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Flow cytometric analysis for adhesion of *Vibrio cholerae* to human intestinal epithelial cell

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Abstract. The adhesion of *Vibrio cholerae* O1 strains to human intestinal epithelial cell, Intestine 407, was analyzed by flow cytometer. According to positive percentages of Intestine 407 cells adhered by *V. cholerae*, two groups of *V. cholerae* strains were classified as follows: more adhesive (more than 50%), less adhesive (less than 50%) strains. In addition, the fluorescence intensity after attachment of *V. cholerae* was directly correlated to the number of the microorganisms. It was concluded that flow cytometry is a useful and objective method for analyzing adhesion of *V. cholerae* to cultured cells.

Key words: Adhesion, Flow cytometry, Intestinal epithelial cell, *Vibrio cholerae*

Introduction

Cholera is a serious epidemic disease that has killed millions of people and continues to be a major health problem world wide. It is well known that cholera disease is caused by the action of cholera toxin, which stimulates fluid secretion by activating adenylate cyclase in intestinal epithelial cells [12, 14]. Colonization of the small intestine by *Vibrio cholerae* is a necessary step in the pathogenesis of cholera; however, the mechanisms by which colonization occurs are not fully understood.

Different putative adherence factors of *V. cholerae* O1 have been proposed to be involved in colonization [8–10, 16, 20]. One of these, a toxin-coregulated pilus, has been shown to be important for colonization of classical *V. cholerae* [11, 18–20]. However, the importance of the toxin-coregulated pilus as a colonization factor for EI Tor vibrio has been questioned [10, 13, 19]. It has been suggested that there are colonization factors other than the toxin-coregulated pilus that may be involved in colonization of intestine by El Tor *V. cholerae*. Moreover, various animal models have been developed to study the pathogenesis of cholera [4, 21]. Infant mice, who lack a complete immune system, are highly susceptible to natural infection with *V. cholerae*. Development of an in vitro model for objective study on the adherence properties of *V. cholerae* is consequently essential to understand colonization factors.

Flow cytometric analysis is an alternative modality for studying the attachment of bacteria. Although immunofluorescent staining of bacteria followed by microscopic observation is well established, many investigators have reported that flow cytometry provided a potentially powerful tool for analyzing bacterium-host cell interactions [5, 6, 22, 23].

Therefore, we addressed ourselves to develop a method for quantitative assessment of the adherence of *V. cholerae* to human cultured intestinal epithelial cells, using immunofluorescence and flow cytometer.

Materials and methods

Bacterial strains and culture conditions. Ten strains of *V. cholerae* O1 of either classical or El Tor biotype, each of which includes serotypes Inaba and Ogawa, were used; four classical biotypes are TC001 (Inaba), 569B (Inaba), Vc214 (Ogawa) and P13 (Ogawa) strains, and six El Tor biotypes are V86 (Inaba), TVC178 (Inaba), TVC138 (Inaba), V81 (Ogawa), VL5962 (Ogawa) and TVC8 (Ogawa) strains. The VL5962 had been isolated from environmental water, and the other strains had been isolated from patients with traveler's diarrhea. These *V. cholerae* strains were kindly provided by D. Y. Kudoh, Tokyo Metropolitan Research Laboratory of Public Health, Japan. The TVC8 strain is a phenotypically non-toxigenic and the VL5962 strain is a genotypically non-toxigenic. All strains were grown in Erlen-Meyer flasks containing Tryptic soy broth (Difco Laboratories, Detroit, USA) at 37 °C for 18 hours with shaking, and harvested followed by washing with phosphate-buffered saline (PBS; pH 7.4). The microorganisms were suspected in Hanks' balanced salts solution containing 0.1% gelatin (HGS), at $0.3–1.6 \times 10^8$ CFU/ml, and applied for the examination of the adhesion to cultured cells.

Cell culture methods. Intestine 407, human intestinal epithelial cell line, was provided by Dr Kanamori, Kyorin University School of Health Sciences. The cells were cultured in minimum essential medium (MEM) with Hanks' salt and L-glutamine (Gibco, New York, USA) supplemented with 5% fetal bovine serum (Wako Pure Chemical Industries Ltd., Osaka, Japan). The stock cultures were passaged at an interval of 4 days. The cultured cells were transferred to the 6 well-culture plates (∅ = 35 mm, Corning, New York, USA) prior to the experiment of the adhesion of *V. cholerae*.

