INFECTION AND IMMUNITY, Mar. 1976, p. 876-883
Copyright © 1976 American Society for Microbiology
Vol. 13, No. 3
Printed in U.S.A.

Cytotoxic Effect of the Thermostable Direct Hemolysin Produced by *Vibrio parahaemolyticus* on FL Cells

JUN SAKURAI, TAKESHI HONDA, YOKO JINGUJI, MICHIKO ARITA, AND TOSHIO MIWATANI*

Department of Bacteriology and Serology, and Laboratory for Culture Collection, Research Institute for Micribial Diseases, Osaka University, Yamada-kami, Suita, Osaka, Japan*

Received for publication 26 September 1975

The thermostable direct hemolysin produced by *Vibrio parahaemolyticus* showed cytotoxic activity on FL cells derived from human amniotic membrane. Scanning electron micrographs of the whole cells showed that the microvilli on the cell surface decreased in number and changed in shape on treatment with the hemolysin. Most of the microvilli disappeared before death of the cells, as judged from the results of staining the cells with trypan blue and measuring release of alkaline phosphatase from the cells. Electron micrographs of thin sections of the cells showed that the cytoplasm of the cells was not significantly affected by treatment with sublethal amounts of hemolysin, even when the microvilli on the cell surface were significantly affected. Lethal amounts of hemolysin affected the cytoplasm and caused disappearance of the nucleus. These results suggest that the microvilli on the cell surface are affected by treatment with the hemolysin before cytotoxic effects develop.

The thermostable direct hemolysin of *Vibrio parahaemolyticus* is produced only by Kanagawa phenomenon-positive strains (23), and its close relation with human pathogenicity has been demonstrated (17). The hemolysin has been isolated and purified extensively and its physicochemical properties have been characterized (9, 22, 27). It lyses human but not horse erythrocytes (16, 25), and the process of its lytic effect on human erythrocytes has been characterized (21).

We have reported the lethal activity of the hemolysin to small experimental animals (9), and we have also demonstrated the cardiotoxicity of the hemolysin by electrocardiographic studies and by using cultured mouse heart cells (7). This paper reports studies on the cytotoxic activity of the hemolysin on FL cells and on the morphological changes of FL cells treated with lethal and sublethal amounts of the hemolysin.

MATERIALS AND METHODS

Preparation of thermostable direct hemolysin. The thermostable direct hemolysin of *V. parahaemolyticus* WP-1 was purified as described previously (16, 22).

Culture of FL cells. FL cells, provided by Y. Okada of this Institute, were cultured in Eagle minimal essential medium (MEM) supplemented with 10% calf serum at 37 C for 5 days. The cells were harvested by replacing the culture medium with a solution containing 0.25% trypsin in 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl (phosphate-buffered saline) for 10 min at 37 C to allow the cells to become detached from the glass and then centrifuging the suspension at 1,000 rpm for 5 min. The precipitated cells were then suspended in Eagle MEM at a final concentration of approximately 6×10^5 cells per ml.

Scanning electron micrography. The FL cells were fixed in 0.1 M phosphate buffer (pH 7.0) containing 1% glutaraldehyde for 1 h at 0 C and then in 0.1 M phosphate buffer (pH 7.0) containing 5% glutaraldehyde for 10 min at 0 C, and postfixed for 10 min in 1% osmium tetroxide (pH 7.0) (15). Then the cells were washed with 0.1 M phosphate buffer (pH 7.0) and dehydrated by passage through a graded series of increasing concentrations of ethanol. To avoid distortions caused by changes in surface tension of the cells on transfer from liquid to air, the cells were treated in a critical-point drying apparatus (Hitachi HCP-1). The cells were examined with a Hitachi HSM-2A scanning electron microscope operated at 20 kV.

Electron micrography. The cells were fixed in glutaraldehyde and osmium tetroxide, and dehydrated in ethanol as described above. Then they were embedded in Epon 812 (13) and thin sections were stained with uranyl acetate (10) and lead citrate (19). Electron micrographs were taken with a Hitachi HU-11DS electron microscope.

Other procedures. Activity of alkaline phosphatase released from FL cells (6, 8) was determined by the method of King and Armstrong (12). Viability of FL cells was measured with trypan blue. For this, 0.1 ml of cell suspension was mixed with 0.2 ml of 0.5% trypan blue solution (14). Then the preparation was observed with a hemocytometer and the per-

centage of cells stained with trypan blue was determined.

RESULTS

Effect of the thermostable direct hemolysin on the viability of FL cells. The viability of FL cells treated with the thermostable direct hemolysin was studied by trypan blue staining. As shown in Fig. 1, when 6.6×10^5 cells were incubated with 5 μg of the hemolysin in 1 ml of the medium at 37 C, death of the cells occurred after 20 min and the percentage of cells stained with trypan blue increased with time to a maximum value after about 60 min.

