Acta Histochem. Cytochem. 33 (2): 89–94, 2000

Induction of Active Invagination of Plasma Membranes by *Helicobacter pylori* Cytotoxin, VacA, in Cultured Gastric Cells: An Immunoelectron Microscopic Study

Shin-ichi Izumi[1], Miyuki Kimura[2,3], Takahiro Kimura[3], Akihiro Wada[3], Toshiya Hirayama[3], Akitoyo Ichinose[4] and Takehiko Koji[1]

[1]Department of Histology and Cell Biology, Nagasaki University School of Medicine, 1–12–4, Sakamoto, Nagasaki 852–8523, [2]Department of Applied Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki 852–8523, [3]Department of Bacteriology, Nagasaki Institute of Tropic Medicine, 1–12–4, Sakamoto, Nagasaki 852–8523 and [4]Central Laboratory, Nagasaki Institute of Tropic Medicine, 1–12–4, Sakamoto, Nagasaki 852–8523

Received February 17, 2000; accepted March 17, 2000

VacA cytotoxin secreted by *Helicobacter pylori* causes cytoplasmic vacuolation in gastric epithelial cells. To elucidate whether VacA directly affects the cell surface, changes in the membrane structure after VacA treatment were examined in a human gastric epithelial cell culture (AZ-521) at an ultrastructural level. When the time-course changes in the localization of VacA were analyzed by immunoelectron microscopy in AZ-521 cells that had been exposed to VacA, VacA was detected predominantly on the plasma membrane at an early stage of the exposure, and later in endocytotic vesicles and/or tubulo-vesicles. Five to 24 hr after exposure, the VacA was distributed in a limestone cavern like-structure, which was formed by elongated surfaces of plasma membranes, as shown by scanning electron microscopy. Therefore, the binding of VacA to the plasma membrane and the subsequent internalization into the enlarged cavern like-plasma membranes may be involved in the induction of vacuolation of gastric epithelial cells by VacA.

Key words: ***Helicobacter pylori*, VacA, Immunohistochemistry, Electron microscopy, Plasma membrane**

I. Introduction

Helicobacter pylori (*H. pylori*) infection causes chronic gastritis, peptic ulcer disease and gastric carcinomas in humans [10, 11]. It is well known that *H. pylori* secretes a potent vacuolating cytotoxin (VacA), which induces cytoplasmic vacuolation in eukaryotic cells, resulting in cell death [1, 3, 12, 15]. Experimental infection of mice with cytotoxin-producing strains of *H. pylori* resulted in gastric epithelium vacuolation, whereas infection with non-cytotoxin-producing strains did not [9]. For the process of cytotoxic vacuolation, it has been proposed that the first step is the binding of VacA to the plasma membrane [4], followed by internalization into the endosomes [4, 14]. The successive fusion of endosomes with lysosomes leads to the formation of vacuoles [14]. In our previous studies, we have shown that VacA caused vacuolation in the human gastric cancer cell line, AZ-521, which expresses a specific protein receptor, i.e., a receptor protein-tyrosine phosphatase (RPTPβ/PTPζ) on the cell surface [16, 17].

In the present study, to clarify the binding site of VacA to the plasma membrane and the mode of entry of VacA with cellular compartments into the cells, we examined the time-course changes in the localization of an inoculated VacA in AZ-521 cultured cells. The VacA in the cultured cells was detected by immunoelectron microscopy.

Correspondence to: Dr. Shin-ichi Izumi, Department of Histology and Cell Biology, Nagasaki University School of Medicine, 1–12–4, Sakamoto, Nagasaki 852–8523, Japan.

II. Materials and Methods

VacA cytotoxin

The purification of VacA cytotoxin from the bacterial high cytotoxin-producing strain *H. pylori* ATCC49503 has been described elsewhere [7, 16].