Antibodies. Rabbits were immunized with either *V. cholerae* strain V86 serotype Inaba or *V. cholerae* strain V81 serotype Ogawa. Vibrio strains (1.0×10^{10} CFU/ml), as the antigens for immunization were fixed with PBS containing 2% glutaraldehyde at 4 °C for 18 hours, and then suspended in normal saline after washing with PBS. The antiserum against the antigen was obtained from rabbit immunized subcutaneously with the antigen in Freund's complete adjuvant (Difco) and thereafter injected intravenously 3 times at intervals of 7 days with the antigen. The antibody titer of anti-Inaba and anti-Ogawa serum was determined by enzyme-linked immunosorbent assay (ELISA) in microtiter plates coated with whole cells of each *V. cholerae* strain. The dilution of the sera showing an optical density (OD) of 0.5 at 490 nm was utilized as the first antibody to detect *V. cholerae* adhering to the cultured cells (Figure 1).

Fluorescein-5-isothiocyanate-conjugated goat affinity purified antibody to rabbit IgG (1:40. Organon Teknika Corporation, Cappel Research Products, Durham, USA) was used as the second antibody for the immunofluorescent staining.

Enzyme-linked immunosorbent assay (ELISA). ELISA for the determination of antibody titer against serotypes Inaba and Ogawa *V. cholerae* was developed by the methods described by Cryz et al. [3] with a slight modification. Vibrio strains (1.0×10^{8} CFU/ml) were fixed with PBS containing 2% glutaraldehyde at 4 °C for 18 hours, and then suspended in 0.1 M carbonate-bicarbonate buffer (pH 9.6) after washing with PBS. Polystyrene microtiter plates (Greiner labortechnik, Germany) were coated with the suspension of cells followed by incubation for 2 hours at 37 °C. The coated plates were washed three times with PBS, and reacted with serially diluted antisera at 37 °C for 2 hours. After the incubation, the plates were washed as described above and reacted with peroxidase-conjugated goat anti-rabbit IgG (1:500, Organon Teknika Corporation) at 37 °C for 2 hours. After 5 washings, the solution of 0.05% H_2O_2 and 0.08% 5-aminosalicylic acid (pH 6.0) in distilled water was added into each well, and the developed color was measured at OD_{490}.

Attachment of V. cholerae *to cultured cells and immunofluorescent staining for flow cytometry.* Three ml of HGS containing each *V. cholerae* strain ($0.3–1.6 \times 10^{8}$ CFU/ml) was added to Intestine 407 cells grown in 6-well culture plates ($1–5 \times 10^{6}$ cells/well). After incubation at 37 °C for 1 hour in the presence of 5% CO_2, the cells were washed with HGS 3 times and reacted with the first antibodies for 30 min at room temperature. After washing with HGS 4 times, the cells were subsequently reacted with the second antibody for 30 min at room temperature. In addition to washing with HGS 5 times, the monolayer cells were treated with HGS containing 0.025% trypsin and 0.01% EDTA. The cell suspension was immediately placed on an ice box and analyzed for bacterial adhesion by flow cytometer.

Analysis of bacterial adhesion by flow cytometer. Fluorescence intensity of the stained cells was measured with an EPICS-CS flow cytometer (Coulter Electronics, Hialeah, Florida, USA), which was equipped with an argon laser set at 500 mW and tuned to the excitation wavelength (488 nm). Fluorescence data were acquired in log mode on 256-channel scale (70 channels equivalent to 1 log decade) and processed by a Coulter Easy 88 microcomputer system linled to the flow cytometer. Cellular debris, unbounded bacteria, and cell clumps were excluded from further analysis by volume gating. The mean fluorescence channel number of a peak representing cell alone (autofluorescence) or cell with adherent bacteria was determined. And the percentage of the cells in the spectrum over the limit of the autofluorescence, manifesting the adhesion of bacteria to the cells, was also determined. For the flow cytometric analysis, $4–5 \times 10^{3}$ cells were scored, and at least three independent experiments were performed for each strain.

Adhesion assay. A number of vibrios to adhere to Intestine 407 cell was essentially examined by the method of Cravioto et al. [2]. Subconfluent cultures of Intestine 407 cells on plastic coverslips (∅ = 30 mm, Sumitomo Bakelite Co., Tokyo, Japan) were placed in the wells of the 6 well-culture plates (∅ = 35 mm, Corning) prior to the experiment of the adhesion of *V. cholerae*. Three ml of HGS containing 569B, TVC138, V86 or P13 strain ($1.2–1.6 \times 10^{8}$ CFU/ml) was added to Intestine 407 cells and incubated at 37 °C for 1 hour in the presence of 5% CO_2. After incubation, the coverslips were washed 3 times with PBS, fixed with 70% methanol, and stained with a 10% solution of Giemsa. The stained coverslips were mounted on glass slides and examined with a light microscope. Thirty cells were selected at random and the number of adhering microorganisms were counted. At least three independent experiments were performed for each strain.