The effect of the hemolysin on the release of alkaline phosphatase from FL cells was also studied (Fig. 1). Release of alkaline phosphatase occurred in parallel with death of the cells. When 6.6×10^5 cells were sonicated and centrifuged, about 600 units of alkaline phosphatase activity were found in the supernatant fluid. On the other hand, when the same number of cells had been treated with 5 μg of the hemolysin, only 108 units of alkaline phosphatase were found in the supernatant. However, the time course of release of alkaline phosphatase from the cells was quite similar to that of death of the cells measured by trypan blue staining, indicating that the release of alkaline phosphatase from the cells can be used as a parameter of cellular damage by the hemolysin (Fig. 1).

The cytotoxic effect of the hemolysin on FL cells was compared to its hemolytic effect on human erythrocytes (Fig. 2). Cytotoxicity was measured as the release of alkaline phosphatase from the cells. When 6.6×10^5 cells were treated with less than 0.5 μg of the hemolysin in 1 ml of the medium, no cytotoxicity was

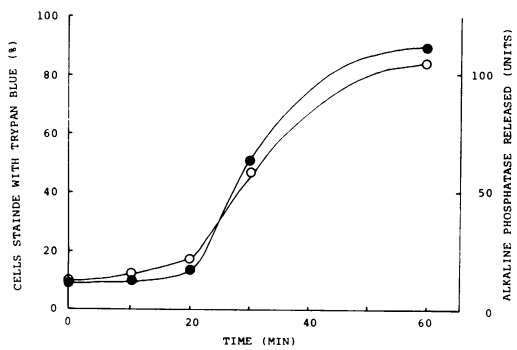

FIG. 1. *Effect of the hemolysin on FL cells. Five micrograms of the hemolysin and FL cells (6.6×10^5 cells) was incubated in 1 ml of Eagle MEM at 37 C. At the times indicated, the percentage of cells stained with trypan blue (●) and the amount of alkaline phosphatase released (○) were measured as described in the text.*

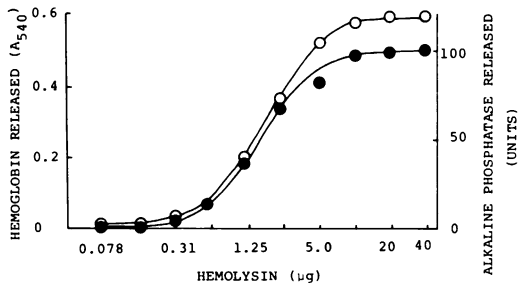

FIG. 2. *Effect of various amounts of the hemolysin on FL cells and human erythrocytes. Various concentrations of the hemolysin were incubated with either FL cells (6.6×10^5 cells) or human erythrocytes (6.4×10^7 cells) and the effect of the hemolysin was measured. The cytotoxicity on FL cells was assayed as release of alkaline phosphatase (●) as described in the text. The hemolytic activity of the hemolysin (○) was measured by incubating the mixture at 37 C for 60 min, centrifuging it at 3,000 rpm for 10 min, and measuring the absorbance of the supernatant fluid at 540 nm.*

observed, but at concentrations of more than 1 μg of the hemolysin, cytotoxicity was proportional to the amount of hemolysin added and maximal cytotoxicity was observed with 5 to 10 μg of the hemolysin. When 6.4×10^7 human erythrocytes were incubated with less than 0.5 μg of the hemolysin, no significant hemolysis was observed and maximal hemolysis was observed with 5 to 10 μg of the hemolysin. The patterns of the dose-response curves in these two experiments were very similar (Fig. 2).

Morphological changes of FL cells treated with the hemolysin. Morphological changes of FL cells induced by treatment with the hemolysin were examined by scanning electron microscopy. Control cells (Fig. 3A) were seen to be completely covered with microvilli. When the cells were treated with the hemolysin (5 μg of the hemolysin per 6.6×10^5 cells) at 37 C, the most marked change was in the morphology of these microvilli. After 5 min of incubation, the number of microvilli on the cell surface had decreased and their shape had also altered significantly (Fig. 3B). After 20 min of incubation, almost all the microvilli had disappeared (Fig. 3C). Under these conditions, the cells were not killed as judged from the results of trypan blue staining and release of alkaline phosphatase (Fig. 1). After 60 min of incubation, more than 95% of the cells had been killed and degradation of the cell surface was observed (Fig. 3D).

