed in CHO cells up to 10,000 ng/ml. In the presence of 0.1 M lactose, the cytotoxicity of CEL-III was strongly inhibited. The binding studies using FITC-labeled CEL-III revealed that the amount of CEL-III bound to MDCK cells was about 2-fold greater than that in the case of CHO cells. The cytotoxicity of CEL-III increased with decreasing temperature. The surviving fractions of Vero cells exposed to CEL-III at 4°C were immediately decreased, and more than 90% of exposed cells were killed within 20 min, whereas at 37°C much longer exposure period (more than 10 h) was required to kill 50% of the cells. CEL-III induced the release of carboxyfluorescein (CF) from CF-loaded MDCK cells and this activity was markedly increased at alkaline pH (pH 10) and at lower temperature (4°C). Even in CHO cells, considerable CF release was induced by CEL-III at 4°C and at pH 10 but not at pH 7.5 at both temperatures. In agreement with these results, CHO cells exposed to CEL-III at 4°C and at pH 10 were killed in a dose-dependent manner. These results suggest that CEL-III exhibits cytotoxicity through damaging the plasma membrane by pore-formation in a temperature- and pH-dependent manner. Different susceptibility of each cell line to CEL-III cytotoxicity may be due to differences in the processes leading to pore-formation after binding to cell-surface carbohydrates.

Key words: cytotoxicity, hemolysis, lectin, pore-forming protein.

Lectins are a group of structurally diverse proteins which recognize specific carbohydrate structures and induce various biological effects including agglutination of animal cells by binding to cell surface glycoproteins or glycolipids. Recent studies have demonstrated that some lectins such as phytohemagglutinin (PHA), concanavalin A (Con A), and wheat germ agglutinin (WGA) show cytotoxic effects on certain cell lines (*1*). Although it remains unknown how these lectins induce cell lysis by binding to cell surface carbohydrates, it has been hypothesized that some lectins lyse the cells by triggering apoptosis (programmed cell death) through activation of endogenous endonucleases, leading to DNA fragmentation (*2*). Many plant lectins have been extensively studied, and some of them have been used as tools in studies of cell surface structures (*3*). A number of animal lectins have also been found in various organisms, and studied along with their physiological functions (*4, 5*). Most animal lectins have been classified as Ca^{2+}-dependent (C-type) or independent (S-type or galectin) (*6, 7*). The C-type animal lectins have a characteristic carbohydrate-recognition domain (CRD) consisting of 120-130 residues, which exhibits some degree of sequence homology between species.

Several C-type lectins have been isolated from marine invertebrates and their roles in the organisms studied (*8-11*). Recently Hatakeyama *et al.* purified four galactose/GalNAc-specific Ca^{2+}-dependent lectins with different molecular masses (CEL-I-CEL-IV) from the marine invertebrate, *Cucumaria echinata* (Holothuroidea) (*12, 13*). Interestingly, one of these lectins, CEL-III caused hemolysis of rabbit and human erythrocytes, while it 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

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Abbreviations: CRD, carbohydrate-recognition domain; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TBS, Tris-buffered saline; FBS, fetal bovine serum; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; F-CEL-III, FITC-labeled CEL-III; MEM, minimal essential medium; PBS, phosphate-buffered saline; CFDA, 6-carboxyfluorescein diacetate; CF, carboxyfluorescein.

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 (*13*). Some bacterial protein toxins have been reported to exhibit their toxic effect by pore-formation in the target cell membrane (*14-17*). Therefore, it is of interest to investigate the cytotoxic activity of CEL-III in cultured cells.

In the present study, we examined the toxic effect of CEL-III as well as its interaction with plasma membranes in various cultured cell lines. The results demonstrate that CEL-III is highly toxic to several cell lines, especially MDCK, and the cytotoxic effect is strongly influenced by temperature and pH.

MATERIALS AND METHODS

Materials—The *Cucumaria echinata* samples were generously provided by N. Ikeda (Fukuoka Fisheries and Marine Technology Research Center). The samples were stored at −30°C until use. CEL-III was purified from the body fluid of *C. echinata* using column chromatography on lactosyl-Cellulofine, GalNAc-Cellulofine, and Sephacryl S-200 as previously described (*12*). The molecular mass of purified CEL-III is estimated at 45 kDa (*12*). 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. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was obtained from Sigma Chem. (St. Louis, MO). FITC isomer I was from Dojin Chemical Laboratories, Kumamoto. 6-Carboxyfluorescein diacetate was from Lambda Probes & Diagnostics, Austria.