Cell culture

The human gastric adenocarcinoma cell line, AZ-521

cells (Culture Collection of Health Science Research Resources Bank, Japan Health Science Foundation), were used [16, 17]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum in a humidified cell incubator at 37°C in 5% CO_2-95% air. For electron microscopic immunohistochemistry, 2×10^4 cells/ml were seeded per well of Lab-Tek 8-well chamber slide (Nalge Nunc International, IL) in 300 μl culture medium and were grown at 37°C in 5% CO_2 in air.

Immunoelectron microscopy

The AZ-521 cells were incubated in DMEM with or without 120 nM VacA, which was activated by a brief exposure to acidic solutions followed by neutralization [2]. After 0, 1, 3, 5, 10 or 24 hr of incubation at 37°C, the AZ-521 cells were washed with 0.01 M sodium phosphate buffered saline, pH 7.2 (PBS), and permeabilized by air-drying for 1 hr. The cells were then fixed with a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in PBS for 10 min at 4°C. The fixed cells were incubated with 1% goat serum in PBS to prevent non-specific adhering of immunoglobulins. The cells were then incubated with specific rabbit anti-VacA IgG (5 μg/ml) [16] in PBS containing 1% bovine serum albumin (BSA/PBS) for 1 hr at room temperature, followed by horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (MBL, Nagoya) diluted 1 : 100 in BSA/PBS. After incubation with the second antibody, post-fixation with 1% glutaraldehyde in PBS for 10 min at 4°C was performed to improve the stability of complexes of VacA and the antibodies. The HRP sites were visualized with DAB and hydrogen peroxide [8]. The cells were then osmified with 2% OsO_4 in PBS for 1 hr at room temperature, after which they were dehydrated in ethanol and inversely mounted in gelatin capsules filled with epoxy embedding resin. The resin was polymerized at 60°C for 2 days and the ultrathin sections were viewed in the transmission electron image mode with an accelerating voltage of 60 kV of a transmission electron microscope (JEOL 1200EX, JEOL Ltd., Tokyo). For the negative staining controls, inoculated AZ-521 cells were reacted with nonimmune IgG (5 μg/ml) instead of the anti-VacA IgG. For another negative staining control, uninoculated AZ-521 cells were reacted with the anti-VacA IgG (5 μg/ml) to specify the reaction products.

