Induction of Fimbriated *Vibrio cholerae* O139

MASAHIKO EHARA, 1+ MAMORU IWAMI, 1 YOSHIIO ICHINOSE, 1 TOSHIYA HIRAYAMA, 1 M. JOHN ALBERT, 2 R. BRADLEY SACK, 3 AND SHOICHI SHIMODORI 4

Department of Bacteriology, Institute of Tropical Medicine, Nagasaki University, 1-chome 12-4 Sakamoto, Nagasaki 852, 1 and Department of Microbiology, School of Health Sciences, Kyushu University, Maizahi 3-1-1, Higashi-ku, Fukuoka 812, 2 Japan; Laboratory Sciences Division, International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka-1000, Bangladesh 3; and Division of Geographic Medicine, School of Public Health, Johns Hopkins University, Baltimore, Maryland 21205 4

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Several fimbriated phases of *Vibrio cholerae* O139 strains were selectively induced and compared immunologically and biochemically with those of *V. cholerae* O1. Fimbrial antigens were detected on the surfaces of fimbriated vibrios colonization of the epithelial cells of a rabbit small intestine. Convalescent-phase sera from six individuals infected with *V. cholerae* O139 revealed the development of antibody against the fimbribin. These findings suggest that the fimbriae of *V. cholerae* O1 and O139 are expressed in vivo during infection and that this consideration must be given to the use of fimbrial antigens as components of vaccines against cholera.

Cholera is an acute diarrheal disease of humans, and the responsible bacteria are toxigenic *Vibrio cholerae* O1 and O139. Like other diarrheal diseases, a cholera infection is initiated through the fecal-oral route, usually by means of contaminated food and water supplies. The development of oral rehydration solution has dramatically reduced the number of fatalities from cholera, but inappropriate health education or adequate provision of safe water supplies appear to be a major stumbling blocks in eradicating this and several other diarrheal diseases. Cholera is still a serious public health problem in developing countries, particularly those in tropical regions. The persistence of cholera in developing countries and the recent outbreak of *V. cholerae* O139 have stimulated considerable research into the molecular analysis of the pathogenesis of *V. cholerae* O1 or O139, resulting in the identification of a number of critical components required for both colonization of the intestinal mucosa and manifestation of disease symptoms. However, no entirely satisfactory vaccine is in widespread use, and our understanding of the pathogenesis of the disease is still far from complete. Like *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa*, *V. cholerae* O1 and O139 have type 4 fimbriae. It is not yet known whether vibriob cells colonizing epithelial cells are fimbriated. It is also not clear whether some other factors participate in the pathogenesis of cholera. We have already reported on the fimbriated phase of *V. cholerae* O1 by using Bgld17 strain (classical biotype and Inaba serotype) isolated in Bangladesh in 1982 (5). The fimbriate of *V. cholerae* O1 are hydrophobic and have hemagglutination activity that is sensitive to D-glucose and D-mannose (6). We applied the same method for the selective induction of fimbriated cells described previously (5) to strains of *V. cholerae* O139. In this report, we describe the fimbriated phase of *V. cholerae* O139 and the direct detection of fimbriae on the surfaces of vibrio cells colonizing the epithelial cells, together with the results of an analysis of paired serum specimens from six individuals infected with *V. cholerae* O139.

**MATERIALS AND METHODS**

**Bacterial strains.** The AI1841 and AI1855 strains of *V. cholerae* O139 were used for the selective induction of fimbriated cells.

**Media.** TCG medium (1% Bacto Tryptone, 0.2% yeast extract, 0.5% NaCl, 0.3% NaHCO3, 0.02% thioproline, 0.1% myo-inositol, 5 mM J-glutamate, 1 mM EGTA) was used for the selective induction of fimbriated cells in the presence of 0.5% chitin. Fimbriated vibrios were grown in alkaline tryptone broth (1% Bacto Tryptone, 0.3% yeast extract, 0.5% NaCl, 0.2% NaHCO3).

**Selective induction of fimbriated *V. cholerae* O139.** Two strains of *V. cholerae* O139, AI1841 and AI1855, were selectively induced to the fimbriated phase by following the methods described previously (5). Briefly, vibriob cells were subcultured in 200-ml conical flasks containing 50 ml of TCG broth at 37°C under static conditions in the presence of chitin. After every subculture, vibrio cells adhering to chitin were extensively washed with normal saline by shaking vigorously. This subculturing process was repeated until the vibriob cells started to form pellets.

**Purification of fimbriae.** Fimbriae were purified from the fimbriated strain of AI1855 by following the methods described previously (6).

**Ligated rabbit ileal loop test.** Ligated loops produced by the method of De and Chatterje (2) were inoculated with 0.1 ml of an overnight alkaline tryptone broth culture of fimbriated vibrio strains. The rabbit was killed at 8 h postinfection.