FITC Labeling of CEL-III—Fluorescein isothiocyanate (FITC)-labeled CEL-III (F-CEL-III) was prepared by essentially the same method as described previously (*18*). 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—Vero (African green monkey kidney), MDCK (Madin-Darby canine kidney), XC (rat sarcoma), CHO (Chinese hamster ovary), HeLa (human epithelia carcinoma), and PtK_1 (Potoroo rat kangaroo kidney) 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 (*19*). These cell lines, especially Vero, MDCK, PtK_1, and CHO, have been used to study the cytotoxicity of ricin, a galactose-specific toxic lectin (*20, 21*), and it has been shown that CHO cells are more than 100-fold more resistant to ricin cytotoxicity than Vero and other cell lines (*22*).

Measurement of Cytotoxicity of CEL-III—Cytotoxicity (LD_{50}) of CEL-III was measured in terms of the inhibition of colony formation as described previously, with slight modification (*20*). In brief, 100-150 cells per well in a 24-well plate were cultured with varying concentrations of CEL-III in the growth medium for 5-8 days. The number of colonies formed was counted after staining with 1% methylene blue in 50% methanol. Clusters of 40 or more cells were considered as colonies.

Measurement of Cell Lysis—CEL-III-induced cell lysis was assessed using the MTT tetrazolium cytotoxicity assay (*23*). In brief, $1\text{-}2\times10^4$ cells per well in a 96-well plate were cultured with varying concentrations of CEL-III in α-MEM containing 35 μM bovine serum albumin (BSA) for 24 h, and then incubated with MTT for 4 h. After aspiration of the medium, dimethylsulfoxide was added to dissolve the MTT formazan reaction product and the optical density was measured at 570 nm using a multiwell scanning spectrophotometer.

Binding of FITC-Labeled CEL-III—For time-course analysis, cell monolayers (1×10^5 cells/well in 48-well plate) were incubated with 25 μg/ml of FITC-labeled CEL-III at 37°C for 2 h in α-MEM containing 10% FBS. After removal of the medium by aspiration, cells were washed three times with ice-cold PBS. Washed cells were solubilized in 1 ml of 20 mM Tris-HCl buffer, pH 8.5, containing 0.2% sodium dodecyl sulfate. The fluorescence intensity of the solubilized cell solution was measured with a fluorescence spectrophotometer (Hitachi Model 650-40) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. In this condition no quenching of fluorescence due to binding to cells was observed. The amount of cell-associated fluorescent CEL-III (F-CEL-III) was determined from the relationship between the concentration and fluorescence intensity of F-CEL-III. In these binding experiments, the amounts of F-CEL-III bound to cells in the presence of 0.1 M lactose were subtracted from each value as the nonspecific binding.

Assay for the Release of Carboxyfluorescein—Membrane integrity was measured based on the previously described method (*24*) using the nonfluorescent, lipophilic, membrane-permeable ester derivative of 6-carboxyfluorescein (CF), 6-carboxyfluorescein diacetate (CFDA), which crosses the cell membrane and is hydrolyzed by intracellular esterases to afford the membrane-impermeable fluorophore, CF (*25*). For loading with CFDA, cells grown in 48-well plates (1×10^5 cells/well) were incubated with 40 μg/ml of CFDA in TBS (10 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 10 mM $CaCl_2$) at 37°C for 1 h. The loading solution was prepared just prior to use from 10 mg/ml stock solution of CFDA in acetone stored at −20°C. After loading, cells were washed twice with PBS and incubated with the indicated concentration of CEL-III in saline solution of pH 7.5 or pH 10 containing 10 mM $CaCl_2$ at 4°C or at 37°C in the presence or absence of 0.1 M lactose. After 1 h incubation, 0.2 ml aliquots of culture supernatants were collected and mixed with 0.5 ml of TBS (pH 7.5) and their fluorescence at 520 nm excited at 490 nm was measured with a fluorescence spectrophotometer (Hitachi Model 650-40). Total cellular content of CF was measured after treatment with 0.2% SDS in TBS.

RESULTS

Cytotoxicity of CEL-III to Various Cell Lines—The cytotoxicity of CEL-III was measured in terms of the inhibition of colony formation by Vero, HeLa, MDCK, PtK_1, XC, and CHO cells. Adherent cells were incubated with varying concentrations of CEL-III in growth medium for 5-8 days. The numbers of colonies formed were counted after staining with 1% methylene blue. As shown in Fig. 1, the susceptibility of each cell line to CEL-III cytotoxicity was quite different and no toxic effect of CEL-III was observed in CHO cells up to 10,000 ng/ml, while more than 50% of MDCK cells were killed by less than 100 ng/ml of CEL-III. Different susceptibility to CEL-III cytotoxicity between MDCK and CHO cells was also observed in MTT assay (data not shown). It is known that CEL-III-induced hemolysis is prevented by specific carbohydrates containing galactose (*12*). Thus, we examined the effect of lactose on CEL-III cytotoxicity in these cell lines. The LD_{50}s of CEL-III estimated from the dose-response curves in the presence or absence of lactose are summarized in Table I. In the presence of 0.1 M lactose, the cytotoxicty of CEL-III was strongly inhibited. These results suggest that the binding of CEL-III to specific carbohydrate chains on the cell-surface is essential for its cytotoxicity. As shown in Table II, the cytotoxicity of CEL-III was also inhibited by several galactose-related carbohydrates, as measured by MTT assay.

