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Electron Microscopic Study of In Vitro *Vibrio vulnificus* Infection to Tissue Culture Cells and Peripheral Blood Cells

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Summary. ***Vibrio vulnificus*** **is the most pathogenic species among the genus *Vibrio*, and such a fatal *V. vulnificus* infection case occurred in Niigata in 2003. In this study, we investigated the in vitro characteristics of *V. vulnificus* infection to tissue culture cells (HEp-2, HeLa, and HCT-8) and human peripheral blood cells by scanning and transmission electron microscopy. *V. vulnificus* damaged those cells, especially HeLa cells. Moreover, *V. vulnificus* adhered to cells possessed unique pearl-like structure, unlike *V. cholerae* O1 or O139 and *V. parahaemolyticus*. When human peripheral blood cells were infected with *V. vulnificus*, leukocytes and platelets were markedly adhered with *V. vulnificus*, and platelet was most seriously damaged. Separation of the outer and inner nuclear membranes was observed in some blood cells such as leukocytes. Pilus-mediated *V. vulnificus* adherence to blood cells was also observed. Invasion (or uptake) of *V. vulnificus* into blood cells was not obvious, in contrast to methicillin-resistant *Staphylococcus aureus*. The data suggest that *V. vulnificus* may use unique appendages for infection, and may damage blood cells such as platelets and leukocytes by producing toxins rather than invading cells, and prevent phagocytosis by bacterium-engulfing cells by toxins.**

Key words — *Vibrio vulnificus*, ultrastructure, in vitro infection, intestinal cells, blood cells.

INTRODUCTION

Vibrio vulnificus is a gram-negative, curved rod with a capsule and a polar monotrichous flagellum. It thrives in warm seawater (15°C or higher) like *V. parahaemolyticus*. *V. vulnificus* frequently isolated from seafish and shellfish including shrimps or zooplanktons, and causes infection in humans via oral ingestion of contaminated sea fish and shellfish[1-5]. Many cases of infections by ingestion of raw oysters have occurred in the United States, and it has been reported that *V. vulnificus* causes half of cases of *Vibrio* infection[1]. Infection can also occur via eels and freshwater fish[6] or even via open wound that is exposed to seawater[5].

In Japan, *V. vulnificus* infection is more likely to occur between June and September when seawater temperature increases, and more incidences occur on the West side of Japan, especially around a bay which is filled in, such as Ariakekai[7-10]. Niigata is located in the North-West of Honshu island, however, a *V. vulnificus* infection case was reported in Niigata in 2003[11].

V. vulnificus is a highly invasive pathogen, and is the most pathogenic *Vibrio* species. Its infection causes necrosis of soft tissues of the extremities, sepsis, and shock, and may lead to death within a few days[1-5]. However, *V. vulnificus* appears to be an opportunistic pathogen, and human infections are relatively rare. Infected patients are generally heavy alcohol drinkers or immunocompromised patients such as those with

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Abbreviations — CFU, colony forming unit; MRSA, methicillin-resistant *Staphylococcus aureus*.

underlying liver diseases[1-5], but the cause is unknown in some cases.

V. vulnificus produces several possible virulence factors such as capsular polysaccharide (acting against phagocytosis by neutrophils and macrophages and against bacteriolysis by complement)[12-15], metallo-protease[16,17], hemolysin-cytolysin[18,19], pili (playing a role in colonization and persistence in oysters)[20], and a factor(s) responsible for apoptosis induction in macrophages[21] or THP-1 human monocytic cells[22],. In this study, we investigated the manner of *Vibrio vulnificus* infection to tissue culture cells and human peripheral blood cells.

MATERIALS AND METHODS

Bacterial strains

V. vulnificus strain NV1B, which was isolated from a patient in Niigata, Japan[11], *V. vulnificus* strains CB1, CB2, and OI1[22], *V. cholerae* O1 strain EO8[23], *V. cholerae* O139 strain T16[23], *V. parahaemolyticus* strain 100B[23], and methicillin-resistant *Staphylococcus aureus* (MRSA) strain E6[24] were used.

Media and bacterial growth

For bacterial growth, we used Luria-Bertani (LB) broth (Difco Laboratories, Detroit. Mich., USA) as liquid media which was inoculated and incubated at 37°C for 12-18 h with agitation. LB agar (Difco), 5% sheep blood agar (Becton Dickinson, Tokyo), and tryptic soy agar (TSA, Difco) containing 5% human erythrocytes were used as solid media. For the growth of *V. vulnificus* and *V. parahaemolyticus*, sodium chloride (at 3%) was added to LB or LB agar media.

In vitro infection to tissue culture cells

The infection of *V. vulnificus* to human ileocecal colorectal epithelial cells (HCT-8), larynx squamous cells (HEp-2), and cervical cells (HeLa) was examined by a method as described previously[25]. Those cells were grown on a plastic coverslip (diameter 13.5 mm, Sumitomo Bakelite, Tokyo) at ~ 50% confluence in Eagle MEM (Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal bovine serum (FBS), or in RPMI 1640 medium (Gibco BRL, Grand Islands, NY) supplemented with 10% FBS. The cells on a plastic coverslip in 1 ml of MEM supplemented with 6% FBS were incubated with 5 µl of bacterial cultures for 1 to 2 h at 37°C. In case of 2 h incubation, the culture medium was changed 1 h after the initial incubation. The cells and bacteria on the plastic coverslips were washed with phosphate-buffered saline (PBS, pH 7.4).