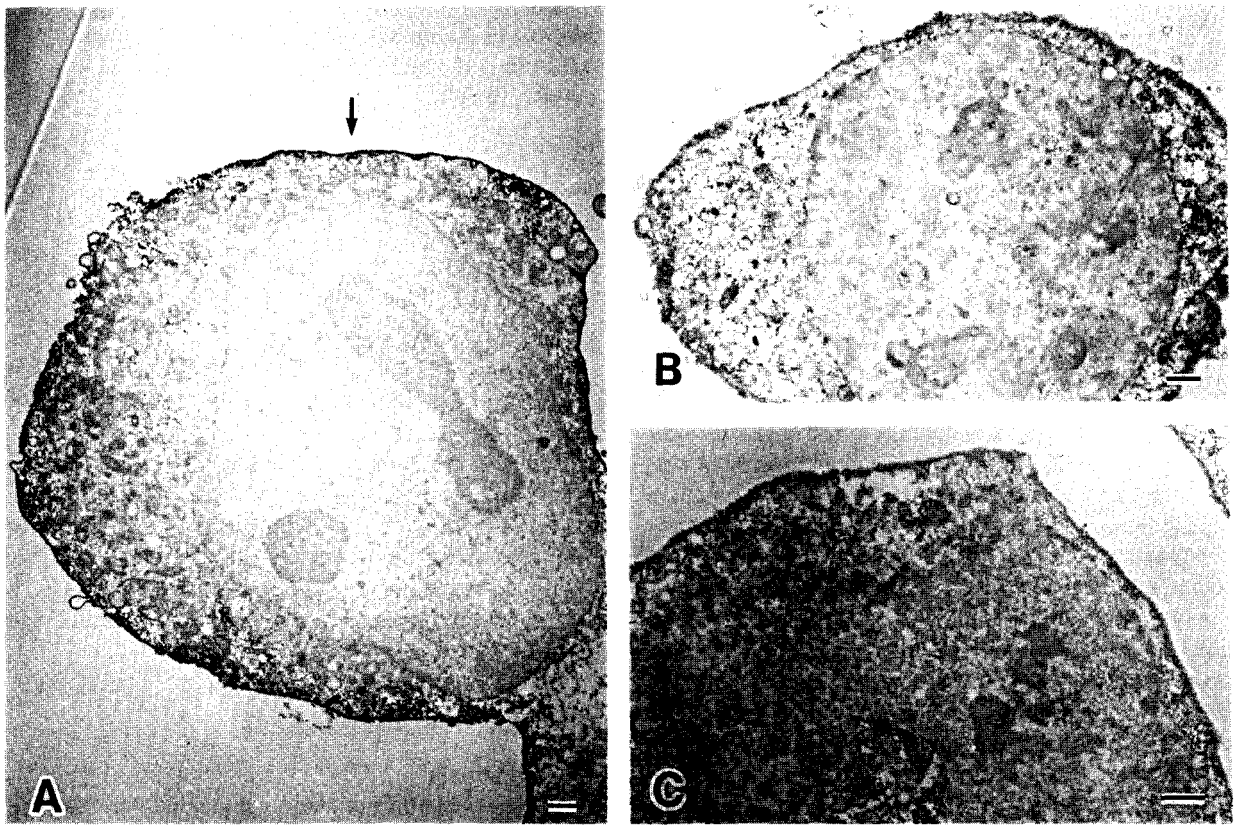

Fig. 1. Immunoelectron micrographs of VacA in AZ-521 cells. **(A)** After 5 hr of incubation with VacA, immunoelectron microscopy using anti-VacA IgG was performed. The plasma membrane and a part of the cytoplasm are positive for VacA (arrow). **(B)** After 5 hr of incubation with VacA, immunoelectron microscopy using nonimmune IgG instead of anti-VacA IgG was performed as the negative control of **(A)**. No reaction products are seen. **(C)** After 5 hr of incubation without VacA, immunoelectron microscopy using anti-VacA IgG was performed as the negative control of **(A)**. No reaction products are seen. Bars = 1 μm.