Binding of CEL-III to Cultured Cells—To ascertain whether or not differential cytotoxicity of CEL-III to cultured cells is due to differential binding efficiency of CEL-III to these cell lines, we examined the binding of FITC-labeled CEL-III (F-CEL-III) to Vero, HeLa, MDCK,

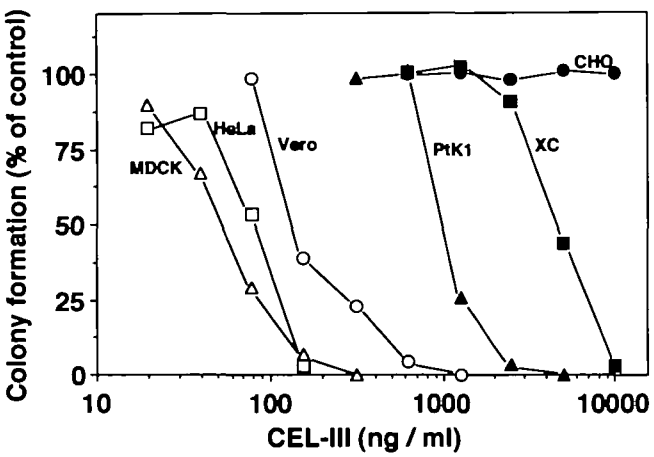

Fig. 1. **Dose-response curves of the cytotoxicity of CEL-III to MDCK (△), HeLa (□), Vero (○), PtK_1 (▲), XC (■), and CHO (●) cells.** The cytotoxicity of CEL-III was measured in terms of the inhibition of colony formation. Adherent cells (100-150 cells/well in 24-well plate) were cultured with varying concentrations of CEL-III in α-MEM supplemented with 10% FBS, pH 7.4 at 37°C for 5-8 days, then the numbers of colonies formed were counted as described in "MATERIALS AND METHODS." Each point represents an average of duplicate measurements.

TABLE I. **The cytotoxicity of CEL-III in various cell lines.** The cytotoxicity of CEL-III was measured in terms of the inhibition of colony formation (LD_{50}). Cells were cultured with varying concentrations of CEL-III in α-MEM supplemented with 10% FBS, pH 7.4 at 37°C for 5-8 days. The LD_{50} value of CEL-III in each cell line was estimated from dose-response curves in the presence or absence of 0.1 M lactose. All measurements were performed in duplicate.

Cells	LD_{50} (ng/ml)	
	−lactose	+0.1 M lactose
MDCK	53	>10,000
HeLa	82	4,500
Vero	130	>10,000
PtK_1	990	>10,000
XC	4,550	>10,000
CHO	>10,000	>10,000

TABLE II. **Effect of simple carbohydrates on the cytotoxicity of CEL-III in MDCK cells.** The cytotoxicity of CEL-III was measured by MTT assay (IC_{50}). Cells grown in 96-well plates (2×10^4 cells/well) were incubated in α-MEM containing 35 μM BSA, pH 7.4 with varying concentrations of CEL-III for 24 h at 37°C in the absence or presence of the indicated saccharides at 0.1 M and subjected to MTT assay as described under "MATERIALS AND METHODS." Each IC_{50} value of CEL-III was estimated from the dose-response curve. All measurements were performed in duplicate.

Saccharide	IC_{50} (ng/ml)
N-Acetylgalactosamine	>10,000
Galactose	>10,000
Lactose	>10,000
Raffinose	3,400
N-Acetylglucosamine	120
Glucose	430
Mannose	300
Ribose	520
Sucrose	490
None	290