In vitro infection to human blood cells

Blood cells were obtained from three healthy adults. In some experiments, blood cells were directly centrifuged and washed in PBS, and then suspended in RPMI 1640 medium. Peripheral blood mononuclear cells (PBMCs) were isolated as previously described[26]. PBMCs were isolated with Ficoll-conray (specific gravity: 1.077) density graduent centrifugation and washed twice in PBS. The cells were finally suspended in the RPMI 1640 culture medium at a cell concentration of 1 X 10^6 to 1 X 10^7cells/ml. For infection, 5 µl of bacterial cultures was added to each 1 ml cell suspension, and incubated for 1 to 2 h at 37°C.

Scanning electron microscopy

The washed plastic coverslips were fixed with 2.5% (vol/vol) glutaraldehyde in PBS (pH 7.4) for 2 h at room temperature and subsequently postfixed in 1% (wt/vol) osmium tetroxide for 1 h at 4°C. The samples were then dehydrated in acetone, critical-point dried, and coated with gold-palladium. The samples were finally analyzed by scanning electron microscop[25].

Transmission electron microscopy

The fixed samples were dehydrated in acetone and embedded in EPOK 812 (Oukenn Inc., Tokyo). The embedded block was cut with an ultramicrotome (MT-500) diamond knife and stained with uranyl acetate and lead citrate. The stained samples were analyzed by transmission electron microscopy[25]. In some experiments, bacteria grown on agar plates and suspended in water, were negatively strained with 1% uranyl acetate for 3 min and analyzed by transmission electron microscopy.

RESULTS

Bacterial characteristics of *V. vulnificus*

V. vulnificus strain NV1B was β-hemolytic (against human erythrocytes) on blood agar plates (Fig. 1, A and B); other *V. vulnificus* clinical isolates were also β-hemolytic (data not shown). *V. vulnificus* strain NV1B had a comma-shaped cell body, enclosed in a capsular wrinkle-like structure, and had a polar monotrichous flagellum (Fig. 1, C). And it occasionally had pili (Fig. 1, D and E).