Next the morphological changes of FL cells were studied by electron microscopy. In thin sections of control cells, microvilli were covering the surface of the cells (Fig. 4). When 6.6×10^5 cells were treated with 5 μg of the hemoly-

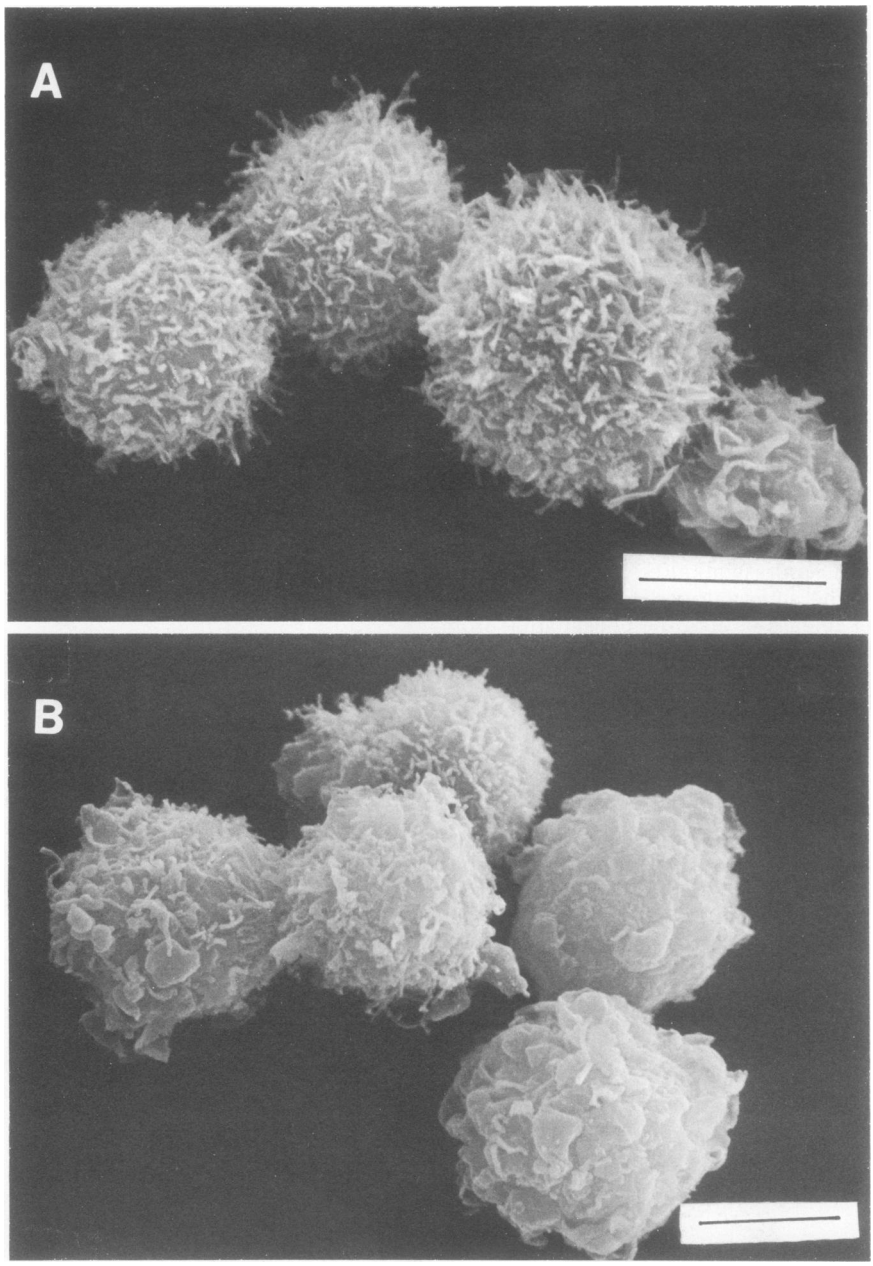

FIG. 3. *Scanning electron micrographs of FL cells treated with the hemolysin. Five micrograms of the hemolysin and FL cells (6.6 × 10^5 cells) was incubated at 37 C in 1 ml of Eagle MEM and the hemolysin-treated cells were harvested by centrifugation (800 rpm, 5 min). Samples for scanning electron microscopy were prepared as described in the text. (A) FL cells incubated at 37 C for 60 min in the absence of the hemolysin; (B) cells incubated with the hemolysin for 5 min, (C) 20 min, and (D) 60 min. The bar represents 10 μm.*