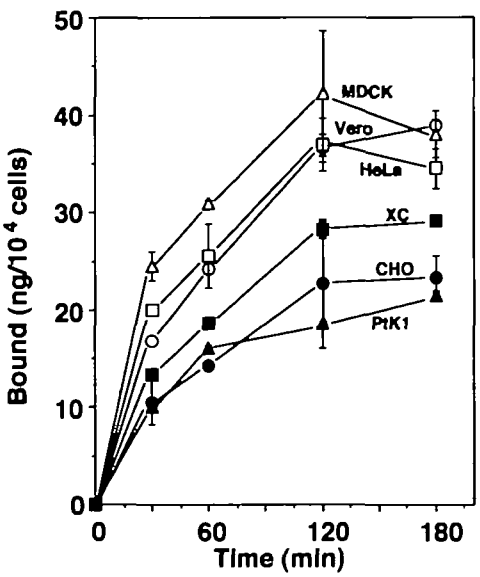

Fig. 2. **Time-courses of binding of F-CEL-III to MDCK (△), HeLa (□), Vero (○), PtK_1 (▲), XC (■), and CHO (●) cells.** Cell monolayers (1×10^5 cells/well in 46-well plate) were incubated with 25 μg/ml of F-CEL-III for various times at 37°C in the growth medium containing 10% FBS. After incubation, cells were washed three times with PBS and the amounts of cell-associated F-CEL-III were determined as described in "MATERIALS AND METHODS." The measurements were performed in duplicate.

PtK_1, XC, and CHO cells. Figure 2 shows the time course of the binding of F-CEL-III to six cell lines at 37°C. During the binding experiments, no significant loss of cells was observed. It was found that F-CEL-III can bind to all these cell lines and the binding of F-CEL-III proceeded rapidly for the initial 30 min and then reached equilibrium after 2 h. After the plateau level had been reached, the amount of F-CEL-III bound to MDCK cells was about 2.0-fold greater than that to CHO cells. Thus, it is reasonable that CEL-III tends to bind more efficiently to sensitive MDCK cells than resistant CHO cells. Since the difference in LD_{50} value between MDCK and CHO cells was more than 180-fold, it is likely that there is some additional factor which results in different susceptibility of these cell lines to CEL-III cytotoxicity.

Effect of Temperature on the Cytotoxicity of CEL-III in Various Cell Lines—It has been shown that the hemolytic activity of CEL-III is dependent on temperature. The highest activity was observed at 10°C, but the activity decreased with increasing temperature, suggesting that the hemolytic activity of CEL-III is not due to an enzymatic reaction (*13*). To examine the effect of temperature on CEL-III cytotoxicity, adherent cells were incubated with

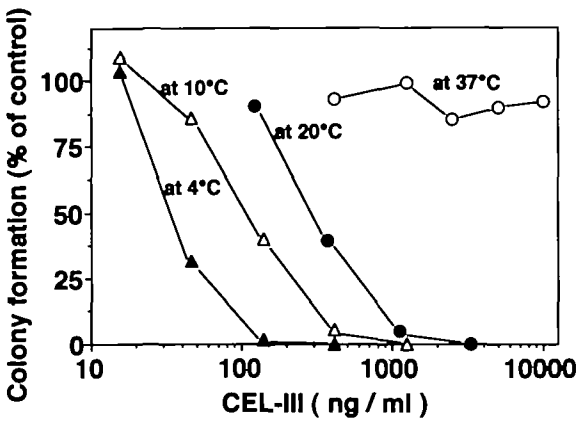

Fig. 3. **The effect of temperature on the cytotoxicity of CEL-III in Vero cells.** Adherent cells (100-150 cells/well in 24-well plate) were incubated with varying concentrations of CEL-III in α-MEM containing 20 mM HEPES (pH 7.4) and 10% FBS at 4°C (▲), 10°C (△), 20°C (●), or 37°C (○). After 2 h incubation, the medium containing CEL-III was removed and replaced with the growth medium containing 0.1 M lactose, and cells were cultured for 6 days for colony formation. The numbers of colonies formed were counted as described in "MATERIALS AND METHODS." Each point represents an average of duplicate measurements.

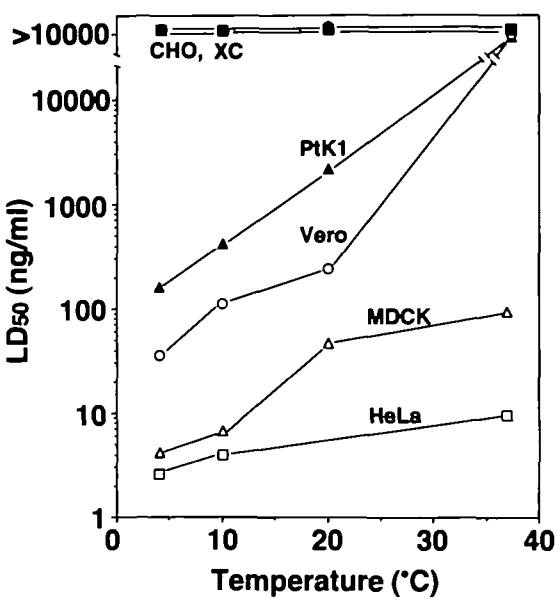

Fig. 4. **The effect of temperature on the cytotoxicity of CEL-III in various cell lines.** The effect of temperature on the cytotoxicity of CEL-III in MDCK (△), HeLa (□), Vero (○), PtK_1 (▲), XC (■), and CHO (●) cells was examined as described in the legend to Fig. 3. From the dose-response curves, LD_{50} values of CEL-III at each incubation temperature were obtained and plotted against temperature.