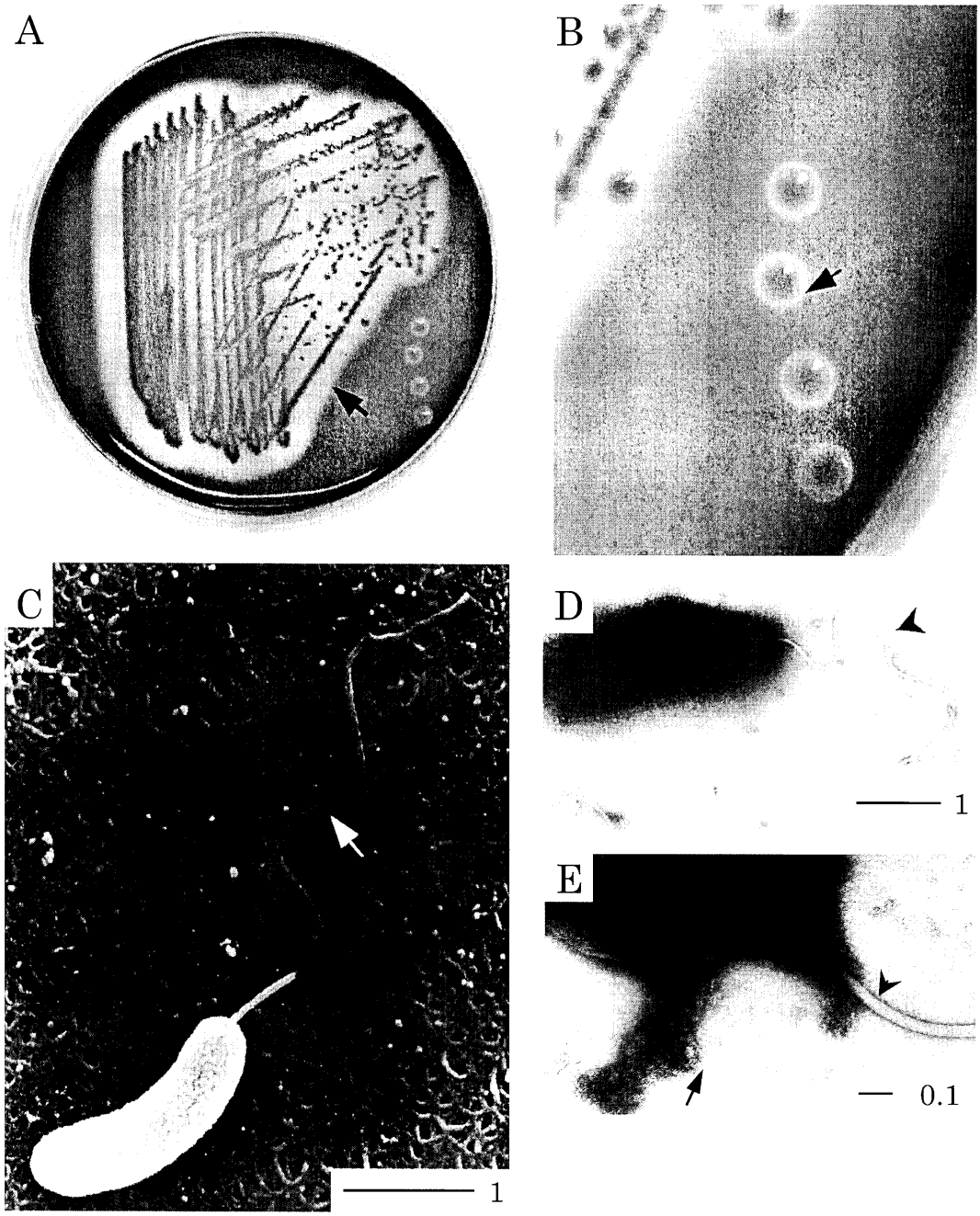

Fig. 1. Bacteriological characteristics of *V. vulnificus* strain NV1B. *V. vulnificus* strain NV1B was grown on TSA agar containing 5% human erythrocytes at 37°C overnight. An arrow points to β-hemolysis (A and B). An arrow in C points to *V. vulnificus* flagella (scanning electrom micrograph). An arrow and arrowhead in D and E, respectively, mark pili and flagella of the bacterium. Bars = 1 μm in C and D, and 0.1μm in E.

In vitro *V. vulnificus* infection to tissue culture cells

V. vulnificus adhered to HeLa, HEp-2, and HCT-8 cells (Fig. 2). Among those tissue culture cells, HeLa cells were most susceptible to the cytotoxic attack by *V. vulnificus*, and the cell surface of HeLa cells was seriously damaged (Fig. 2, A and B). In the case of HEp-2 cells (Fig. 2, C to E), only some adherent *V. vulnificus* displayed a tight interaction with cells (Fig. 2, E). A similar bacterial adherence was also observed with HCT-8 cells (Fig. 2, F to H).

V. vulnificus, adhered to HCT-8 cells, occasionally possessed a unique pearl-like structure, which expanded from the bacterial pole and seemed to be adhesive to the cell surface (Fig. 3). Such a unique pearl-like structure was not observed with *V. cholerae* O1 or O139 and *V. parahaemolyticus* (data not shown).