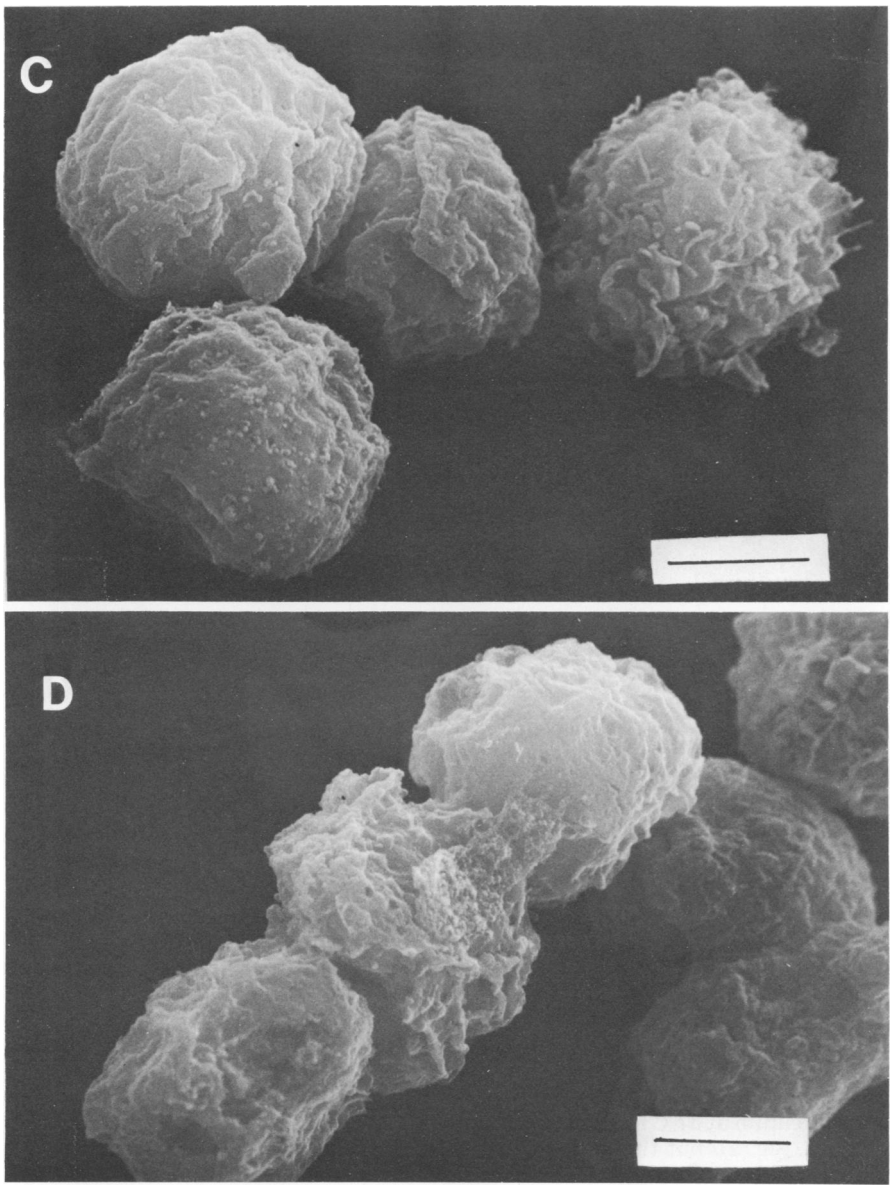

FIG. 3. C-D

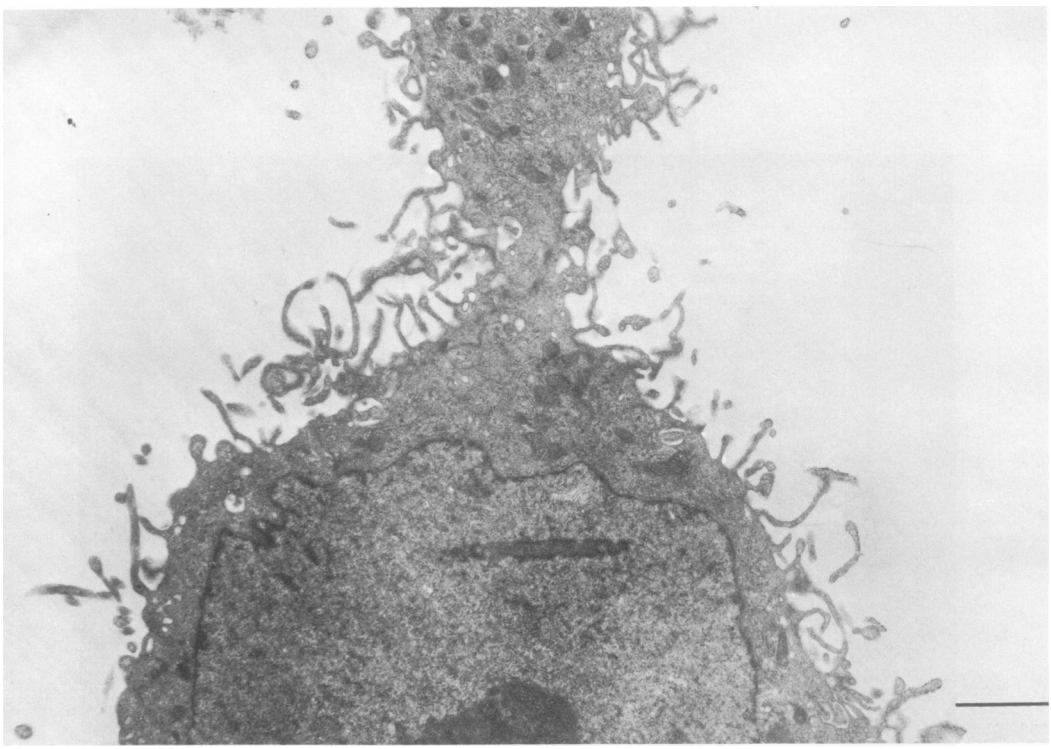

FIG. 4. *Electron micrograph of FL cells. The sample was prepared as described in the text. The bar represents 1 μm.*

sin in 1 ml of the medium at 37 C for 5 min, significant changes were observed in the shape of their microvilli, but no significant changes were observed in their cytoplasm (Fig. 5A). After 30 min of incubation, an electron micrograph showed the disappearance of microvilli as well as significant changes of the cytoplasm and disintegration of the nucleus (Fig. 5B), and after 60 min of incubation, complete degradation of the cytoplasm and loss of the nucleus were observed (Fig. 5C).

Morphological changes of the microvilli on the cell surface were also observed when the cells were treated with sublethal amounts of the hemolysin. For instance, when 6.6×10^5 cells were treated with 0.5 μg of the hemolysin in 1 ml of the medium at 37 C for 60 min, no cell death occurred (Fig. 2), but the number of microvilli on the cell surface decreased significantly as shown by both scanning electron microscopy (Fig. 6A) and electron microscopy (Fig. 6B). Under these conditions no significant changes were observed in the cytoplasm of the cells (Fig. 6B).

DISCUSSION

The present data showed that the thermostable direct hemolysin of *V. parahaemolyticus* has cytotoxic activity on FL cells. Scanning electron micrographs of whole cells and electron micrographs of thin sections of the cells showed that morphological changes of the microvilli covering the surface of the cells were the primary changes induced by the hemolysin. These changes were followed by degradation of the cytoplasm and nucleus. The latter changes might be due to death of the cells, but the changes in the microvilli occurred on treatment with sublethal amounts of the hemolysin.

Several workers have reported that hemolysins produced by various kinds of microorganisms affected various kinds of cells (1–5, 11). The hemolysin of *V. parahaemolyticus* affected not only FL cells but also other cultured cells, such as HeLa cells (20; Sakurai et al., unpublished observations), L cells (20), Ehrlich tumor cells (Sakurai et al., unpublished observations), and cultured mouse heart cells (Goshima et al., manuscript in preparation). Among these cells, Ehrlich tumor cells were the most resistant to the hemolysin (about 50 times more resistant than FL cells). Different sensitivities of different kinds of erythrocytes to the hemolysin were also reported (16, 25). Previously we demonstrated that hemolysis of human erythrocytes by the hemolysin was initiated by the