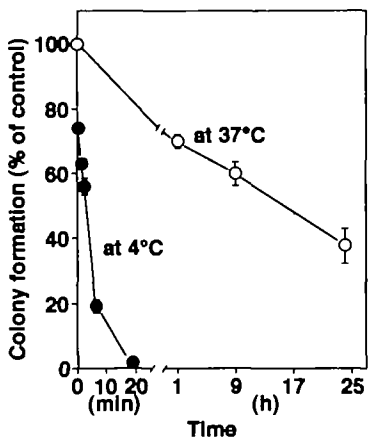

Fig. 5. **Cytotoxicity of CEL-III in Vero cells after various incubation times.** Vero cells (100-150 cells/well in 24-well plate) were incubated with 1,000 ng/ml of CEL-III for various time periods at 4 or 37°C. The medium was then removed and replaced with growth medium containing 0.1 M lactose. After culture for 6 days, the numbers of colonies were counted as described in "MATERIALS AND METHODS." The measurements were performed in duplicate.

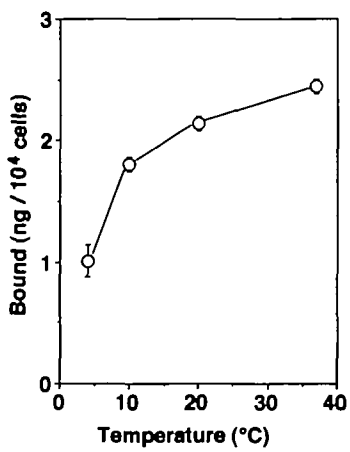

Fig. 6. **The effect of temperature on the binding of CEL-III to Vero cells.** Vero cells (1×10^5 cells/well in 46-well plate) were incubated with 10 μg/ml of F-CEL-III for 1 h at indicated temperature in α-MEM containing 20 mM HEPES (pH 7.4) and 10% FBS. After incubation, cells were washed three times with PBS and the amounts of cell-associated F-CEL-III were determined as described in "MATERIALS AND METHODS." The measurements were performed in duplicate.

varying concentrations of CEL-III at different temperatures. After 2 h incubation, the medium containing CEL-III was removed and replaced with growth medium containing 0.1 M lactose to prevent further toxic effect of CEL-III, and the cells were cultured under the normal growth conditions for 5-7 days. During the 2 h incubation with CEL-III, no loss of cells was observed. Figure 3 shows the dose-response curves of CEL-III in Vero cells at different temperatures. The cytotoxicity of CEL-III increased with decreasing temperature and the highest toxicity was observed at 4°C, whereas no toxic effect was observed up to 10,000 ng/ml at 37°C under these conditions. As shown in Fig. 4, the LD_{50} values of CEL-III in PtK_1, MDCK, and HeLa cells also decreased with decreasing temperature as seen in Vero cells. Among the cell lines tested, the susceptibility of Vero cells to CEL-III cytotoxicity was strongly affected by temperature, while only a slight effect of temperature was observed in the case of HeLa cells. However, no significant toxic effect of CEL-III was observed in XC and CHO cells even at 4°C. To characterize further the temperature-dependent cytotoxicity of CEL-III, Vero cells were exposed to a fixed concentration of CEL-III (1,000 ng/ml) for various times at 4 or 37°C. The medium containing CEL-III was removed and replaced with growth medium containing 0.1 M lactose, and cells were allowed to grow to form colonies. As shown in Fig. 5, at 4°C the surviving fractions exposed to CEL-III immediately decreased, and almost all cells were killed within 20 min. In contrast, at 37°C a prolonged exposure period was required to achieve a given toxic effect. These results suggest that CEL-III can more quickly induce membrane damage leading to cell death at lower temperature.