**Analysis of convalescent-phase sera from cholera patients infected with V. cholerae O139.** Paired serum specimens were obtained from six cholera patients who were bacteriologically diagnosed as having cholera and who were admitted to the International Centre for Diarrhoeal Disease Research, Bangladesh, Hospital and were examined by immunoblotting to determine whether or not immunoglobulin G (IgG) antibodies against fimbriae had developed in the peripheral blood. Preimmune sera were collected 3 days after the onset of clinical symptoms. Crude fimbrial fractions, obtained by centrifugation, were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were electroblotted onto a nitrocellulose membrane (Bio-Rad, Richmond, Calif.) by using a Bio-Rad electroblotting apparatus (70 V for 1 h). Convalescent-phase sera were diluted 20-fold. A sheet of nitrocellulose membrane was incubated either with polyclonal antibody against fimbriae as a positive control for fimbriob or with convalescent-phase sera. It was then developed with peroxidase-conjugated goat anti-human IgG.

**SDS-PAGE and immunoblotting.** SDS-PAGE was performed as described by Laemml (9). The samples were boiled for 5 min in final sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.001% bromophenol blue with 5% (vol/vol) β-mercaptoethanol prior to electrophoresis through 5% stacking and 15% separating gels. The gels were then either stained in Coomassie brilliant blue or transferred electrophoretically to 0.2-mm- pore-size nitrocellulose membrane for immunoblotting (13).

**Electron microscopy.** For negative staining, one drop of the sample was placed on a sheet of Parafilm (American National Can, Greenwich, Conn.), and a Formvar-coated copper grid was floated on the drop for 2 min. The excess liquid was removed with filter paper. The specimen was washed with distilled water three times for 10 s each time and was then stained with 1% uranyl acetate for 30 s. The excess stain was removed by using the tip of a piece of filter paper. The specimen was examined by an JEM 100CX electron microscope operated at 80 kV.

For sample preparation for scanning electron microscopy, a part of the washed loop culture of fimbriated vibrio strains was used as a positive control for fimbrillin or with convalescent-phase sera. It was also not clear whether some other factors participate in the pathogenesis of cholera. We have already reported on the fimbriated phase of *V. cholerae* O1 by using Bgld17 strain (classical biotype and Inaba serotype) isolated in Bangladesh in 1982 (5). The fimbriate of *V. cholerae* O1 are hydrophobic and have hemagglutination activity that is sensitive to D-glucose and D-mannose (6). We applied the same method for the selective induction of fimbriated cells described previously (5) to strains of *V. cholerae* O139. In this report, we describe the fimbriated phase of *V. cholerae* O139 and the direct detection of fimbriae on the surfaces of vibrio cells colonizing the epithelial cells, together with the results of an analysis of paired serum specimens from six individuals infected with *V. cholerae* O139.
reacted with monoclonal antiserum against the fimbriae of *V. cholerae* O1 (monoclonal antibody 42; IgG) for 15 min. Normal mouse serum was used as a control. The specimen was washed a further three times and was reacted for 15 min with a few drops of 30-nm colloidal gold-labeled anti-mouse IgG (heavy and light chains) goat serum (E. Y. Laboratories, Inc., San Mateo, Calif.). The sample was rinsed as described above and was dehydrated through a series of ethanol baths, dried to the critical point, and coated with gold-palladium. Post-fixation with osmium tetroxide was omitted. The specimen was examined with a JSM 840A scanning electron microscope operated at 10 kV.

**RESULTS**

**Induction of fimbriated cells.** Fimbriated cells of *V. cholerae* O139 were selectively induced by five serial subcultures in the presence of chitin. These fimbriated cells form pellicles when they are cultured in a liquid medium under static conditions and autoagglutinate in normal saline (Fig. 1). When cultured on a solid agar plate, fimbriated cells form rough surface colonies with irregular edges (Fig. 2). The same studies were also applied to strain AI1841, with similar results.

**Immunoelectron microscopy of fimbriated cells.** A portion of the pellicle formed by fimbriated strain AI1855 was suspended in normal saline containing 1% glucose to separate autoagglutinated cells. The hemagglutinating activity of the fimbriated cells is also inhabitable with 1% glucose and D-mannose. The separated cells were detected immunologically by using a monoclonal antibody against the fimbriae of *V. cholerae* O1 (monoclonal antibody 42) (Fig. 3).

**Purification of fimbriae.** The subunit of fimbriae purified from fimbriated strain AI1855 is 17.5 kDa, as estimated by

![FIG. 1. Electron micrograph showing autoagglutinated cells of *V. cholerae* O139 AI1855. Bar, 0.5 μm.](image1)

![FIG. 2. Comparison of colonial characteristics of nonfimbriated cells and fimbriated cells. Strains of nonfimbriated and fimbriated AI1855 were streaked onto BTB agar plates and were examined with transmitted oblique illumination after 48 h at 37°C.](image2)
SDS-PAGE, and is immunologically and biochemically identical to that of *V. cholerae* O1 (Fig. 4). The N-terminal amino acid sequence of the fimbrillin of fimbriated strain AI1855 is completely identical to that of the fimbrillin of *V. cholerae* O1 (data not shown).

**Direct detection of fimbral antigens in vivo.** Fimbrial antigens were detected on the surfaces of vibrio cells colonizing epithelial cells of the rabbit small intestine. Fimbrial antigens appeared as lumpy structures, possibly due to drying to the critical point (Fig. 5). These antigens were not detected on the surfaces of vibrio cells when normal mouse serum was used as a control.

