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Observation of Cholera Toxin in *Vibrio cholerae* by Using Enzyme Labeled Antibody

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Abstract : Pleomorphic changes were previously observed in *Vibrio cholerae* during toxin production in the syncase medium. These changes are mainly characterized by large round cells, and the release of membrane-enveloped particles following budding and pinching off of the cell walls. To understand the relationship between toxin production and these morphological changes, localization of the toxin in the organism was examined using horseradish peroxidase labeled antibody. Toxin was detected mainly in the periplasmic space of rod shape organism, and occasionally in large round cells, but not in the membrane-enveloped particles. Toxin was also found in the mesosomal structures and outer surface materials in some of these cells. Besides, a densely stained, branching network was observed first time in *V. cholerae* without the fixation with glutaraldehyde.

INTRODUCTION

Vibrio cholerae produces an exotoxin (cholera toxin) which was purified and crystalized by Finkelstein and LoSpalluto in 1972 (6). Although the biological mechanism of toxin action is well understood, the mechanism of toxin production and secretion by the organism needs further investigation. It is well known that the secretion of cholera toxin (CT) is not due to cell lysis but to active transport during cell growth (14). To understand the mechanism of CT secretion, several workers have investigated the morphological changes of *V. cholerae* during toxin production. In 1967, Chatterjee and Das suggested that the budding of small particles from the cell wall could be a process of CT secretion, and that these small particles would contain the toxin (2). But in 1969, Kennedy and Richardson reported a result contradicting the data of Chatterjee and Das. They noted an increase of extracellular surface material during toxin production only in whole-mount preparations, and a reduced ribosome content and increased nuclear area in

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sectional preparations. They also described that no other changes were visible at this time in sectional material (11). Fernandes *et al.* reported morphological changes of *V. cholerae* during CT production under anaerobic conditions in 1977, and stressed the relation of large round cells to CT production (5). In our studies, the release of small membrane-enveloped particles and the presence of large round cells were predominant during CT production (8). On the other hand, it was observed that these morphological changes were also induced by glycin without CT production (9).

The purpose of this study was to clarify the location of CT in *V. cholerae*, especially in membrane-enveloped particles and large round cells by electron microscopy using horseradish peroxidase conjugated anti-choleratoxin antibody.

MATERIALS AND METHODS

Organism: *Vibrio cholerae* 569B, a highly toxigenic strain, was the principal organism used. *Vibrio cholerae* El Tor K-25 was used as a non (or weakly) toxigenic control strain.

Preparation of antibody to CT and its conjugation to horseradish peroxidase (HRP): Rabbits were inoculated subcutaneously with 50 μg of purified CT (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) mixed with Freund's complete adjuvant. Three weeks after the initial dose, an additional 50 μg of CT was injected intramusculary without adjuvant. Sera pooled from these rabbits 2 weeks after the second dose had an antibody titer of 1:10,240 as determined by passive hemagglutination method (PHA). The CT-sensitized sheep erythrocytes used in this assay were kindly supplied by Dr. Ohtomo and Dr. Muraoka of the Chemo-Sero-Therapeutic Research Institute. The IgG fraction of the sera was purified by using ammonium sulfate and DEAE sephadex A-50 gel filtration, and then concentrated to 20 mg/ml. This concentrate had a PHA titer of 1 : 5,120. HRP-IgG conjugates were prepared by the method of Avrameas and Ternynck (1), using HRP (SIGMA Chemical Co., RZ: 3.1). The antibody specificity and enzymatic activity of the conjugate were determined by Ouchterlony double diffusion against CT and by peroxidase reaction of the precipitin band with diaminobenzidine (DAB) and hydrogen peroxide. The antibody titer of this conjugate was 1 : 1,024 as determined by PHA.