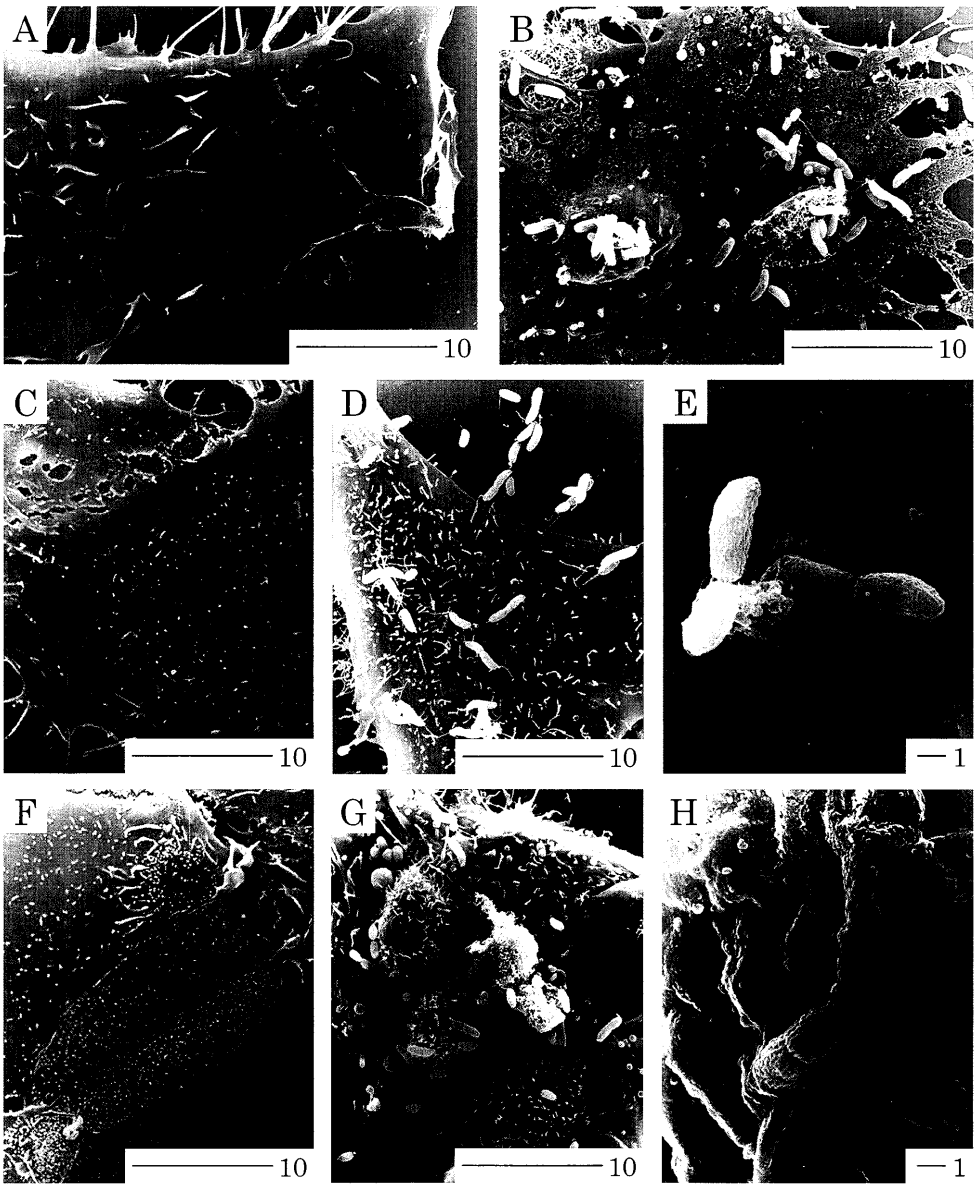

Fig. 2. Scanning electron micrograph showing *V. vulnificus* adherence to HeLa (A and B), HEp-2 (C to E), and HCT-8 (F to H) cells. Cells were infected with *V. vulnificus* strain NV1B in RPMI 1640 medium for 1 h at 37°C. Cells in A, C , and F are those (control cells) not infected with *V. vulnificus*. Tight interaction of *V. vulnificus* with cells, occasionally seen, is shown in E and H. Bars = 10 μm in A to D, F and G, and 1μm in E and H.