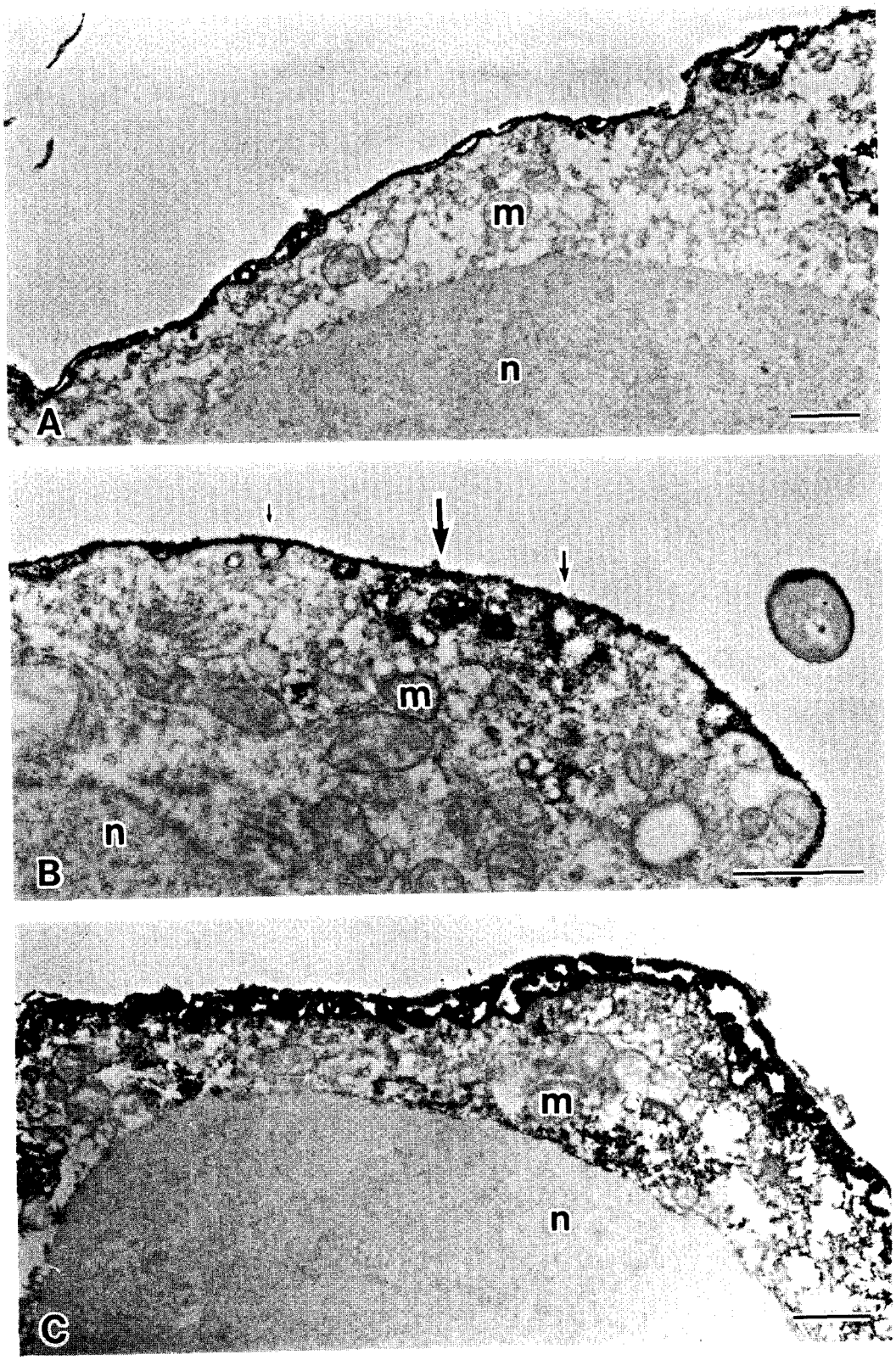

Fig. 2. Immunoelectron micrographs of VacA in AZ-521 cells at various incubation times. **(A)** After 1 hr of incubation, VacA is seen in the plasma membrane and in a narrow tramline-like plasma membrane structure. **(B)** After 5 hr of incubation, VacA is seen in the plasma membrane, in the plasma membrane attached-endocytotic vesicles (small arrow), in the fused-endocytotic vesicle (arrow), and in the tubularly dilated- and invaginated-vacuole (large arrow). **(C)** After 10 hr of incubation, VacA is seen in the tubular architecture between a wide tramline-like plasma membrane structure or in a limestone cavern-like structure. m, mitochondria; n, nucleus. Bars = 1 μm.