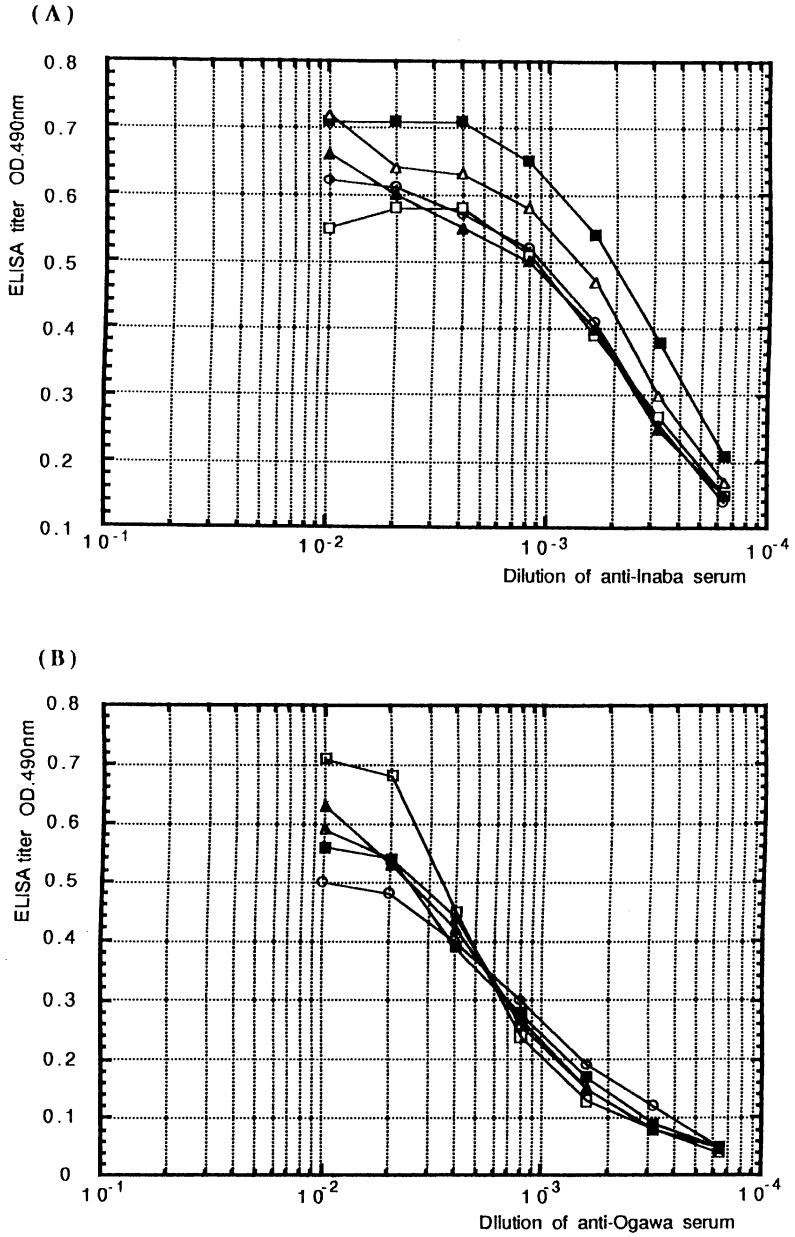

Figure 1. Reactivitis of anti-*V. cholerae* serotypes Inaba and Ogawa sera in ELISA. (A) Whole cells of *V. cholerae* serotype Inaba strains (□, TVC 178; △, 569B; ○, TVC 138; ■, TC001; ▲, V86) coated onto polystyrene microtiter plates were reacted with the serially diluted antiserum against *V. cholerae* strain V86 serotype Inaba. (B) Whole cells of *V. cholerae* serotype Ogawa strains (□, VL 5962; △, V81; ○, Vc 214; ■, P13; ▲, TVC 8) coated onto polystyrene microtiter plates were reacted with the serially diluted antiserum against *V. cholerae* strain V81 serotype Ogawa. The plates were reacted with peroxidase-conjugated goat anti-rabbit IgG, and substrate for the enzyme was added, followed by a measurement of OD490 nm.

