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A Role of Protease Produced by *Vibrio cholerae* in Its Adherent Mechanism

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Abstract: The relationship between protease activity *in vitro* and the degree of colonization of *Vibrio cholerae* in the ileal loop test was investigated using 35 strains of *V. cholerae* (biotype classical, El Tor and non-O1 *V. cholerae*, 10, 15, and 10 respectively). The mean protease activity in non adherent group was 9.15 mm, higher than that in adherent (+) and (++) group, 6.74mm and 6.19mm, respectively in agar-gel single-diffusion method. The difference between the mean protease activity in non-adherent and adherent (++) groups was statistically significant (T-test, $p<0.05$). This suggests that the amount of protease produced *in vitro* does not necessarily correlate with the adherent potential of the vibrios to the intestinal epithelial cells.

Key words: *Vibrio cholerae*, Protease activity, Colonization factor

Extracellular proteases have been thought to play an important role in the pathogenesis of diarrheal disease caused by *Vibrio cholerae*. Protease-deficient mutants are known to be less virulent (Schneider and Parker, 1978). Pathogenic *V. cholerae* produce a hemagglutinin/protease with the ability to activate the A subunit of cholera enterotoxin and cleave other physiologically important substrates, including mucin, fibronectin, and lactoferrin (Finkelstein *et al.*, 1983).

Furthermore, its mucinase activity could also be important in allowing penetration of the mucous layer which may be essential for successful colonization of cholera vibrio in the intestine. Thus, we investigated the relationship between protease activity and the degree of colonization of *V. cholerae* in the ileal loop test to clarify the role of protease in the mechanism of adherence.

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Ten strains of *Vibrio cholerae*, biotype classical, 15 strains of *V. cholerae*, biotype El Tor and 10 strains of *V. cholerae* non-O1 were used in this study. Bacteria were cultured in 2 ml heart infusion broth at 37℃ overnight with resting. Rabbits each weighing 2−3 kg were starved but provided water for 48 h prior to challenge. Rabbits were anesthetized intramuscularly with ketamine hydrochloride and intravenously with pentobarbital. One tenth ml bacterial culture (2×10^7 bacteria) was inoculated into each loop. Control loops were injected with 0.5 ml of normal saline solution. The rabbits were killed and the intestine was removed 13h after inoculation. The length of each loop was measured and the volume of fluid accumulated in the loop was recorded. The extent of fluid accumulation (FA) was expressed by the FA ratio as the volume (in milliliters) of accumulated fluid per length (in centimeters) of the loop. The resected rabbit ileal loops were opened under a normal saline solution and 1.5cm segments of each loop were removed. The pieces of intestine were washed by agitation consecutively in three beakers of normal saline solution to remove debris and free bacteria, not firmly bound to the surface of epithelial cells. The samples were fixed with 2% glutaraldehyde in PBS (10 mM, pH 7.0), then washed with PBS and post-fixed with 2% osmium tetroxide solution. The samples were replaced into iso-amyl acetate overnight after dehydration with a series of alcohol, dried by a critical point drying method and coated with platinum-palladium.

Comma-shaped organisms with unipolar flagellum attached to the surface of villi were counted with a JOEL-100CX scanning electron microscope. The criteria to show the degree of attachment of organism are as follows: numerous organisms were easily found attached on the surface of villi (++), several organisms were found (+), and no organism was found (−) (Fig. 1a & 1b).

Protease activity was detected by using a single-diffusion technique in agar gel (0.75%) containing skim milk (1.5%) as a substrate as previously described by Honda, *et al.* (1987). Becteria were inoculated in 50 ml tryptic soy broth (Difco Laboratories) in 300ml Erlenmeyer flasks and cultured with shaking at 30℃ for 20 h. Culture supernatants obtained by centrifugation at 40,000xg, for 20 min were used as the sample. A sample solution (20 μl) was added to wells 3.5 mm in diameter, and plates were incubated for 12 h at 37℃. Zones of clearing were measured.

The attached organisms were counted and all the strains were categorized into three groups, i.e., non-adherent, adherent (+) and adherent (++) as described before. No organisms were found in the specimen inoculated with normal saline solution. Fig. 2 gives a comparison of FA ratio in each group. The mean FA ratio in the rabbit ileal loop test in the non-adherent group was 0.52 and lower than that in the adherent (+) and (++) group, 0.95 and 0.97, respectively (Table 1). The difference between the mean FA ratio in each group, however, was not statistically significant (T-test). The FA ratio can be separated into two groups, more than 1.2 and less than 0.9. Fig. 3 shows the distribution of protease activities produced by *V. cholerae* in each group. The mean protease activity in the non-adherent group is 9.15 and higher than that in the adherent (+) and (++) groups, 6.74 and 6.19, respectively (Table 1). The difference between the mean protease activity