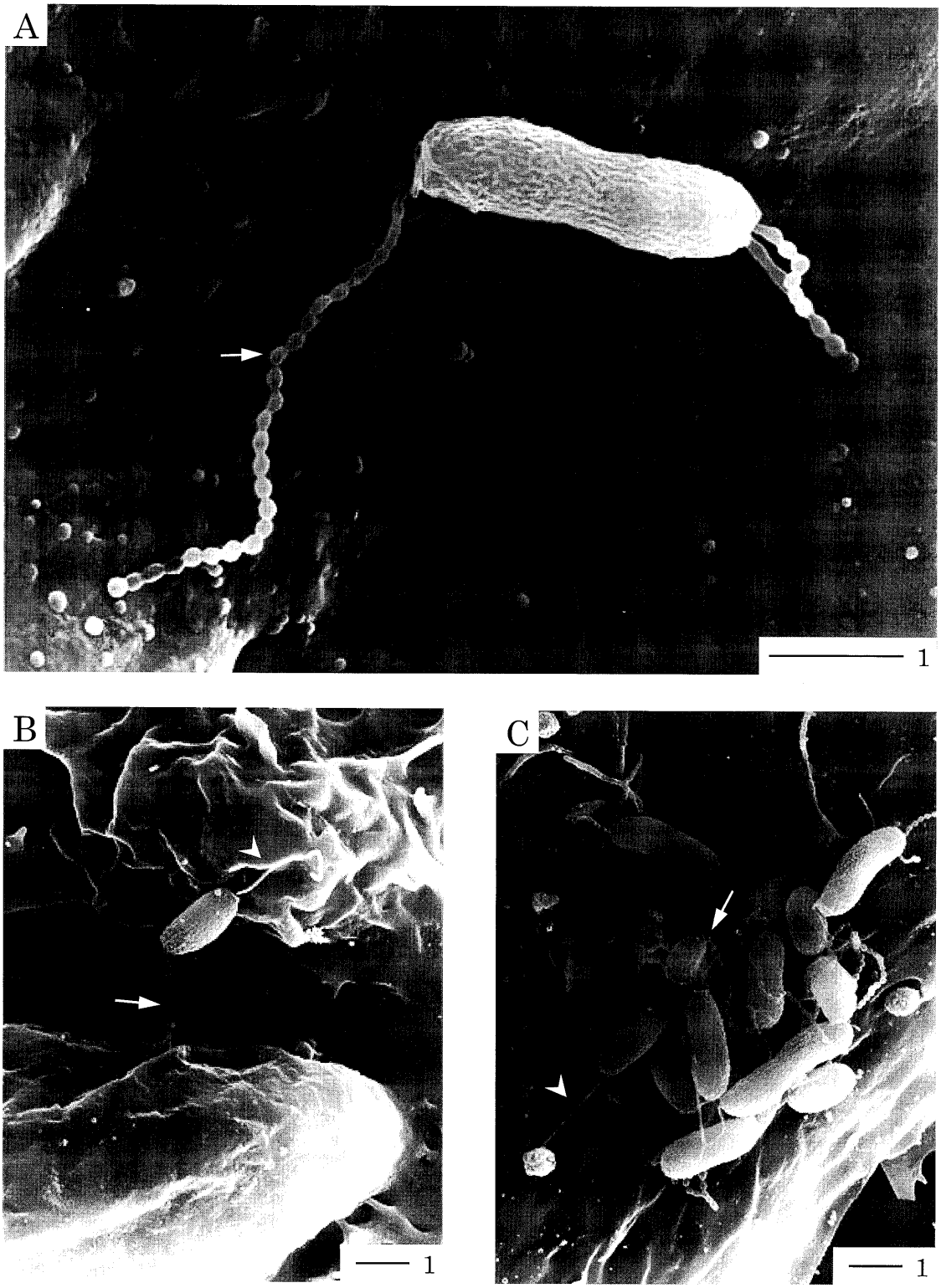

Fig. 3. Scanning electron micrograph showing the pearl-like structure of *V. vulnificus* (strain NV1B) on HCT-8 cells. Infection condition was the same as those in Fig. 2. Arrows and arrowhead, respectively, point to the pearl-like structure and flagella of *V. vulnificus*. Bars = 1 μm.

In vitro *V. vulnificus* infection to human blood cells

Human blood cells were infected with *V. vulnificus* and then analyzed by scanning and transmission electron microscopy (Figs. 4 to 6). *V. vulnificus* adherence to cells was observed with red cells, platelets, granulocytes, lymphocytes, and monocytes. Among those blood cells, platelets were most susceptible to the cytotoxic effect of *V. vulnificus*, and serious damages were obviously seen at both the surface and inside of cells (Fig. 4).

V. vulnificus adherence to red cells was barely seen, but occasionally pilus-mediated adherence was observed (Fig. 5, A). In contrast, cells morphologically identified as granulocytes (probably neutrophils, Fig.

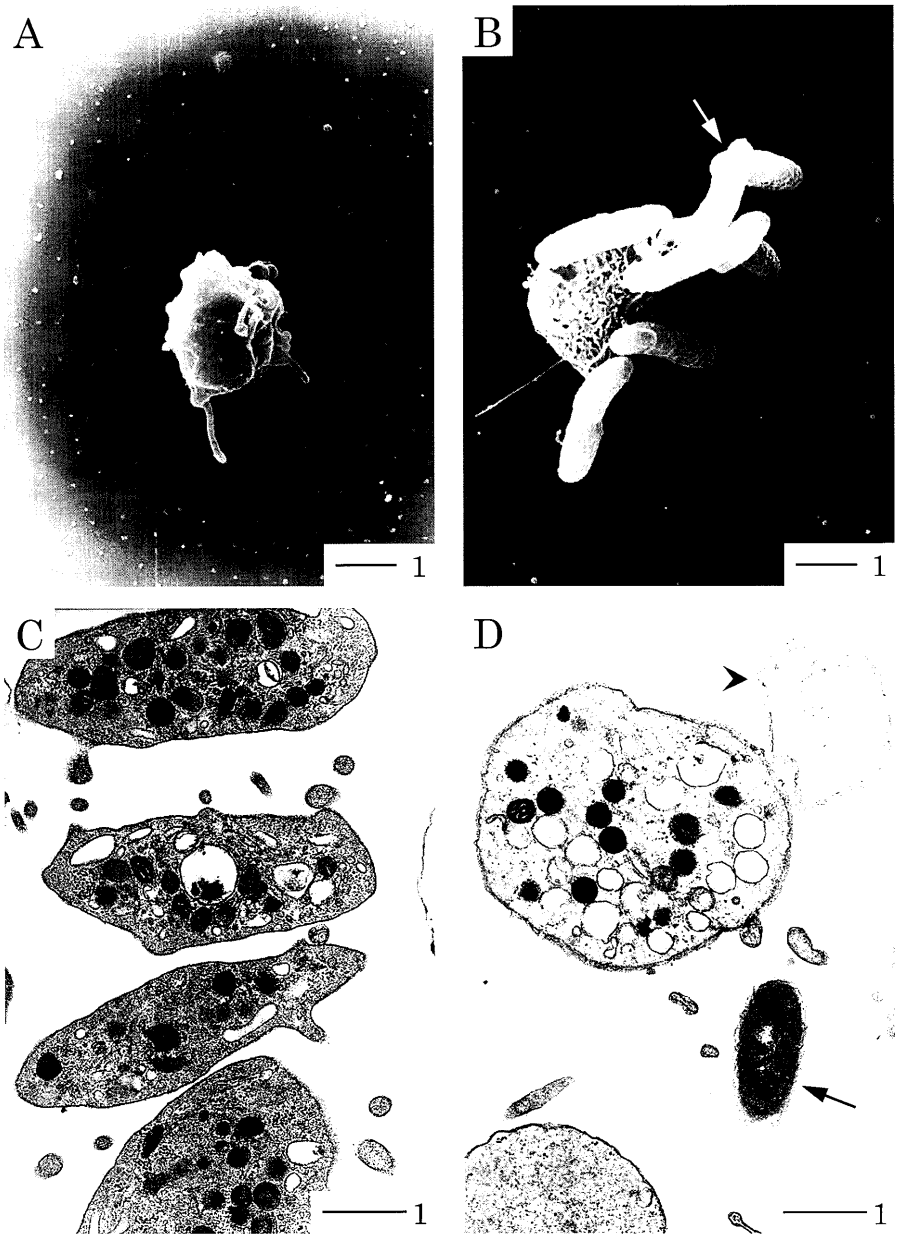

Fig. 4. Scanning (A and B) and transmission (C and D) electron micrographs showing infection to platelets of *V. vulnificus* strain NV1B. Infection was performed in RPMI 1640 medium for 1 h at 37°C. Cells in A and C are those (control cells) not infected with *V. vulnificus*. Arrows in B and D point to *V. vulnificus*. An arrowhead in D points to platelet ghosts. Bars = 1 μm.