Immunoperoxidase staining of CT in *V. cholerae*: The staining was performed by the method described by Kawaoi and Nemoto (10). *V. cholerae* strain 569B was cultured in syncase medium with shaking in a 30°C water bath for 8 hr. 100 ml of the culture was centrifuged at 8,000×g for 15 min. The cell pellet obtained was washed in phosphate buffer (0.1 M, pH 7.3) and then mildly fixed with 0.5% glutaraldehyde for 12 hr at 4°C. The fixed pellet was washed 3 times and resuspended in 1.5 ml of the buffer. 0.5 ml of HRP-IgG conjugate was then added, and the mixture was incubated at 4°C for 5−10 hr. After this reaction, cells were again washed 3 times and suspended in

0.2% DAB in tris buffer (0.05 M, pH 7.6), and incubated at room temperature for 1 hr. Hydrogen peroxide was added to a final concentration of 0.005%. After 5 min, the mixture was diluted with Tris buffer and centrifuged. The pellet obtained was washed 2 more times with phosphate buffer. The washed pellet was then postfixed by 1% osmium tetroxide in phosphate buffer for 2 hr. On the other hand, labeled cells without glutaraldhyde fixation were also prepared.

Preparation of specimens for electron microscopy: The cell pellet fixed with osmium tetroxide was ordinarily dehydrated by serially increased concentrations of alcohol and acetone, and embedded in Epon. Thin sections were cut by Reichert OM2 ultramicrotome with a glass knife. Some of the sections, in addition, were stained by uranyl acetate and lead citrate. They were observed by a JEOL-JEM, 100B electron microscope.

Control samples: The following samples were used as controls. 1. Cells, treated with non-conjugated HRP instead of HRP-IgG conjugate. 2. Cells, treated with non-conjugated anti-CT IgG before treating with HRP-IgG conjugate. 3. Non toxigenic *V. cholerae*, strain K-25. All control samples were examined with and without glutaraldehyde fixation.

RESULTS

Electron-opaque regions resulting from the HRP-IgG conjugate were mainly seen in the periplasmic spaces and cytoplasmic membranes, including mesosomal structures (Fig. 1). The bulging area of the cell wall and the membrane-enveloped particles which were reported in our previous paper were negative for the presence of CT (Fig. 1, Fig. 2). In the large round cells electron dense staining was especially evident at the inner surface of the widened periplasmic spaces, which have an appearance similar to vacuoles (Fig. 3). Generally, CT was more frequently detected in rod shape organisms than in large round cells. Three different patterns of CT staining were noted in the organisms. In one population of cells, most of the toxin was intracellular, with small amounts of toxin on the cell surface (Fig. 4-A). In another group, cells were coated with large amounts of toxin, and intracellular toxin was also present (Fig. 4-B). In a third group of cells, toxin was detected only at the surface of the cell, and the periplasmic space was devoid of staining (Fig. 4-C).

The control study revealed that the samples treated with non-conjugated HRP instead of HRP-antibody conjugate and the samples treated with non-conjugated antibody before treating with HRP-antibody conjugate were negative for staining. Strain K-25, which is a non toxigenic organism, did not show a specific reaction.

Toxin was not detected in the organisms which were not fixed with glutaraldehyde (Fig. 5). A densely stained, branching network with cisterns was observed in this samples, and also in the samples treated with non-conjugated HRP instead of HRP-antibody conjugate, and in the samples from a non toxingenic strain.