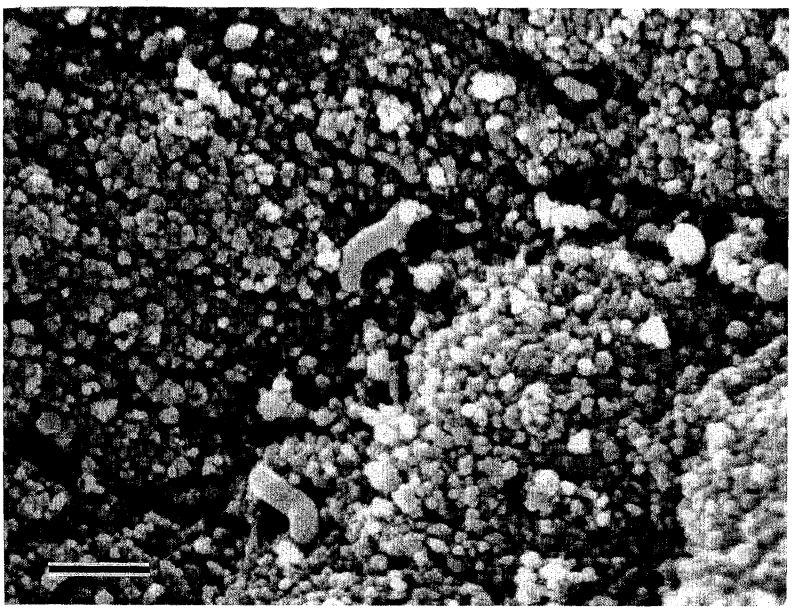

Fig. 1a. Scanning electron micrograph of *V. cholerae* classified adherent (+) Bar represent 1μm.

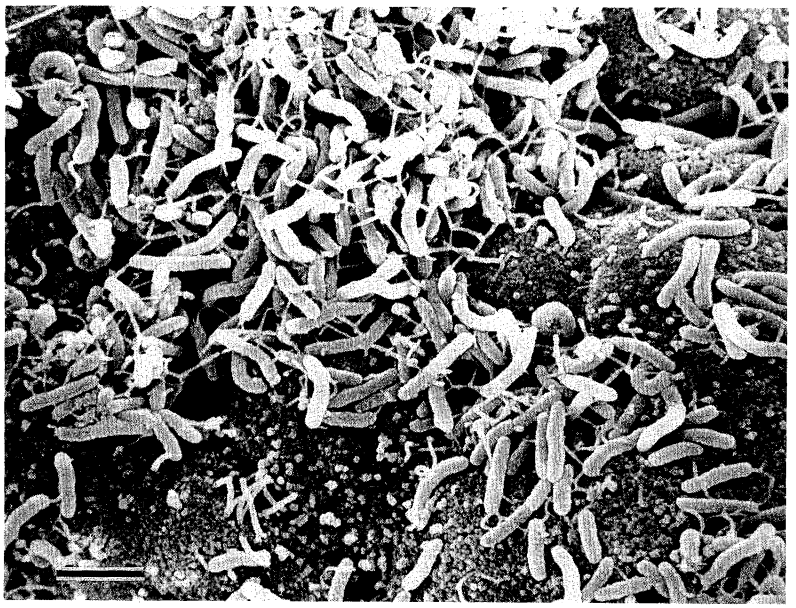

Fig. 1b. Scanning electron micrograph of *V. cholerae* classified adherent (++) Bar represents 1μm.

Table 1. FA ratio and protease activity (PA) in each group

group	(No. of strains)	FA ratio	PA
non adherent	(5)	0.22±0.56	9.15±2.24
adherent (+)	(21)	0.95±0.61	6.74±2.95
adherent (++)	(9)	0.97±0.56	6.19±1.04

Value represent means ± standard deviations.
FA ratio: ml/cm, PA:diameter (mm) of translucent zone, adherent (+): several organisms were found attached on the surface of villi, adherent (++): numerous organisms were found.

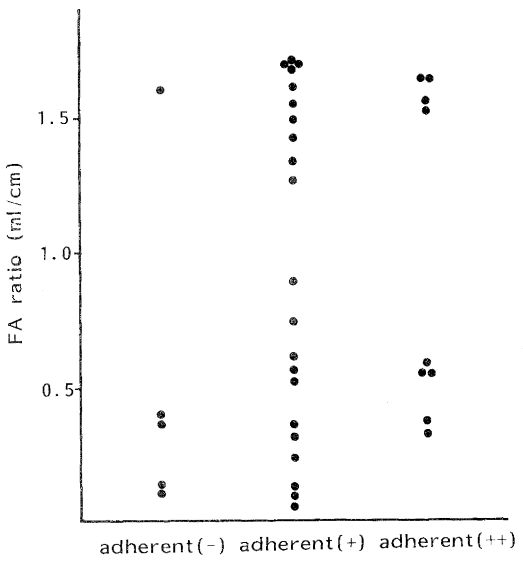

Fig. 2. The comparison of FA ratio in each group.