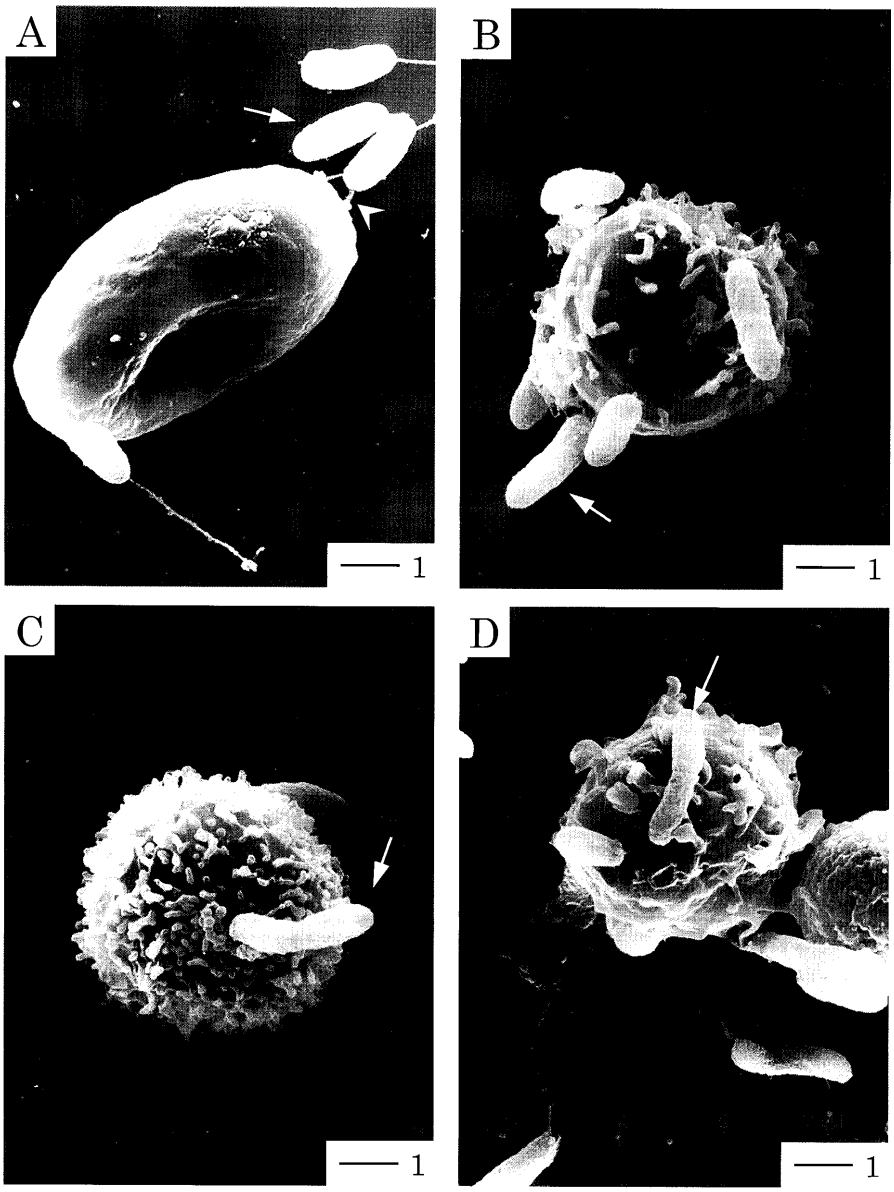

Fig. 5. Scanning electron micrographs showing infection to red cells (A) and white blood cells (B to D) of *V. vulnificus* strain NV1B. Infection was performed in RPMI 1640 medium for 1 h at 37°C. Cells in B, C, and D are most probably identified as granulocytes (probably neutrophils), lymphocytes (probably T cells), and monocytes (or macrophages). Arrows point to *V. vulnificus*. An arrowhead points to *V. vulnificus* pili. Bars = 1 μm.

5B), lymphocytes (probably T cells, Fig. 5C), and monocytes (or macrophages, Fig. 5C) provided a better adherence target for *V. vulnificus*.

V. vulnificus occasionally caused serious cytotoxic damages to some cells such as lymphocytes (Fig. 6). Chromatin was condensed, and separation of the outer and inner nuclear membranes was obviously seen. In those cells, the cellular membrane as well as cytoplasm was also damaged.