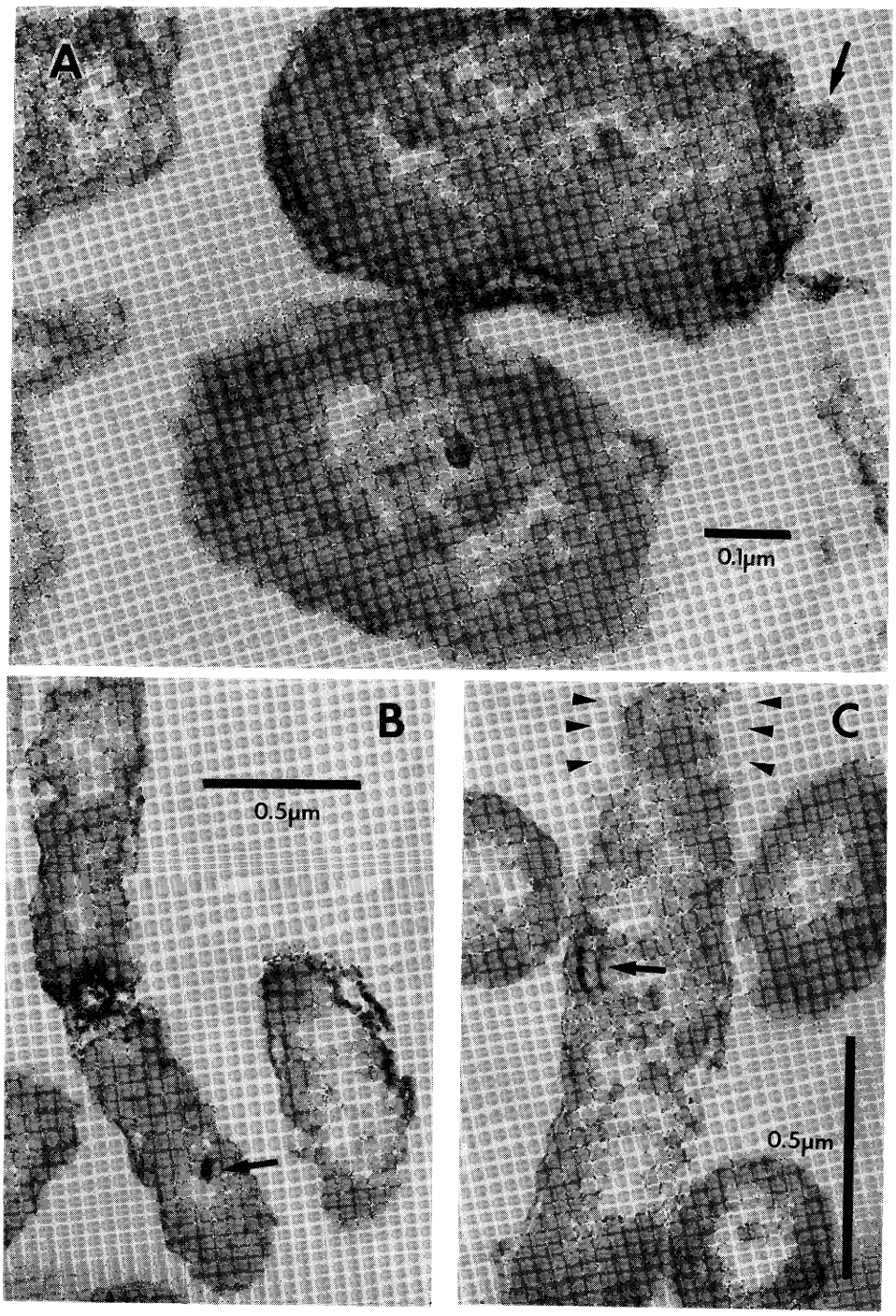

Fig. 1

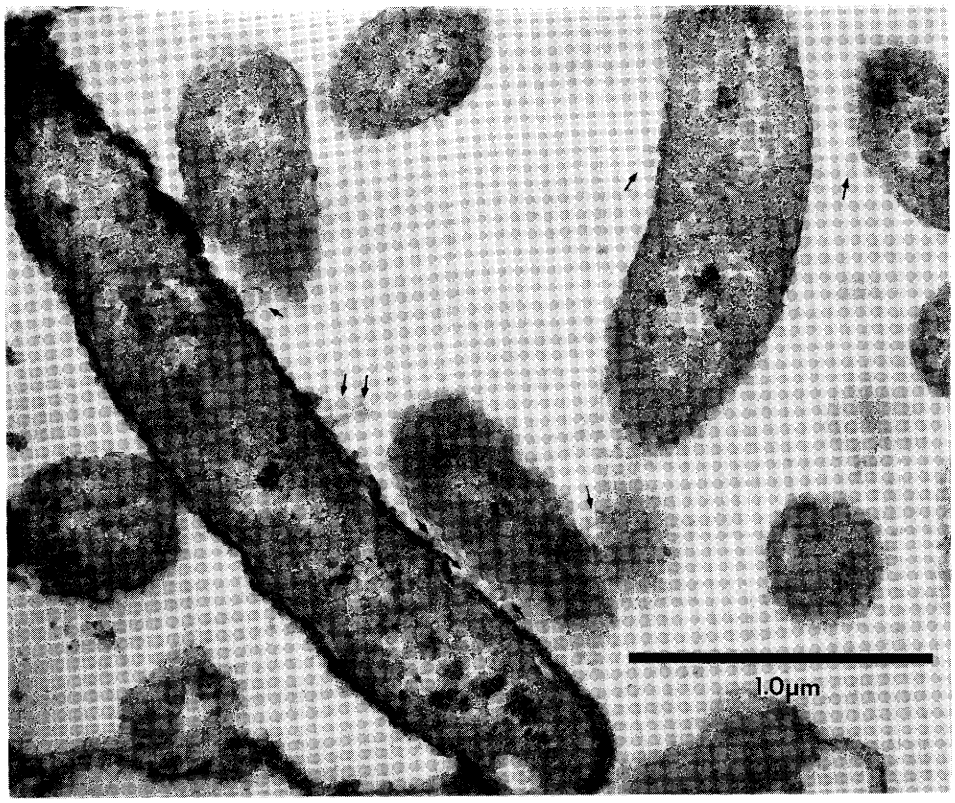

Fig. 2

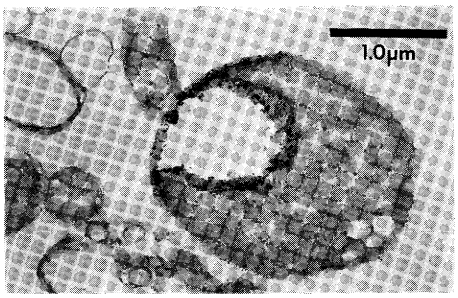

Fig. 3

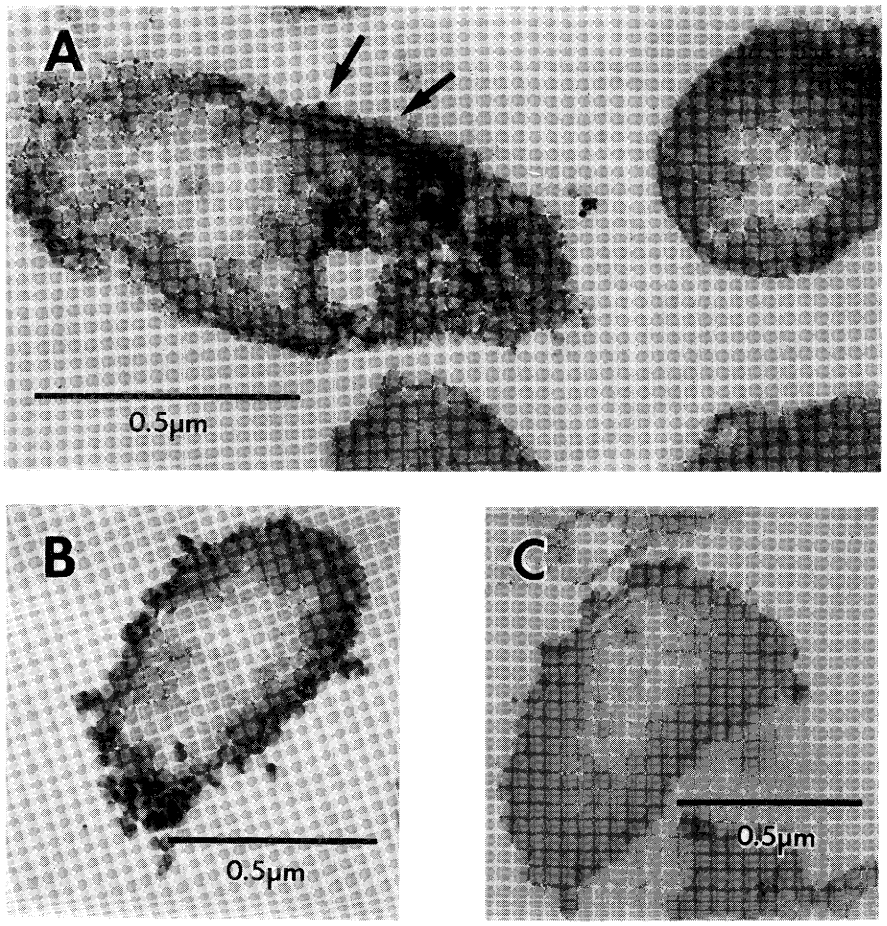

Fig. 4

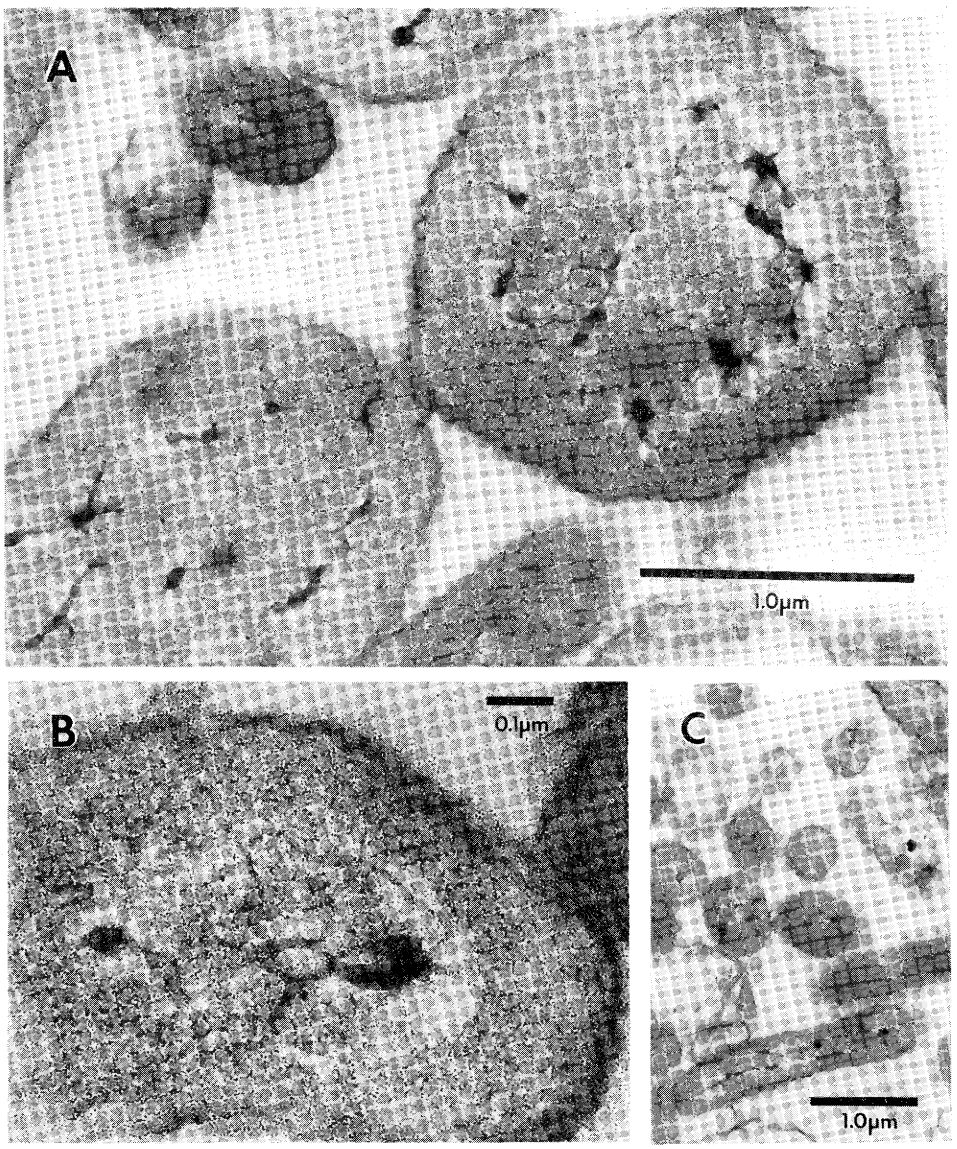

Fig. 5

DISCUSSION

The morphological changes of *V. cholerae* during CT production have been investigated, and it has been suggested that development of large round cells and membrane-enveloped particles originating from the cell wall had an important relation to the secretion of CT (2, 5, 8). The results of this study do not support this hypothesis. By using HRP-conjugated anti-CT antibody, CT was absent in the particles and was more frequently detected in rod shape organism than in large round cells. The membrane-enveloped particles and large round cells might appear during a process of cell degeneration in such suboptimal