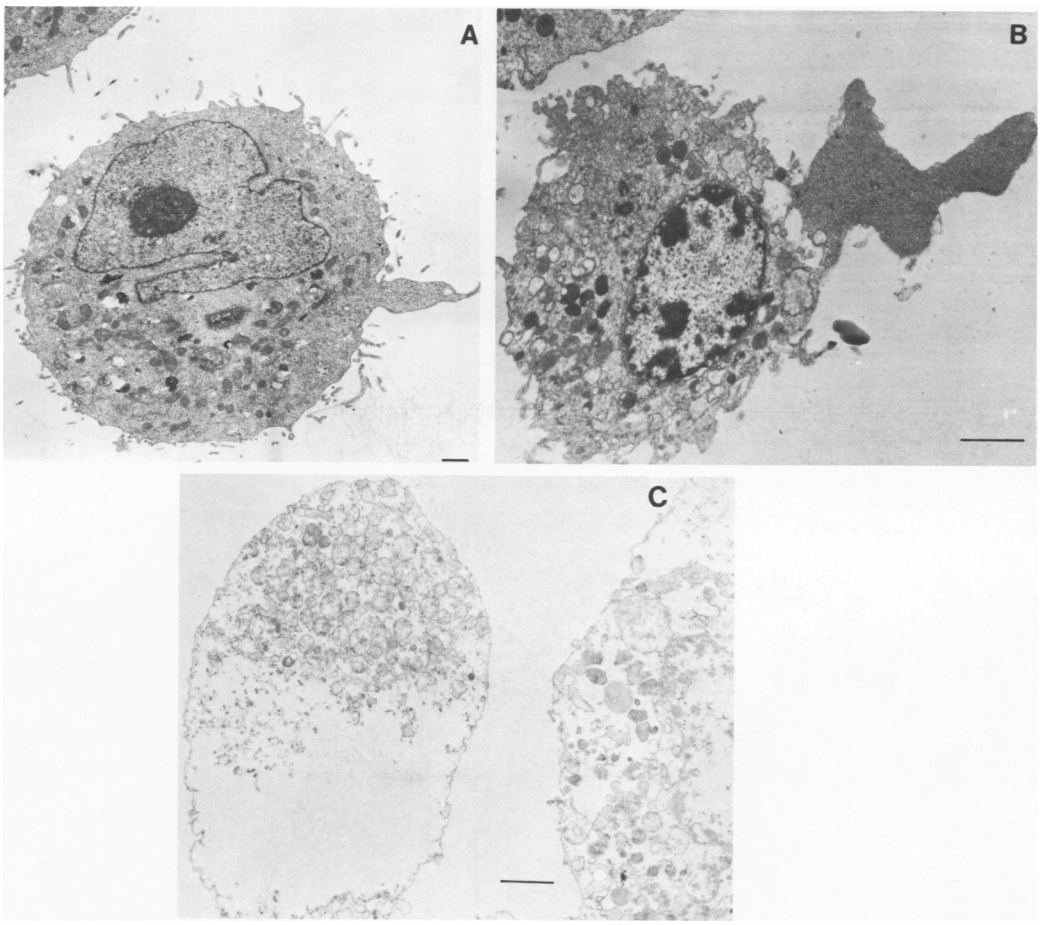

FIG. 5. *Electron micrographs of FL cells treated with the hemolysin. Five micrograms of the hemolysin and FL cells (6.6 × 10^5 cells) was incubated in 1 ml of Eagle MEM at 37 C and the hemolysin-treated cells were harvested. Thin sections of the treated cells were prepared as described in the text. (A) Cells incubated with the hemolysin for 5 min, (B) 30 min, and (C) 60 min. The bar represents 1 μm.*