Effect of Temperature on the Binding of CEL-III to Vero Cells—We investigated the effect of temperature on the binding of F-CEL-III to Vero cells. As shown in Fig. 6, the binding was temperature-dependent and the amount of bound CEL-III increased with increasing temperature. This increase is probably due to the temperature-dependent internalization of bound CEL-III into the cells. Since the cytotoxicity of CEL-III decreased with increasing temperature (Fig. 4), internalized CEL-III may not be responsible for the cytotoxicity. Recent studies have indicated that CEL-III interacts with the lipid bilayer as well as with specific saccharides (unpublished data), suggesting that complicated mechanisms may be involved in the interaction of CEL-III with the cell membrane leading to membrane damage. Further studies are required on the interaction of CEL-III with the cell membrane.

CF Release from MDCK and CHO Cells Treated with CEL-III—It has been considered that the hemolytic activity of CEL-III is manifested by damage to the erythrocyte

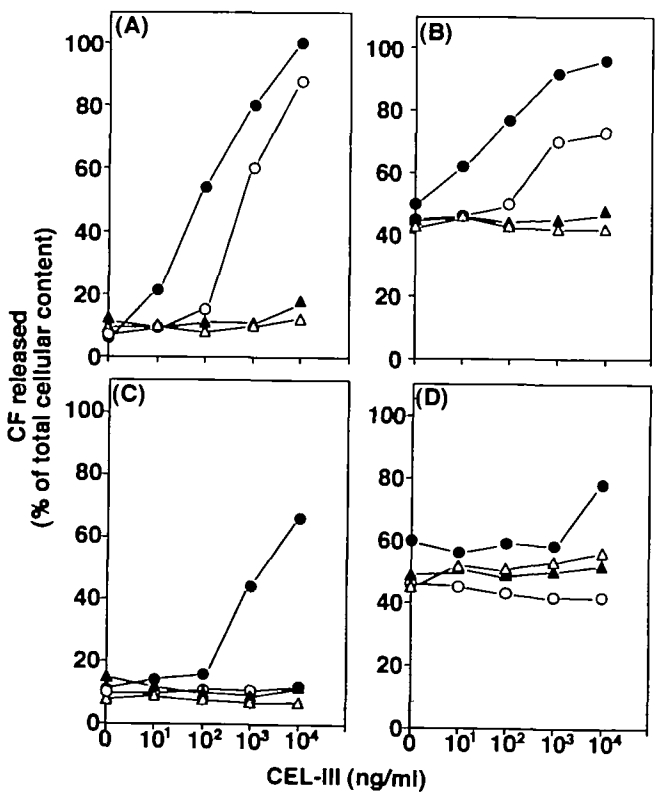

Fig. 7. **CEL-III-induced CF release from CF-loaded cells.** CF-loaded MDCK (A, B) or CHO (C, D) cells were incubated with varying concentrations of CEL-III in 10 mM Tris-HCl (pH 7.5) (○, △) or 10 mM sodium borate (pH 10) (●, ▲) buffer containing 0.15 M NaCl and 10 mM $CaCl_2$ in the presence (△, ▲) or absence (○, ●) of 0.1 M lactose at 4°C (A, C) or 37°C (B, D) for 1 h. The CF released from the cells was measured as described in "MATERIALS AND METHODS." The measurements were performed in duplicate.

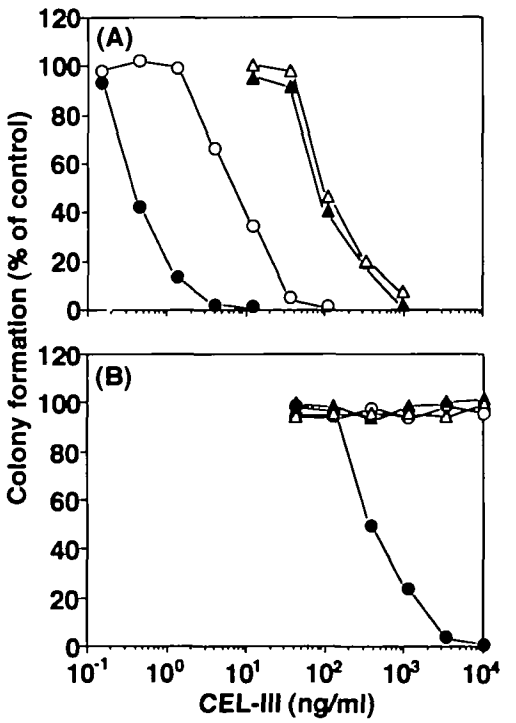

Fig. 8. **The effect of pH on the cytotoxicity of CEL-III to MDCK (A) and CHO (B) cells.** Adherent cells (100-150 cells/well in 24-well plate) were incubated with varying concentrations of CEL-III in 10 mM Tris-HCl (pH 7.5) (○, △) or 10 mM sodium borate (pH 10) (●, ▲) buffer containing 0.15 M NaCl and 10 mM $CaCl_2$ at 4°C (○, ●) or 37°C (△, ▲). After 1 h incubation, the medium containing CEL-III was removed and replaced with the growth medium containing 0.1 M lactose and cells were cultured for 7 days for colony formation. The numbers of colonies formed were counted as described in "MATERIALS AND METHODS." Each point represents an average of duplicate measurements.