conditions, and it can be said that they have no relation to CT formation and secretion. The amount of CT in each of the organisms was variable, probably because the phase of CT production was different from cell to cell. CT at the cell surface was seen in only a few cells. It is possible that surface CT was removed during the washing procedures employed after each reaction to remove surplus materials such as glutaraldehyde, HRP-IgG conjugate, DAB, etc. Ohtomo *et al.* (1977) reported that CT subunit A is produced in the cytoplasm, and subunit B is produced at the cytoplasmic membrane or nearby. They may be assembled into a whole CT molecule during secretion from the cells (12). The observation of CT at the surface of the cells which made the surface appear ragged, suggests that the accumulated CT in the periplasm was secreted by the changes in cell wall permiability.

In organisms not fixed with glutaraldehyde, CT was not detected even in the periplasmic space, due to removal during repeated washings. But in these samples, a branching network in the cell was densely stained by osmium. These structures were also seen in the control samples. A few reports concerning microtubules in bacteria have been seen (3, 4, 7, 13), but the tubules was found in a small proportion of the examined cells, and were few in number. The branching network found in this study was also seen in some other bacteria including *Escherichia coli* and *Enterobacter cloacae*. But a triple layered unit membrane was not confirmed as a component of the structure. This branching network appears to be connected to the cytoplasmic membrane, and may be a tubular structure, because HRP and DAB were ingested when by unfixed cells but not by cells fixed with glutaraldehyde.