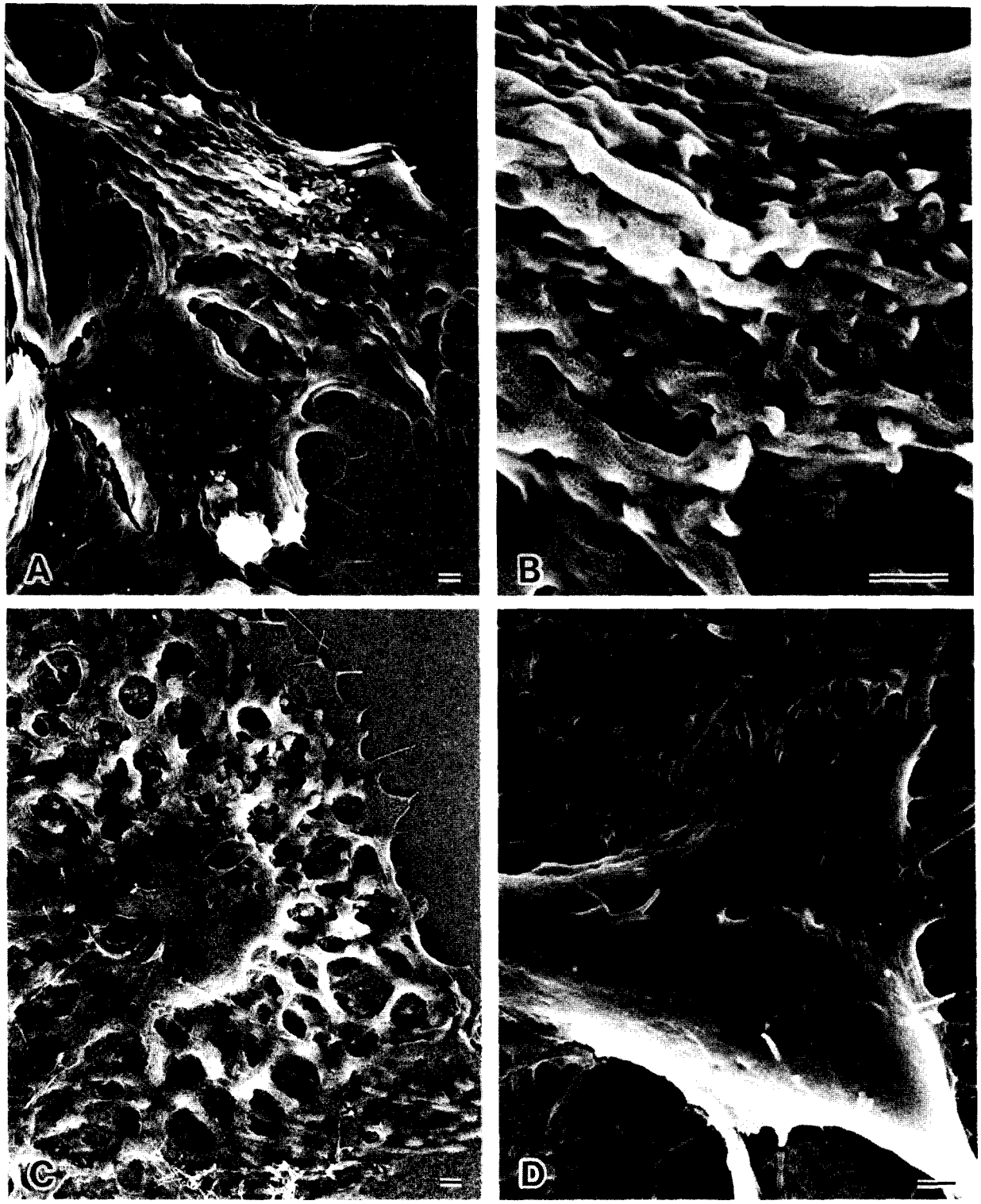

Fig. 3. Secondary electron micrographs of AZ-521 cell surfaces. AZ-521 cells were incubated with or without VacA for 10 hr. **(A)** VacA-treated cells. Remarkable wrinkling of plasma membrane is seen. **(B)** Enlargement of **(A)**. A cavern-like structure with a variety of holes in addition to the wrinkles is seen. **(C)** VacA-treated cells. Remarkable holes or vacuoles with a cavern-like structure of plasma membrane are seen. **(D)** VacA-nontreated cells. Smooth surfaces of the plasma membrane with small vesicles are seen. Bars = 1 μm.

Scanning electron microscopy

The AZ-521 cells that were incubated for 10 hr with or without VacA were dried, ion-spattered with gold palladium (20 nm thickness), and examined by secondary electron imaging of a scanning electron microscope (JSM-040AN, JEOL Ltd., Tokyo) at an accelerating voltage of 10 kV.

III. Results

Immunoelectron microscopy showed that the externally added VacA was specifically localized in the plasma membrane and cytoplasmic vesicles (Fig. 1A). In the negative control cells, no reaction products were seen (Fig. 1B, C).