membrane through pore-formation. In fact, it has been shown that CEL-III induced the release of ATP from rabbit erythrocytes osmotically protected by dextran 8 (*13*). To ascertain whether or not the cytotoxic effect of CEL-III to cultured cells is due to the formation of transmembrane pores, as in the hemolytic mechanism, we examined the CEL-III-induced release of CF from CF-loaded MDCK and CHO cells which were preincubated with CFDA. In these cell lines, CEL-III was found to induce CF release in a pH- and temperature-dependent manner (Fig. 7). At pH 7.5, the release of CF was observed in MDCK cells treated with CEL-III in a dose-dependent manner, whereas no CF release was detected in CHO cells up to 10,000 ng/ml of CEL-III. At pH 10, increased CF release was observed in MDCK cells and significant CF release was also detected in CHO cells, which are very resistant to CEL-III cytotoxicity. At 4°C, CEL-III induced more pronounced CF release from these cell lines than at 37°C, albeit about 50% of intracellular CF was spontaneously released at 37°C. In the presence of 0.1 M lactose, the release of CF with CEL-III from these cell lines was almost completely inhibited. These results are well consistent with the finding that the hemolytic activity of CEL-III is caused by the binding of CEL-III to galactose-containing cell-surface receptors and subsequent pore-formation. The pH effect on CF release with CEL-III in cultured cells is also consistent with the hemolytic activity of CEL-III, which increased with increasing pH up to pH 10 (*12*). Therefore, these results strongly suggest that the cytotoxicity of CEL-III is a result of damage to the plasma membrane through pore-formation, as in the hemolytic mechanism.

Effect of pH on the Cytotoxicity of CEL-III in MDCK and CHO Cells—The results of CF release assay (Fig. 7) indicate that pH is one of the important factors in the action mechanism of CEL-III on cultured cells. Thus, we examined the effect of pH on the cytotoxicity of CEL-III in MDCK and CHO cells (Fig. 8). In MDCK cells, when the cells were incubated at 4°C, CEL-III exhibited about 18-fold stronger cytotoxicity at pH 10 than at pH 7.5, based on the LD_{50} values. However, no significant pH effect on CEL-III cytotoxicity was observed at 37°C. In addition, even CHO cells became susceptible to CEL-III cytotoxicity at pH 10 at 4°C, while no toxic effect of CEL-III was observed under any other conditions tested (Fig. 8B).

DISCUSSION

Recent studies (*13, 29*) have indicated that the hemolytic activity of CEL-III involves the following steps: (i) binding of native CEL-III to *N*-acetylgalactosamine- or galactose-containing carbohydrate chains on erythrocyte membranes in monomer form; (ii) conformational change upon the interaction with specific carbohydrates; (iii) oligomerization of CEL-III to form ion-permeable pores; (iv) colloid-osmotic shock leading to hemolysis. Similar mechanism have been postulated for other pore-forming proteins such as staphylococcal α-toxin, streptolysin O, *Tethya* hemolysin from marine sponge, and complement C5b-9 (*17, 26-28*). Since CEL-III has Ca^{2+}-dependent lectin activity as well as hemolytic activity, it may be possible that the hemolytic activity of CEL-III is more specific to its target cells. In fact, rabbit and human erythrocytes are lysed at very low CEL-III concentrations, whereas CEL-III shows only hemagglutinating activity against chicken and horse erythrocytes even at relatively high concentrations (*12*). In the present study, we found that CEL-III exhibits a toxic effect on several cell lines, albeit with greatly varying efficiency. Among the cell lines tested, high susceptibility to CEL-III cytotoxicity was observed in MDCK, HeLa, and Vero cells, whereas XC, CHO, and PtK_1 cells were relatively resistant. The LD_{50} value of CEL-III in most sensitive MDCK cells was 53 ng/ml, whereas no significant toxic effect was observed in CHO cells up to 10,000 ng/ml. Since lactose effectively inhibited the CEL-III cytotoxicity (Table I), it is suggested that the binding of CEL-III to specific carbohydrate chains on the cell-surface is essential for its cytotoxicity. Binding studies using FITC-labeled CEL-III indicated that the amounts of CEL-III bound to relatively sensitive MDCK, HeLa, and Vero cells were 1.5-2-fold greater than those to CHO, PtK_1, and XC cells, which are resistant to CEL-III cytotoxicity. 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 differences in LD_{50} values between MDCK and CHO cells (>180-fold). The interaction between CEL-III and the cell membrane leading pore-formation may be the key step determining the susceptibility to CEL-III cytotoxicity, rather than binding efficiency. 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. Probably membrane repair mechanisms may exist that are more effective in CHO cells than MDCK cells. Further work is necessary to resolve this problem.