Results

Reactivities of the antibodies against V. cholerae *serotypes Inaba and Ogawa.* The antibodies against *V. cholerae* serotypes Inaba and Ogawa were obtained from rabbits after immunization as described in Materials and methods. The reactivities of the anti-Inaba and anti-Ogawa sera with whole cells of vibrio serotypes Inaba and Ogawa strains were respectively examined by ELISA (Figure 1). The dilutions of sera showing 0.5 of OD_{490} in ELISA were utilized as the first antibody for immunofluorescent staining. The dilutions of anti-V86 (Inaba) antiserum, 1:2000, 1:1500, 1:900, 1:800, and 1:800, were applied for the immunostraiing of TC001, 569B, TVC138, V86 and TVC178 strains, respectively. Similarly, the dilutions of anti-V81 (Ogawa) antiserum, 1:350, 1:250, 1:250, 1:250, 1:250, and 1:100 were applied for VL5962, TVC8, V81, P3, and Vc214 strains, respectively.

Adherence of V. cholerae *to cultured cells*. The adhesion of *V. cholerae* strains to cultured epithelial cells was analyzed by flow cytometer. Positive percentage of the cells in the spectrum over the limit of the autofluorescence, which indicated the adhesion of the microorganisms to cultured cells, and mean fluorescence channel number of the peak are shown in Table 1. According to positive percentages of Intestine 407 cells adhered by *V. cholerae*, two groups of *V. cholerae* strains were classified as follows: more adhesive (more than 50%) and less adhesive (less than 50%) strains.

Figure 2 shows compared histograms of 569B, TVC138, V86 and P13 in the flow cytometric analysis. The former two and the latter two strains represent less and more adhesive strains, respectively. The mean fluorescence channel number of more adhesive strains was significantly higher than that of the autofluorescence of cells. In contrast, the mean fluorescence channel number of less adhesive strains was similar to that of the autofluorescence of cells.

In addition, a number of vibrios adhering to Intestine 407 cells observed by light microscopy was counted by adherence assay (Table 1). It was generally demonstrated that the number of microorganisms adhering to Intestine 407 cells correlated with the mean fluorescence channel numbers of the cells adhered with the strain.

From these results, it was confirmed that the positive percentage in the flow cytometeric analysis manifests the ability of adhesion of *V. cholerae* to Intestine 407 cells, and the fluorescence intensity correlates to the number of organisms adhered to the cell.

Discussion

It is obvious that detailed study at the cellular level on *V. cholerae*-host cell interactions will contribute toward understanding of the colonization by the microorganism. Therefore, in the present study we have evaluated adherence of *V. cholerae* to cultured human intestinal epithelial cells by flow cytometric analysis.

Flow cytometry is a potentially effective tool for analyzing bacterium-host cell interactions [5, 6, 22, 23]. However, flow cytometric analysis requires that individual cells should be suspended in a suitable buffer. Usually, 0.25% trypsin in PBS is utilized to rip up cells from culture plates. In the present study, lower concentrations of trypsin (0.025%) and EDTA (0.01%) were used for preparation of cultured cells to avoid overdigestion of cell surface structures involved in the binding to the microorganisms prior to flow cytometric analysis. In addition, the reactivity of anti-*V. cholerae* antibodies against *V. cholerae* serotype Inaba or Ogawa was examined by ELISA in order to determine the serum dilution used for the flow cytometric analysis. We attempted to develop a method for quantitative assessment of the adherence of *V. cholerae* to cultured intestinal epithelial cells in this manner.

According to the positive percentage of Intestine 407 cells adhered by *V. cholerae*, two groups of the microorganism were classified; more and less adhesive strains. In addition, the mean fluorescence channel numbers of cells adhered with each strain correlated to the number of the adhering microorganisms. In 9 clinical isolates, the adhesion rate ranged from 0.7% to 98.4%. The adherence rate of VL5962 isolated from environmental water was