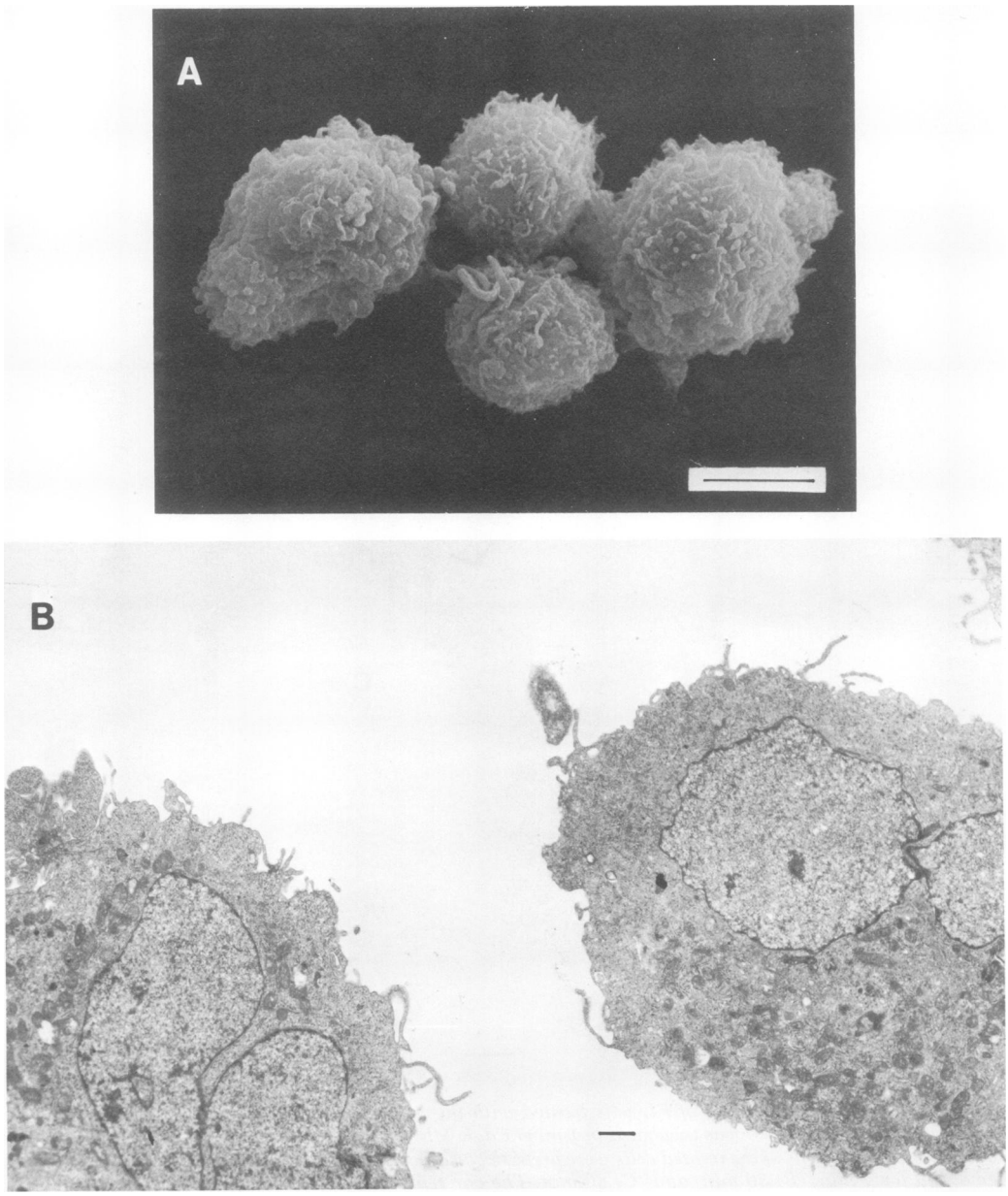

FIG. 6. *Morphological changes of FL cells treated with sublethal amounts of the hemolysin. Five micrograms of the hemolysin and FL cells (6.6 × 10^5 cells) was incubated in 1 ml of Eagle MEM at 37 C and the hemolysin-treated cells were harvested. The samples were prepared as described in the text. (A) Scanning electron micrograph; (B) electron micrograph of a thin section. The bar represents 10 μm in (A) and 1 μm in (B).*

binding of the hemolysin to the cell surface (21) and that the binding site of the hemolysin on the cell surface might be a ganglioside (24). The difference in sensitivities of various cells to the hemolysin may be explained as due to differences in the binding capacities of these cells to the hemolysin, and possibly to differences in the amounts of the ganglioside that binds the hemolysin on their surfaces.

It has been reported that the thermostable direct hemolysin has lethal toxicity (9) and cardiotoxicity (7). Zen-Yoji et al. (26) showed that administration of 500 μg of the hemolysin gave a positive reaction in the rabbit ileal loop test

and proposed that the hemolysin has enterotoxic activity. Obara et al. (18) reported histopathological changes of the cells of the small intestine of suckling mice challenged orally with the purified hemolysin. These observations by Zen-Yoji et al. (26) and by Obara et al. (18) may be explained by the cytotoxic activity of the hemolysin demonstrated in this paper.

ACKNOWLEDGMENTS

We would like to express thanks to T. Wada, T. Watanabe, H. Hidaka, and H. Yamada for help in scanning electron micrography, to Y. Okada for interest and discussion of this work, and to Y. Takeda for help in preparing this manuscript.

