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Adherence of *Vibrio parahaemolyticus* to Human Epithelial Cell Lines

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Light microscopy and a radioassay detected no significant difference in adherence of Kanagawa-positive and Kanagawa-negative strains of *Vibrio parahaemolyticus* to human epithelial cell lines.

Vibrio parahaemolyticus is a marine bacterium that causes gastroenteritis after consumption of contaminated seafood. Isolates of this microorganism that are hemolytic when cultivated on Wagatsuma agar are termed Kanagawa-positive strains (7). With few exceptions (10), food and environmental isolates of *V. parahaemolyticus* are Kanagawa negative, while fecal isolates from cases of gastroenteritis are routinely Kanagawa positive (7, 8). Although the Kanagawa reaction is presently considered to be the best marker for enteropathogenicity, the role of the hemolysin in gastroenteritis or its possible association with other virulence factors is unknown.

Adherence of bacterial cells to mammalian cells correlates with the ability of many bacterial species to cause infections of epithelial surfaces of the intestinal, respiratory, urinary, and genital tracts (4, 9). Using a microscopic assay, Carruthers (3) reported that Kanagawa-positive *V. parahaemolyticus* strains adhered better than Kanagawa-negative strains to human epithelial cell lines. The purpose of this study was to investigate the adherence of *V. parahaemolyticus* to mammalian cells by using light microscopy and a radioassay (1) to study a possible relationship between hemolysin production and adherence.

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MATERIALS AND METHODS

V. parahaemolyticus W-54, A-97, SN-36, and SN-72 were obtained from S. P. De, Cholera Research Centre, Calcutta, India. Strain ATCC 27969 was supplied by R. R. Colwell, University of Maryland, while the remaining strains were obtained from M. J. Voll, University of Maryland, who originally obtained the cultures from B. Wentz, Food and Drug Administration, Washington, D.C. The Kanagawa reaction of all strains was determined on Wagatsuma agar (7) and agreed with the designation of the suppliers. Intestine 407 cells were purchased from the American Type Culture Collection, and human intestine cell line 11000 (Flow 11000) was obtained from Flow Laboratories (Rockville, Md.). Human cell lines were routinely cultured in Dulbecco modified Eagle medium containing 10% fetal calf serum. When used in adherence experiments, intestine 407 cells were trypsinized, and 1.25 $\times 10^5$ cells were added to 15-mm-round Thermonox plastic cover slips (Lux Scientific Corp., Newburg Park, Calif.) which were in 16-mm wells of Costar 24-well cluster plates. Flow 11000 cells were trypsinized and added at a concentration of 2.8×10^4 cells per cover slip. Monolayers were incubated at 37°C in a humidified atmosphere at 5% CO_2 in air. Four to five days later, when monolayers were confluent, the cells were washed twice in Earle balanced salt solution containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.0 to 7.2) and 43 μM unlabeled valine (EBSS-HV). After removal of final wash fluid, monolayers were used in adherence experiments.

Growth medium for *V. parahaemolyticus* contained, per liter: K_2HPO_4, 9.5 g; KH_2PO_4, 3.0 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4$, 0.1 g; NH_4NO_3, 1.0 g; NaCl, 25 g; and glucose, 5 g (J. A. Baross, Ph.D. thesis, University of Washington, Seattle, 1972). Ten milliliters of medium containing 1 μCi of [U-^{14}C]valine (285 mCi/mmol, Amersham) was inoculated and incubated at 37°C in a shaking water bath. Cell numbers were estimated by reference to a standard curve, plotting Klett units versus colony-forming units. Exponentially growing bacteria were harvested by centrifugation and suspended in EBSS-HV to a concentration described in each experiment. To measure total counts incorporated into vibrios, we precipitated duplicate 1.0-ml cell samples with cold 5% trichloroacetic acid, washed them on 0.45-μm (pore size) membrane filters (Millipore Corp.) with 5% trichloroacetic acid and ethanol, dried them at 55°C overnight, and counted them for 20 min in 10 ml of a toluene-based scintillation fluid. One-milliliter samples of labeled vibrios were added to each monolayer, and the mixture was incubated at 37°C on a metabolic shaking incubator (Precision Scientific Co.) at 90 oscillations per min. At each time interval that adherence was measured, unattached vibrios were removed by aspiration from four cover slips. Cover slips were washed twice in the wells, removed, and washed on both sides with EBSS-HV. Each of three cover slips was placed, with monolayer side up, in scintillation vials, dried at 55°C overnight, and counted in 10 ml of toluene-based scintillation

fluid. Percent adherence was expressed as the average of the triplicate counts on monolayers times 100 divided by the total counts in cells added to monolayers (1). The fourth cover slip, which was fixed in methanol and stained with 0.008% crystal violet, was used to microscopically measure adherence and evaluate the confluence of monolayers after incubation with vibrios. In the microscopic assay, the number of attached vibrios was determined by examining a minimum of 50 mammalian cells under oil immersion.

RESULTS

When various concentrations of ^{14}C-labeled vibrios were incubated for 45 min with monolayers of intestine 407 cells, the curves for adherence measured by microscopy and radioassay were parallel up to the highest concentration of vibrios added, at which point visual estimation of numbers of attached vibrios was difficult (Fig. 1). The percentage of vibrios adhering was nearly constant up to an apparent saturation point. In further experiments, strains were tested for adherence below the saturation level of ca. 5×10^6 attached vibrios per monolayer and 30 vibrios per mammalian cell. When the experiment was repeated using Flow 11000 cells, the number of vibrios added was proportional to adherence of up to 6×10^6 vibrios per monolayer and 50 vibrios per mammalian cell.