Table 1. Adherence of *V. cholerae* strains to Intestine 407 cells

Strain	Biotype and serotype		Flow cytometric analysis		Adherence assay
			% adherence[a]	Mean channel No. ± SD[b]	No. of bacteria/cell
TC 001	Classical	Inaba	0.7	62.5 ± 21.2	ND[c]
569B	Classical	Inaba	1.2	60.1 ± 21.5	0.14 ± 0.5
TVC 8[d]	EL Tor	Ogawa	23.0	69.8 ± 28.6	7.1 ± 1.9
TVC 138	El Tor	Inaba	24.7	87.4 ± 50.0	7.7 ± 3.5
Vc 214	Classical	Ogawa	28.3	101.3 ± 34.6	9.6 ± 2.7
V86	El Tor	Inaba	53.1	140.1 ± 64.5	14.8 ± 5.8
TVC 178	El Tor	Inaba	59.2	123.9 ± 48.7	12.2 ± 3.9
P13	Classical	Ogawa	96.0	193.0 ± 40.1	35.3 ± 7.3
VL 5962[e]	El Tor	Ogawa	98.0	203.9 ± 48.7	ND
V 81	El Tor	Ogawa	98.4	215.1 ± 37.7	ND

[a] The percentage of the cells in the spectrum over the limit of the autofluorescence of Intestine 407, manifesting the adhesion of *V. cholerae* to the cells, was determined.
[b] Mean channel No. of control Intestine 407 was 58.0 ± 20.0.
[c] Not done.
[d] Phenotypically non-toxigenic strain.
[e] Genotypically non-toxigenic strain.

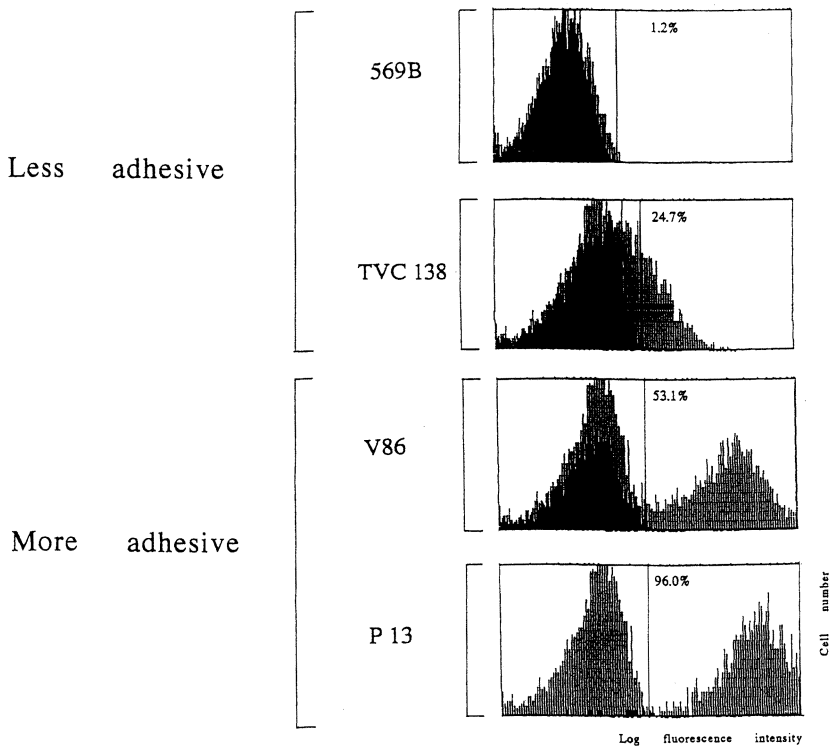

Figure 2. Flow cytometric analysis for adhesion of *V. cholerae* to Intestine 407 cells. Two patterns of compared histograms which manifested the adhesion of *V. cholerae* represent more and less adhesive strains.

98.0%. The occurrence of watery diarrhea by less adhesive *V. cholerae* strains presented in this study might be associated with other factors such as proliferative activity or toxin production after adhesion to epithelial cells.

Attridge & Rowley [1] have reported the role of the flagellum in the adherence of *V. cholerae*. They have shown that V86 strain is well adherent to small intestines of adult mice in in vitro attachment assay, but 569B is not. Our survey demonstrated a similar result that 569B or V86 strain belongs to the less or more adhesive strain, respectively. Therefore, we confirmed that the flow cytometric analysis for adhesion of *V. cholerae* to Intestine 407 cells is an useful and objective method.

In recent communications on adhesion of *Escherichia coli* to cultured cells, three patterns of adherence have been described: localized adherence in which micro colonies are formed at one or more places on the cells [7], diffuse adherence where bacteria cover the cells uniformly [17] and the enteroadherent-aggregative pattern in which the bacteria have a characteristic stacked brick appearance on the surface of the cells and on the glass slide free from the cells [15]. It is possible that the adhesion pattern of *E. coli* can be distinguished by the method presented in this paper.

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