Before and just after VacA inoculation, no VacA was seen in the plasma membrane. At 1 hr of incubation, VacA was seen in the plasma membrane and in a narrow tramline-like plasma membrane structure (Fig. 2A). At 3 hr of incubation, VacA was seen in a wide tramline-like plasma membrane structure with membranous compartments. At 5 hr of incubation, VacA was seen in the plasma membrane, in plasma membrane-bound endocytotic vesicles (Fig. 2B, small arrow) as well as in internalized endocytotic vesicles (Fig. 2B, arrow). The VacA was also seen in a dilated tubulo-vesicular structure, which was derived from the plasma membrane (Fig. 2B, large arrow).

When the cells were incubated for 10 hr in the presence of VacA, complicated structures of plasma membranes were formed. VacA was seen in many vesicles within a wide tramline-like plasma membrane structure or in a tubular limestone cavern-like structure (Fig. 2C). The width of the tramline-like two layers in the cell peripheral region ranged between approximately 50 nm and 600 nm (Fig. 2A–C). In some cells, VacA was seen in a limestone cavern-like structure located deep in the cytoplasm. Scanning electron microscopy revealed that VacA treatment caused wrinkles and holes with a limestone cavern-like structure of the plasma membrane in the cells (Fig. 3A–C), while there were no significant structural changes without treatment (Fig. 3D). The outside diameter of wrinkles of the cell surface ranged between approximately 80 nm and 900 nm (Fig. 3A, B). After 24 hr of incubation, VacA was seen in the vacuoles (Fig. 4).

IV. Discussion

To gain insights into the direct cytotoxicity of VacA from *H. pylori* in gastric epithelial cells, an ultrastructural analysis of the effect of VacA on the vacuolation in the cells would be required. In the present study, we examined the time-course changes in the localization of an inoculated VacA in human gastric AZ-521 cells by immunoelectron microscopy. Consequently, the VacA was localized sequentially in the plasma membrane, in the plasma membrane-derived tubulo-vesicular structures, in the tramline-like plasma membrane structures, in the limestone cavern-like structures, and in the vacuoles in AZ-521 epithelial cells by 24 hr after VacA inoculation. The results indicate that the binding of VacA to the plasma membrane may be required for the induction of vacuolation in the cells.

Regarding the localization of exogenously added substances, autoradiography has also been a useful technique for identifying target cells of the ligand substance, even when the ligand synthesizing cells are intermingled with the target cells [5] or when the purified antibody against the ligand could not be obtained [6]. On the other hand, the non-radioisotopic immunoelectron microscopy technique is easy to handle and has higher resolution, especially at the ultrastructural level, to localize non-labeled