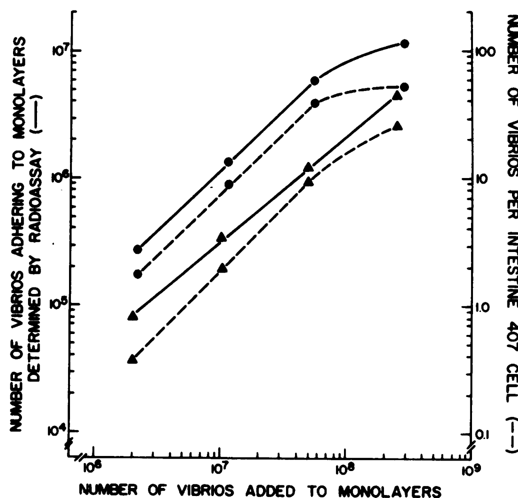

FIG. 1. *Adherence of V. parahaemolyticus to intestine 407 cells measured by radioassay and light microscopy as a function of number of vibrios added to monolayers. Symbols: ●, Strain A-97; ▲, strain 5E-2. Strain A-97 is Kanagawa negative, and strain 5E-2 is Kanagawa positive. The number of vibrios adhering to monolayers was determined in the radioassay by multiplying percent adherence times number of vibrios added to monolayers. The number of vibrios per intestine 407 cell was determined by examining a minimum of 50 intestine 407 cells under oil immersion. The specific activity of both strains, A-97 and 5E-2, was ca. 4×10^{-4} cpm per colony-forming unit.*

To compare the adherence of seven Kanagawa-positive and nine Kanagawa-negative strains, we incubated 4.0 to 10^7 vibrios with intestine 407 cells for 20, 40, and 60 min. Only data for 40 min of incubation are presented (Fig. 2) because adherence for all strains approximately doubled between 20 and 40 min, while a few strains gave decreased adherence between 40 and 60 min with partial destruction of monolayers. The mean of 4.4 for Kanagawa-positive vibrios per mammalian cell and 11.4 for Kanagawa-negative vibrios was not significantly different by Student's t test. Greater than 90% of the vibrios was seen on cells rather than on occasional bare spots on the cover slip. In the radioassay, the mean percent adherence of 2.4 for Kanagawa-positive and 4.4 for Kanagawa-negative strains also was not significantly different. There was considerable overlapping in the amount of adherence of Kanagawa-positive and Kanagawa-negative strains with the greater mean adherence of Kanagawa-negative vibrios due primarily to strains W-54 and A-97. When the vibrio strains were tested for adherence to Flow 11000 cells, the mean percent adherence of 1.0 for Kanagawa-positive strains and 2.1 for Kanagawa-negative strains was not significantly different by Student's t test.

DISCUSSION

Because the amount of adherence observed varied over greater than a 12-fold range for different vibrio isolates, strain selection for ex-

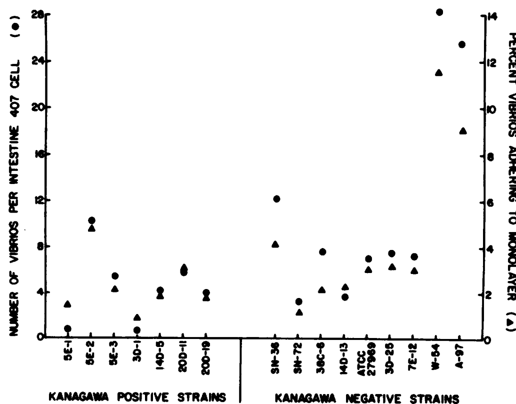

FIG. 2. *Adherence of Kanagawa-positive and Kanagawa-negative strains to intestine 407 cells measured by microscopy and radioassay. The number of vibrios per intestine 407 cells was determined by microscopic assay. The percentage of vibrios adhering to monolayers was measured by radioassay. The 4.0×10^7 vibrios added to monolayers contained ca. 3×10^4 cpm. The standard error for repeated assays was not greater than ±15%.*

periments becomes an important factor in comparison of adherence. Carruthers (3) tested seven Kanagawa-positive and three Kanagawa-negative strains with one cell line and tested one Kanagawa-positive and one Kanagawa-negative strain with a second cell line. The present data indicate that the amount of adherence of Kanagawa-positive and Kanagawa-negative strains overlaps to the extent that better adherence is not consistently associated with the presence or absence of hemolysin.

The results of the radioassay and microscopic assay were in close agreement for individual strains (Fig. 2). Therefore, it is unlikely that release of labeled proteins from vibrios and their subsequent attachment to mammalian cells could have accounted for the similarity in mean adherence of Kanagawa-positive and Kanagawa-negative strains measured in the radioassay. A defined medium was used in these studies to efficiently label vibrios with [^{14}C]valine. Cells from exponentially growing cultures were compared in adherence because the percent adherence varied little when vibrios were harvested at various time intervals during exponential growth but decreased when cells from overnight cultures were tested. It is possible that Kanagawa-positive and Kanagawa-negative strains could differ in adherence if cells were grown under cultural conditions other than those used in the present studies.

The failure to find a correlation between hemolysin production and adherence does not preclude a role for adherence in the virulence of *V. parahaemolyticus*. Differences in pathogenicity among strains may be due to virulence factors that act subsequently to attachment and are unique to Kanagawa-positive strains. This hypothesis is consistent with the report of Boutin et al. (2) that both Kanagawa-positive and Kanagawa-negative strains penetrated the lamina propria of rabbit ligated ileal loops, whereas only Kanagawa-positive strains produced fluid accumulation and inflammation. In addition, Joseph et al. (5) observed by electron microscopy adherence of a Kanagawa-negative strain to the villus of the rabbit ileum. Adherence may play an important role both in virulence and in survival of this bacterium in the marine environment (6).

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