Invasion (or uptake) of *V. vulnificus* into cells was not obvious with any blood cells (Figs. 4 and 6). This was markedly contrast to the infection case of MRSA, in which invasion (or uptake) of MRSA was markedly seen (Fig. 7).

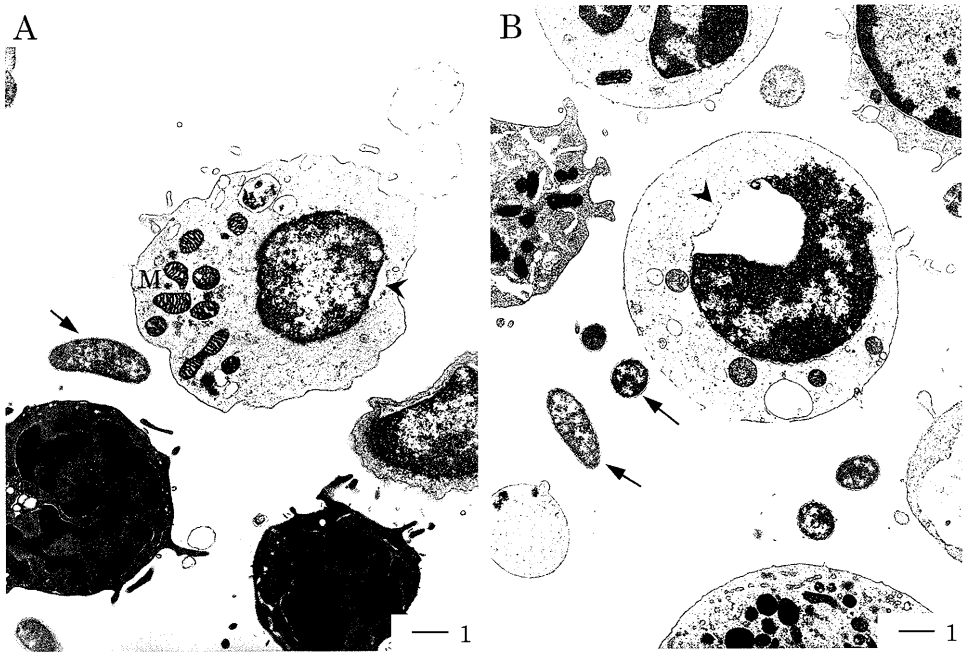

Fig. 6. Transmission electron micrographs showing the damage of white blood cells caused by *V. vulnificus* strain NV1B. Infection was performed in RPMI 1640 medium for 1 h (A) or 2 h (B) at 37°C. Cells are probably identified as lymphocytes. Arrows point to *V. vulnificus*. Arrowheads point to separation of the outer and inner nuclear membranes in the damaged cells. In A, mitochondria (M) is well seen in a dying cell. Bars = 1 μm.

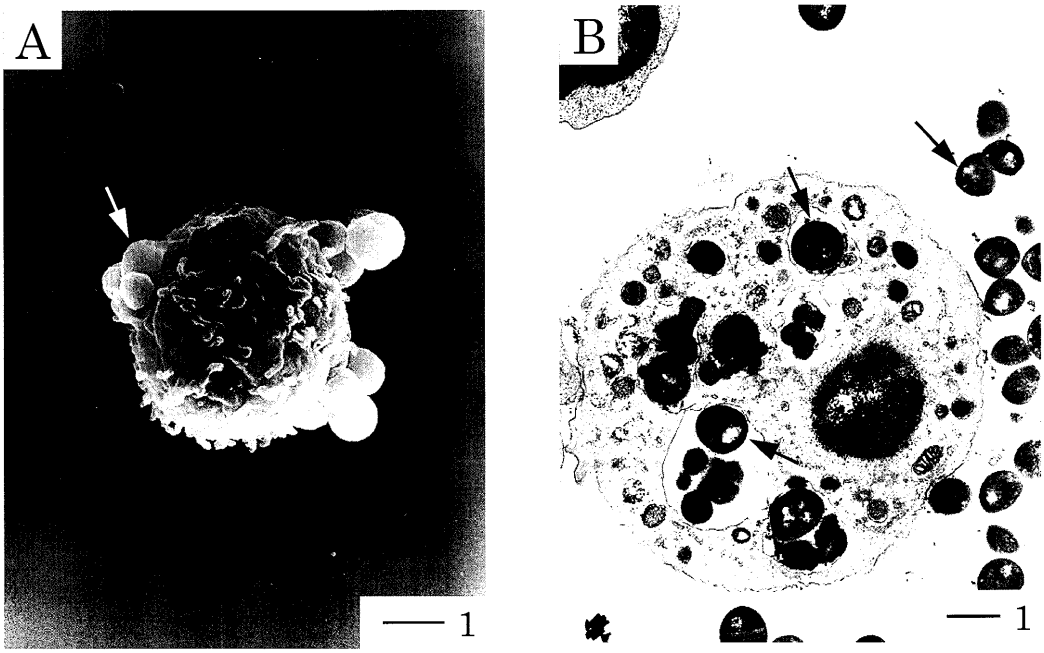

Fig. 7. Scanning (A) and transmission (B) electron micrographs showing infection to (or uptake by) white blood cells of MRSA strain E6. Infection was performed in RPMI 1640 medium for 1 h at 37°C. Cells are probably neutrophils. Arrows point to MRSA. Bars = 1 μm.