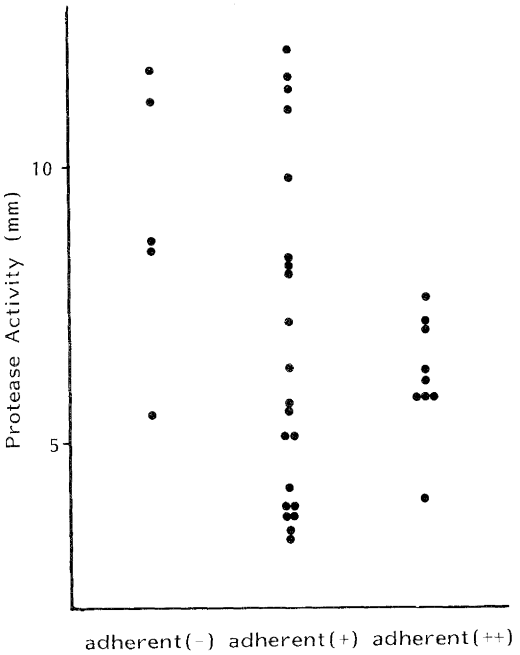

Fig. 3. The comparison of protease activity in each group.

in the non-adherent and adherent (++) groups was statistically significant (T-test, $p<0.05$), but not that between the non-adherent and adherent (+) groups or between the adherent (+) and adherent (++) groups. This means that the strains of *V. cholerae* in the non-adherent group show higher protease activity than the strains in the adherent (++) group, or that the amount of protease produced does not correlate with the adherent potential of the vibrios to the intestinal epithelial cells. On the other hand, no significant correlation was seen between fluid accumulation and protease activity (Ichinose *et al.*, 1987). These facts support the conclusion that neither the hemagglutinin/protease nor the cell associated hemagglutinin are discriminating pathogenic characteristics, although these factors may contribute to virulence as perviously reported by Booth and Finkelestein (1986).

Protease activity was assayed *in vitro* to exclude the effect of indigenous protease and protease inhibitors in the intestine. Therefore, the possibility that the protease activity *in vitro* might not reflect its activity *in vivo* cannot be excluded. A certain amount of protease might be required to destroy the mucosal layer in the intestine as the first step of colonization but high protease activity might promote detachment of *V. cholerae* from the intestinal epithelial cells in the ileal loop test. From this study it is suggested that protease is not an essential factor for colonization but factors other than protease such as fimbriae which was purified by Ehara *et al.* (1987) may play an important role in the association of *V. cholerae* with the small intestinal surface. It might be, of course, unreasonable to make the generalization because of the limited number of strains of *V. cholera* tested, and it is virtually important to reconsider the system to assess the phenomenon of adherence, for example, incubation period in ileal loop test, although the abdomen of rabbit was opened 13 h after inoculation in this study, and to use infant rabbit model as an unligated intestinal experimental animal model.

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REFERENCES

1) Booth, B.A. & Finkelstein R.A. (1986): Presence of the HA/protease and other potential virulence factors in O1 and non-O1 *Vibrio cholerae*. J. Infect. Dis., 154, 183−186.

2) Ehara, M., Ishibashi, M., Ichinose, Y., Iwanaga, M., Shimotori, S. & Naito, T. (1987): Purification and partial characterization of fimbriae of *Vibrio cholerae* O1. Vaccine, 5, 283−288.

3) Finkelstein, R.A., Finkelstein, M.B. & Holt, P. (1983): *Vibrio cholerae* hemagglutinin/lectin/protease hydrolyzes fibronectin and ovomucin: F.M. Burnet revisited. Proc. Natl. Acad. Sci. USA, 80, 1092−1095.

4) Honda, T., Booth, B.A., Finkelstein, M.B. & Finkelstein, R.A. (1987): Comparative study of *Vibrio cholerae* non-O1 protease and soluble hemagglutinin with those of *Vibrio cholerae* O1. Infect. Immun., 55, 451−454.

5) Ichinose, Y., Supawat, K., Ishibashi, M., Ehara, M. & Naito, T. (1987): A study on the enterotoxic factors in *Vibrio cholerae* infection. Trop. Med., 29 (4), 211−219.

6) Schneider, D.R. & Parker, C.D. (1978): Isolation and characterization of protease-deficient mutants of *Vibrio cholerae*. J. Infect. Dis., 138, 143−151.

コレラ菌の定着性とそのプロテアーゼ活性との関連について

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コレラ菌感染における腸管上皮定着性とプロテアーゼ活性との関連を明らかにするためにクラシック型，エルトール型及び non-O1 コレラ菌，各々10，15，10株を用いてプロテアーゼ活性及び成熟ウサギループテストにおける投与後13時間の腸管上皮定着性を検討した．その結果非定着菌群のプロテアーゼ活性は高度定着菌群のそれより高い値を示しその平均値の差は有意であった（T-test，$p<0.05$）即ち，in vitro でのプロテアーゼ産生量はコレラ菌の小腸上皮における定着の程度とは必ずしも相関しないことが示唆された．

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