**Analysis of convalescent-phase sera from patients infected with *V. cholerae* O139.** When sera diluted 200-fold were used to demonstrate the presence of a specific antibody against fimbrae, fimbrillin bands were undetectable by Western blotting, as reported previously for the convalescent-phase sera from patients infected with *V. cholerae* O1 (4). With less diluted sera (20-fold), the fimbrillin bands were visible. There was little difference in the intensity of the reaction to fimbrillin between the convalescent-phase sera infected with *V. cholerae* O1 (4) and sera infected with O139 (Fig. 6). In none of the preimmune sera tested were elevated antibody levels against fimbrillin detected (data not shown).

**DISCUSSION**

This study has described the fimbriated-phase cells of *V. cholerae* O139. Fimbrial antigens were first demonstrated on the surfaces of vibrio cells colonizing the epithelial cells. Combined with the previous data for fimbriated *V. cholerae* O1, *V. cholerae* O1 and O139 have two distinct phases, the flagellated phase and the fimbriated phase. Cells in the flagellated phase are motile and form smooth colonies; on the other hand, fimbriated-phase cells are nonmotile and form rough colonies different from rugose colonies (10). Fimbriated cells of *V. cholerae* O1 and O139 are hydrophobic and form a pellicle when they are cultured in a liquid medium under static conditions. These are common features of fimbriated organisms with type 4 fimbrae. When isolated from stool specimens from cholera patients, all strains of *V. cholerae* O1 and O139 form smooth colonies and are motile (6b). After detachment from epithelial cells, vibrio cells seem to change their phase quickly.
to the flagellate phase. It has not been elucidated whether vibrio cells colonizing epithelial cells in the small intestine of humans are fimbriated. The history of cholera research has provided little information that can be used to answer this question. Only the presence of antibodies to fimbriulin in the convalescent-phase sera from patients suggests that the fimbriae of vibrio cells are expressed in vivo. All preimmune sera used in this study revealed no elevated levels of antibody against fimbriae, suggesting that the infections with strains of *V. cholerae* O139 were primary and not recurrent. Recently, two reports, one by Thelin and Taylor (12) and one by Attridge et al. (1), have indicated the relative contributions of toxin-coregulated pilus (TCP) and cell-associated mannose-sensitive hemagglutinin (MSHA; type 4 fimbriae) to the colonization abilities of *V. cholerae* O1 strains of the El Tor biotype and O139 Bengal strains by using isogenic parental and in-frame deletion mutant pairs in the infant mouse cholera model. In those two studies, little attention was paid to phase variation. It is not yet clear whether vibrio strains change their phase when inoculated into the small intestine of a suckling mouse. These two papers tell us the present situation for stock strains of *V. cholerae* O1 of the El Tor biotype and O139 Bengal. When tested by Western blotting, all strains of the El Tor biotype and O139 Bengal cultured in AKI medium (8) express TCP and MSHA. The TCP was not recognized by the convalescent-phase sera, and solid long-term protection can be engendered in the absence of a detectable anti-TCP immune response (7). Fimbriae (MSHA) of *V. cholerae* O1 and O139 were recognized by the convalescent-phase sera. This is a distinct difference in the reactivity in vivo between these two potential colonization factors. TCP is suggested to be a receptor for the hypothetical filamentous phage CTXf (14). Recently, we found two types of filamentous phage (types fs1 and fs2) (3). These two types of filamentous phage require type 4 fimbriae (MSHA) as receptors (11). Freshly isolated strains of *V. cholerae* O1 biotype El Tor harbor the plasmid (replicative-form [RF] DNA) encoding fs1 or fs2 (3), although most stock strains of *V. cholerae* O1 lack the RF DNA of fs1 or fs2 when the strains are examined by PCR (6a). Furthermore, the filamentous phage fs1 was inducible by gene expression in vivo when freshly isolated strains of *V. cholerae* O1 biotype El Tor were cultured in ligated rabbit ileal loops (3). These findings suggest that the phase variation in vivo, i.e., from the flagellated to the fimbriated phase, may be related to the lysogeny of a filamentous phage. Thus, animal experiments with stock strains lacking the RF DNA of fs1 or fs2 do not necessarily reflect the real colonization potential, because stock strains of *V. cholerae* do not harbor the RF DNA of a filamentous phage. The vibrio strains AI1841 and AI1855 used in this study were found to be lysogenic, producing the filamentous phage fs1. Fimbriated cells of *V. cholerae* O139 harboring the RF DNA of fs1 were easily induced, in contrast to the induction of *V. cholerae* O1
Bgd17 of the classical biotype, which lacks the RF DNA of fs1. It is not known why some strains with the RF DNA of a filamentous phage became fimbriated after a few passages and others lacking the RF DNA required many serial transfers. Whether all cholera patients discharge a certain type of filamentous phage in their stool specimens remains to be elucidated. The relation of a filamentous phage with phase variation also remains to be studied. Our findings presented in this report suggest that consideration must be given to the use of fimbrial antigens of *V. cholerae* O1 or O139 as components of vaccines against cholera.

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