DISCUSSION

The pathogenesis of *V. vulnificus* has not fully been clarified. *V. vulnificus* possesses capsular polysaccharide, which protects *V. vulnificus* from phagocytosis by neutrophils and macrophages and from bacteriolysis by complement[12-15]. Such capsule was seen as a wrinkle-like structure under a scanning electron microscope. *V. vulnificus* produces cytolysin, a well-known extracellular toxin; however, its cytolysin knockout mutants are still virulent for mice[27]. And, metalloprotease-knockout mutants are also not significantly attenuated in animal infection models[28]. All *V. vulnificus* strains used in this study were also β-hemolytic on human erythrocyte-containing agar plates. This β-hemolysin activity may be responsible, in part, for the cytolytic activities of *V. vulnificus* observed with tissue culture cells and blood cells in this study. Oysters are heavily contaminated with *V. vulnificus*, especially in the United States, and *V. vulnificus* possesses pili (type IV pili) , which play a role in colonization and persistence in oysters[20]. Type IV pili are shered by several bacterial pathogengs, and are required for bacterial virulence to cause diseases such as cholera, pneumonia, gonorrhea, and meningitis[29]. For instance, the toxin co-regulated pilus (TCP) is required for *V. cholerae* colonization to human or animal intestines[30,31]. In this study, pilus-mediated *V. vulnificus* adherence to human blood cells was observed, indicating a possible role of *V. vulnificus* pili in human infection.

Interestingly, when *V. vulnificus* was grown with HCT-8 cells, pearl-like structure was observed. Such unique surface structure was not observed when the bacteria were grown in bacterial liquid media or agar plates. Also, such structures were not observed with other *Vibrio* species such as *V. cholerae* O1 and O139 or *V. parahaemolyticus*. The pearl-like structure adhered to HCT-8 cells, just like *V. vulnificus*. This suggests that the the pearl-like structure may play a role in human infection (as e.g., an adherence factor). Morphologically similar structures are reported with *V. campbellii* (a marine vibrio) as the tubular appendages of unknown function[32], although the relatedness of those structures are not known.

We previously reported a *V. vulnificus* infection case occurred in Niigata in 2003[11],. The patient was a 76-yer-old male who had medical history of diabetes, and was a heavy alcohol drinker. He did not eat raw fish or shellfish before the development of the symptoms, and the source of infection was unknown. The patient reported disturbance of consciousness, and mild swelling of the lower leg was observed. The same *V. vulnificus* strains (strains NV1bB and NV1L) were isolated from venous blood and puncture fluid of the lower leg. White blood cells and platelets were decreased, and the patient died.

In this study, we examined in vitro *V. vulnificus* (strain NV1B) infection to human blood cells. In accordance with the patient's laboratory findings, platelets and lymphocytes (especially platelets) were highly susceptible to the cyctolytic activity of *V. vulnificus*. Since no *V. vulnificus* cells were seen in those blood cells, in contrast to MRSA which also causes bacteremia, most probably *V. vulnificus* does not invade blood cells, but could produce toxins (including β-hemolysin) responsible for cell damages. This also suggests a possibility that *V. vulnificus* can prevent phagocytosis by bacterium-engulfing cells (such as neutrophils) by toxins.

One of the remarkable damages in white blood cells such as lymphocytes was the chromatin condensation and separation of the outer and inner nuclear membranes. This damage may be caused by an apoptosis-inducing factor. *V. vulnificus* can induce apoptosis in vitro and in vivo. Kashimoto et al.[21] showed that five clinical isolates, but none of the four environmental isolates induce apoptosis in murine macrophage-like cell line J774, and in mouse peritoneal macrophages. By using the same J774 infection assay, Gulig et al.[33] examined the apoptotic abilities of the set of 50 clinical and environmental *V. vulnificus*. They also found that the most strains have the ability to induce the apoptosis in macrophages[33]. Kashimoto et al.[34] reported that *V. vulnificus* induses lymphocyte, but not neutrophil, depletion in association with apoptosis in vivo. We also previously reported that clinical strains as well as freshly-isolated environmental strains, but not stored-environmental strains of *V. vulnificus*, markedly activated caspase proteolytic activities, such as caspase-3 of THP-1 human monocytic cells[22]. Moreover, the Rtx (repeat in toxin family of toxins) A gene is the most recently identified virulence factor, responsible for the apoptosis during *V. vulnificus* infection[35]. To gain a better understanding of the molecular mechanisms through which *V. vulnificus* causes serious systemic infection, further studies are required.

In conclusion, although *V. vulnificus* appears to be an opportunistic pathogen, it may display a unique mode of adherence and infection using bacterial appendages (pearl-like structure), and may damage blood cells such as platelets and leukocytes by producing toxins rather than invading cells, and prevent phagocytosis by bacterium-engulfing cells by toxins.

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