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酵素抗体法を用いた菌体内コレラ菌毒素の電顕的観察

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毒素産生と非産生の条件下で培養したコレラ菌569B株および毒素非産生株 K25の電顕形態学的比較に引き続き，酵素標識抗毒素抗体を用いて菌体内における毒素の局在を観察しようとした．

使用菌株および2種の培養条件は既報と同様である．choletox で免疫して得た家兎血清（PHA価10,240倍）より IgG を硫安塩析と DEAE-sephadex A-50ゲル濾過で精製，濃縮（同5,120倍），これに Avrameas and Ternynck (1971) の方法で horseradish peroxidase (Sigma RZ : 3.1) を結合させた（同1,024倍）．洗浄菌体を0.5%グルタールアルデヒドで固定，洗浄後 HRP-IgG を，再洗浄後 0.2% diaminobenzidine，ついで過酸化水素を作用させた．充分洗浄後1%オスミウム酸で固定，常法で脱水，エポン包埋，超薄切し，一部では酢酸ウラニルとクエン酸鉛染色を行って，鏡検した．

毒素産生条件下の569B では，全般的に大円型細胞よりも桿状細胞に毒素が証明され，後者では periplasmic space，細胞膜，メソゾーム様構造の部分，前者では空胞様部分の内側表層に認められた．毒素の存在様式は，1)大部分細胞内で少量が表層，2)内部にもあるが大量が細胞表面，3) periplasmic space になく細胞表面，の3種にまとめられた．前報で毒素産生条件にのみ多くみられた，細胞壁の突出部とこれが遊離した粒子，また対照（非結合 HRP の使用，抗毒素 IgG 前処置，K25株）ではすべて毒素染出はみられなかった．

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