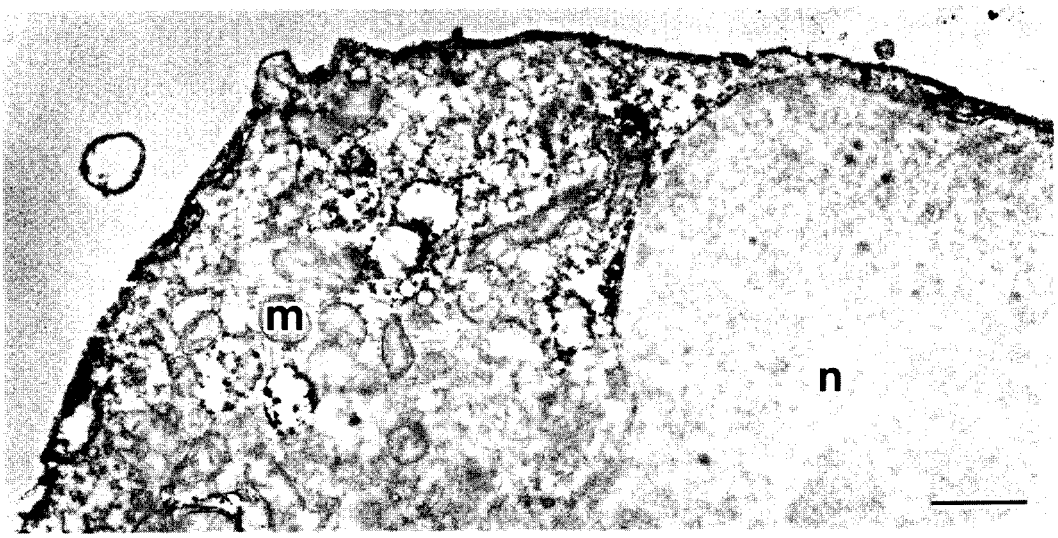

Fig. 4. An immunoelectron micrograph of VacA in cytoplasmic vacuoles. AZ-521 cells were incubated with VacA for 24 hr. VacA is seen in vacuoles and plasma membrane. m, mitochondria; n, nucleus. Bar = 1 μm.

intact ligand substances. Our method described here may be widely applicable to localize exogenously added substances in cultured target cells, when the antibodies are available.

Using the non-radioisotopic immunoelectron microscopy we clearly demonstrated the binding site of VacA to the plasma membrane and the mode of entry of the VacA with cellular compartments into AZ-521 cells. The VacA was distributed in the plasma membrane just after inoculation and the membranous compartment of complicated structures of the cells at 5 hr of incubation. The VacA was then distributed in a tramline-like structure and in a limestone cavern like-structure. These structures were confirmed by scanning electron microscopy as a wrinkle structure of plasma membrane and a hole structure of plasma membrane, respectively. As shown in the results, the value of the width of the tramline-like two layers in the cell peripheral region by transmission electron microscopic immunohistochemistry was nearly the same as that of the diameter of wrinkles in the cell surface by scanning electron microscopy. This fact may indicate that a tramline-like structure is formed by the complicated infoldings or invaginations of the cell membrane.

Our findings indicate that the VacA bound to the plasma membrane may be associated with the endocytotic vesicles. Then the endocytotic vesicles seem to have internalized into the cell by the endocytic pathway and processed to enlarge and fuse together to form tubulo-vesicles. Such expansion of endosomal compartments has been exhibited in biopsies of *H. pylori*-colonized human gastric epithelium [3]. Then, the enlarged tubulo-vesicles seemed to be processed to a tramline-like plasma membrane structure and/or a limestone cavern-like structure, resulting in the formation of vacuoles. Interestingly, the binding of VacA-producing *H. pylori* to human gastric AGS cultured cells resulted in cytoskeletal actin rearrangement and pedesteral formation [13]. Therefore, the complicated membrane structures may be associated with the cytoskeletal rearrangement of AZ-521 cells by the binding of VacA. Thus, the VacA may induce the active invagination of the plasma membranes of cultured gastric cells.

In conclusion, the binding of VacA to the plasma membrane and the subsequent internalization into the tramline-like and cavern-like structures may be involved in the induction of vacuolation of human gastric AZ-521 cells by VacA.