Interestingly, the cytotoxic activity of CEL-III was highly temperature-dependent and the cytotoxicity of CEL-III increased with decreasing temperature. In Vero cells, LD_{50} of CEL-III at 4°C was 34 ng/ml, while at 37°C no toxic effect was observed up to 10,000 ng/ml (Fig. 3) after 2 h exposure to CEL-III. The temperature-dependent characteristic of CEL-III action was also observed in MDCK, HeLa, PtK_1 cells (Fig. 4), whereas CHO and XC cells were not killed by CEL-III at any temperature tested under these conditions. CEL-III may require a longer incubation time than 2 h to produce a toxic effect on the latter cell lines even at 4°C. Kinetic analysis regarding incubation time demonstrated that the surviving fractions of Vero cells exposed to CEL-III at 4°C decreased much more rapidly than at 37°C, suggesting that CEL-III can induce lethal damage on the target cell membranes at a faster rate at lower temperature (Fig. 5). These results are consistent with the finding that the hemolytic activity of CEL-III is dependent on temperature; the highest activity was observed at 10°C, but the activity decreased with increasing temperature (*13*). After the binding to specific carbohydrates on the cell surface, the processes leading to ion-permeable pore formation may be extremely temperature-dependent. Although the details of the mechanism of pore-formation by CEL-III are still unclear, immunoblotting analysis of proteins from the erythrocyte membrane after treatment with CEL-III demonstrated that CEL-III oligomers were irreversibly bound to the membrane, suggesting that CEL-III monomers bound to cell-surface carbohydrates spontaneously oligomerize in the membrane

to form ion-permeable transmembrane pores (*13*). Since oligomer formation of CEL-III has been observed when CEL-III was incubated with SDS (*13*), hydrophobic interaction with the membrane lipid-bilayer may trigger the formation of CEL-III oligomers. CEL-III pore formation may be caused by binding to specific carbohydrates and subsequent conformational change in the hydrophobic environment at the surface of the membrane. This hypothesis is supported by the recent finding (*29*) that the surface hydrophobicity of CEL-III increases with the binding of specific carbohydrates such as lactose under certain conditions, and this change was accompanied by the formation of a CEL-III oligomer.

Another characteristic feature of CEL-III is pH dependence. It has been shown that the hemolytic activity of CEL-III increases with increasing pH from neutral to 10, but almost no hemolysis is observed at acidic pH. In agreement with these findings, it was found that CEL-III induced the release of carboxyfluorescein (CF) from CF-loaded cells to a greater extent at pH 10 than at pH 7.5 in both MDCK and CHO cells, albeit MDCK cells were more sensitive than CHO cells (Fig. 7). This activity was also temperature-dependent and stronger activity was observed at 4°C than at 37°C, as with the cytotoxic activity described above, suggesting that the release of CF is compatible with the cytotoxic activity of CEL-III. In addition, the results showed that even CHO cells become sensitive to CEL-III cytotoxicity at 4°C and at pH 10, while no toxic effect of CEL-III was observed in CHO cells under any other conditions tested (Fig. 8). These results suggest that the biological activity of CEL-III through the cell membrane is markedly facilitated at higher pH and at lower temperature. In contrast to CEL-III, it has been shown that membrane damage through pore-formation by Staphylococcal α-toxin is enhanced at low pH (*30, 31*). Some other protein toxins also appear to require acidic pH for their activity. For example, the facilitation of membrane penetration of protein toxins in acidic pH has been observed for diphtheria toxin (*32, 33*) and tetanus toxin (*34*). Low pH is also required by certain viruses to penetrate the cell membrane and induce membrane fusion (*35-37*). These viruses and diphtheria toxin have all been shown to enter cells through the receptor-mediated endocytotic pathway. A unique conformational change of CEL-III in a hydrophobic environment may be responsible for its characteristic behavior in the interaction with the cell membrane leading to pore formation, which is not seen with other protein toxins. Because of these characteristic features, CEL-III may become a useful tool for controlled permeabilization of cell membranes, as has already been proposed for other pore-forming protein toxins (*38*). Although the role of CEL-III in the organism is still unclear, CEL-III may be involved in defense mechanisms through directly acting on invading pathogens as a toxic protein. Further studies are required to elucidate in more detail the mechanism of pore formation of CEL-III, as well as its role in the organisms.

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