LITERATURE CITED

1. **Bernheimer, A. W.** 1968. Cytolytic toxins of bacterial origin. Science 159:847-851.
2. **Bernheimer, A. W.** 1974. Interactions between membranes and cytolytic bacterial toxins. Biochim. Biophys. Acta 344:27-50.
3. **Fauve, R. M., J. E. Alouf, A. Delaunay, and M. Raynaud.** 1966. Cytotoxic effects in vitro of highly purified streptolysin O on mouse macrophages cultured in a serum-free medium. J. Bacteriol. 92:1150-1153.
4. **Gladstone, G. P., and A. Yoshida.** 1966. The cytopathic action of purified staphylococcal δ-hemolysin. Br. J. Exp. Pathol. 48:11-19.
5. **Ginsburg, I., and N. Grossowicz.** 1960. Effect of streptococcal haemolysins on Ehrlich ascites tumor cells. J. Pathol. Bacteriol. 80:111-119.
6. **Higashino, K., S. Kudo, Y. Yamamura, T. Honda, and J. Sakurai.** 1975. Possible identity between the hepatoma alkaline phosphatase and an isozyme of human amniotic membrane (FL cells). Clin. Chim. Acta 60:267-272.
7. **Honda, T., K. Goshima, Y. Takeda, Y. Sugino, and T. Miwatani.** 1976. Demonstration of the cardiotoxicity of thermostable direct hemolysin (lethal toxin) produced by *Vibrio parahaemolyticus*. Infect. Immun. 13:163-171.
8. **Honda, T., T. Kurabori, S. Ishigami and J. Sakurai.** 1973. Alkaline phosphatase of FL cells (in Japanese). Igaku No Ayumi 86:313-314.
9. **Honda, T., S. Taga, T. Takeda, M. A. Hasibuan, Y. Takeda, and T. Miwatani.** 1976. Identification of lethal toxin with thermostable direct hemolysin produced by *Vibrio parahaemolyticus* and some physicochemical properties of the purified toxin. Infect. Immun. 13:133-139.
10. **Huxley, H. E., and G. Zubay.** 1961. Preferential staining of nucleic acid-containing structures for electron microscopy. J. Biophys. Biochem. Cytol. 11:273-296.
11. **Jeljaszewicz, J., S. Szmigielski, M. Korbecki, and C. Zak.** 1965. Histochemical demonstration of changes in enzymatic activity of KB cells produced by staphylococcal alpha and beta hemolysin. J. Infect. Dis. 115:421-428.
12. **King, E. J., and R. Armstrong.** 1934. A convenient method for determining serum and bile phosphatase activity. Can. Med. Assoc. J. 31:376-381.
13. **Luft, J. H.** 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9:409-414.
14. **McLimans, W. F., E. V. Davis, F. L. Glover and G. W. Rake.** 1957. The submerged culture of mammalian cells: the spinner culture. J. Immunol. 79:428-433.
15. **Millonig, G.** 1962. Further observations on a phosphate buffer for osmium solutions in fixation, electron microscopy, p. 8. *In* S. S. Bresses (ed.), 5th Int. Congr. electron microscopy, Vol. 2. Academic Press Inc., New York.
16. **Miwatani, T., J. Sakurai, A. Yoshihara, and Y. Takeda.** 1972. Isolation and partial purification of thermolabile direct hemolysin of *Vibrio parahaemolyticus*. Biken J. 15:61-66.
17. **Miyamoto, Y., T. Kato, Y. Obara, S. Akiyama, K. Takizawa, and S. Yamai.** 1969. In vitro hemolytic characteristic of *Vibrio parahaemolyticus:* its close correlation with human pathogenicity. J. Bacteriol. 100:1147-1149.
18. **Obara, Y., S. Yamai, T. Nikkawa, Y. Miyamoto, M. Ohashi, and T. Shimada.** 1974. Histopathological changes in the small intestine of suckling mice challenged orally with purified hemolysin from *Vibrio parahaemolyticus*, p. 253-257. *In* T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda (ed.), International symposium on *Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
19. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17:208-212.
20. **Sakazaki, R., K. Tamura, A. Nakamura, T. Kurata, A. Ghoda, and Y. Kazuno.** 1974. Enteropathogenic activity of *Vibrio parahaemolyticus*, p. 231-235. *In* T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda (ed.), International symposium on *Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
21. **Sakurai, J., M. A. Bahavar, Y. Jinguji, and T. Miwatani.** 1975. Interaction of thermostable direct hemolysin of *Vibrio parahaemolyticus*. Biken J. 18:187-192.
22. **Sakurai, J., A. Matsuzaki, and T. Miwatani.** 1973. Purification and characterization of thermostable direct hemolysin of *Vibrio parahaemolyticus*. Infect. Immun. 8:775-780.
23. **Sakurai, J., A. Matsuzaki, Y. Takeda, and T. Miwatani.** 1974. Existence of two distinct hemolysins in *Vibrio parahaemolyticus*. Infect. Immun. 9:777-780.
24. **Takeda, Y., T. Takeda, T. Honda, J. Sakurai, N. Ohtomo, and T. Miwatani.** 1975. Inhibition of hemolytic activity of thermostable direct hemolysin of *Vibrio parahaemolyticus* by ganglioside. Infect. Immun. 12:931-933.
25. **Zen-Yoji, H., H. Hitokoto, S. Morozumi, and R. L. LeClair.** 1971. Purification and characterization of a hemolysin produced by *Vibrio parahaemolyticus*. J. Infect. Dis. 123:665-667.
26. **Zen-Yoji, H., Y. Kudoh, H. Igarashi, K. Ohta, and K. Fukai.** 1974. Purification and identification of enteropathogenic toxins "a" and "a'" produced by *Vibrio parahaemolyticus* and their biological and pathological activities, p. 237-243. *In* T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda (ed.), International symposium on *Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
27. **Zen-Yoji, H., Y. Kudoh, H. Igarashi, K. Ohta, K. Fukai, and T. Hoshino.** 1974. Further studies on characterization and biological activities of an enteropathogenic toxin of *Vibrio parahaemolyticus*. Proc. 4th Int. Symp. Animal Plant Microbiol. Toxins, Tokyo.