V. References

1. de Bernard, M., Arico, B., Papini, E., Rizzuto, R., Grandi, G., Rappuoli, R. and Montecucco, C.: *Helicobacter pylori* toxin VacA induces vacuole formation by acting in the cell cytosol. *Mol. Microbiol.* 26; 665–674, 1997.
2. de Bernard, M., Papini, E., de Filippis, V., Gottardi, E., Telford, E., Manetti, R., Fontana, A., Rappuoli, R. and Montecucco, C.: Low pH activates the vacuolating toxin of *Helicobacter pylori*, which becomes acid and pepsin resistant. *J. Biol. Chem.* 270; 23937–23940, 1995.
3. Fiocca, R., Luinetti, O., Villani, L., Chiaravalli, A. M., Capella, C. and Solcia, E.: Epithelial cytotoxicity, immune responses, and inflammatory components of *Helicobacter pylori* gastritis. *Scand. J. Gastroenterol.* 29 (Suppl 205); 11–21, 1994.
4. Garner, J. A. and Cover, T. L.: Binding and internalization of the *Helicobacter pylori* vacuolating cytotoxin by epithelial cells. *Infect. Immun.* 64; 4197–4203, 1996.
5. Hasegawa, H., Mizuhira, V., Notoya, N., Iwanaga, K., Mizojiri, K., Kitamura, K., Eto, T., Kangawa, K. and Matsuo, H.: Autographic study on the binding site of ^{125}I-adrenomodullin in rat tissues. *Acta Histochem. Cytochem.* 31; 335–343, 1998.
6. Izumi, S., Slayden, O. V., Rubin, J. S. and Brenner, R. M.: Keratinocyte growth factor and its receptor in the rhesus macaque placenta during the course of gestation. *Placenta* 17; 123–135, 1996.
7. Kimura, M., Goto, S., Wada, A., Yahiro, K., Niidome, T., Hatakeyama, T., Aoyagi, H., Hirayama, T. and Kondo, T.: Vacuolating cytotoxin purified from *Helicobacter pylori* causes mitochondrial damage in human gastric cells. *Microb. Pathogen.* 26; 45–52, 1999.
8. Komuta, K., Kanematsu, T., Nakane, P. K. and Koji, T.: Localization of epidermal growth factor receptor enhancer protein in A431 epidermoid carcinoma cells by Southwestern histochemistry. *Acta Histochem. Cytochem.* 31; 267–273, 1998.
9. Marchetti, M., Arico, B., Burroni, D., Figura, N., Rappuoli, R. and Ghiara, P.: Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science* 267; 1655–1658, 1995.
10. Nomura, A., Stemmerman, G. N., Chyou, P. H., Kato, I., Perez-Perez, G. I. and Blaser, M. J.: *Helicobacter pylori* infection and gastric carcinoma among Japanese-Americans in Hawaii. *N. Engl. J. Med.* 325; 1132–1136, 1991.
11. Peterson, W. L.: *Helicobacter pylori* and peptic ulcer disease. *N. Engl. J. Med.* 324; 1043–1048, 1991.
12. Ricci, V., Sommi, P., Fiocca, R., Romano, M., Solcia, E. and Ventula, U.: *Helicobacter pylori* vacuolating toxin accumulates within the endosomal-vacuolar compartment of cultured gastric cells and potentiates the vacuolar activity of ammonia. *J. Pathol.* 183; 453–459, 1997.
13. Segal, E. D., Falkow, S. and Tompkins, L. S.: *Helicobacter pylori* attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins. *Proc. Natl. Acad. Sci. USA* 93; 1259–1264, 1996.
14. Sommi, P., Ricci, V., Fiocca, R., Necchi, V., Romano, M., Telford, J. L., Solcia, E. and Ventula, U.: Persistence of *Helicobacter pylori* VacA toxin and vacuolating potential in cultured gastric epithelial cells. *Am. J. Physiol.* 275; G681–G688, 1998.
15. Telford, J. L., Giara, P., Dell'Orco, M., Comanducci, M., Buroni, D., Bugnoli, M., Tecce, M. F., Censini, S., Covacci, A., Xiang, Z., Papini, E., Montecucco, C., Perente, L. and Rappuoli, R.: Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. *J. Exp. Med.* 179; 1653–1658, 1994.
16. Yahiro, K., Niidome, T., Hatakeyama, T., Aoyagi, H., Kurazono, H., Padilla, P. I., Wada, A. and Hirayama, T.: *Helicobacter pylori* vacuolating cytotoxin binds to the 140-kDa protein human gastric cancer cell lines, AZ-521 and AGS. *Biochem. Biophys. Res. Commun.* 238; 629–632, 1997.
17. Yahiro, K., Niidome, T., Kimura M., Hatakeyama, T., Aoyagi, H., Kurazono, H., Imagawa K., Wada, A., Moss, J. and Hirayama, T.: Activation of *Helicobacter pylori* VacA toxin by alkaline or acid conditions increases its binding to a 250-kDa receptor protein-tyrosine phosphatase β. *J. Biol. Chem.* 274; 36693–36699, 1999.