INFECTION AND IMMUNITY, Nov. 1978, p. 387-392
0019-9567/78-0022-0387$02.00/0

Vol. 22, No. 2
Printed in U.S.A.

Role of Motility in Experimental Cholera in Adult Rabbits

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Received for publication 29 August 1978

The role of motility in the pathogenesis of cholera was evaluated in ligated ileal loops of adult rabbits. Four strains of *Vibrio cholerae* (including both Inaba and Ogawa serotypes of both classical and El Tor biotypes) were compared with their aflagellated, but fully toxigenic and prototrophic, isogenic derivatives as to their ability to produce fluid accumulation in the rabbit gut. The nonmotile mutants required an at least 100-fold-higher dose than their respective wild-type strains to produce comparable fluid accumulation responses. The decreased ability of nonmotile strains to produce a fluid response was not due to their failure to multiply in vivo, since they increased in numbers in the rabbit ileum at the same rate as the wild-type strains, but probably was related to their inability to associate with the intestinal mucosa. After 3 h of incubation, 45 to 53% of motile, [^{35}S]-labeled cells adsorbed to the intestinal wall, whereas only 3 to 15% (depending upon the strain) of the nonmotile bacteria were associated.

Although undeniably a toxigenic disease, there are other factors involved in the pathogenesis of cholera (3). With the infant mouse model, introduced by Ujiiye and co-workers (18, 19), Guentzel and Berry (10) found that *Vibrio cholerae* must be motile to produce apparent illness. Nonmotile but toxigenic and prototrophic derivatives of highly virulent Ogawa and Inaba serotypes of classical and El Tor strains showed greatly reduced virulence for suckling mice as compared with the motile parental strains. The loss of virulence of these aflagellated strains was characterized by their inability to colonize the small intestines of the orally challenged infants (11). Motile organisms were found by fluorescent-antibody staining to be able to penetrate into the intervillous spaces and deep within the crypts of LieberKühn. Nonmotile organisms, when present, remained confined to the intestinal lumen. Nonmotile strains were also less virulent than parental strains for adult mice infected intraperitoneally with the organisms suspended in hog gastric mucin (6).

Other investigators also have reported that motility is related to the virulence of vibrios. Verwey (20) suggested that the organism must be motile to migrate into the intervillous spaces of the intestinal mucosa and to produce disease. In studies of isolated ileal loops in adult rabbits, Williams et al. (21) observed that a nonmotile strain was less efficient in producing positive loops than was its motile parent.

Lankford (14) and Lankford and Legsombur-

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ana (15) postulated an important role of adhesive factors in cholera infection. Freter, Jones, and Abrams (9, 12, 13) studied adhesion of vibrios to isolated brush borders of the rabbit ileum and found that unless the organisms were motile they failed to adhere in vitro. Neither motility nor the flagellum was identified as a primary requirement for adherence, but loss of motility was believed to be associated with the simultaneous loss of adhesions from the vibrio surface (13).

The present report is an extension of these studies and strengthens the evident that motility plays a significant role in the pathogenicity of *V. cholerae* as evidenced in ligated ileal loops of adult rabbits. The results also confirm those obtained in infant mice and suggest that association of vibrios with the intestinal mucosa is necessary for the onset of cholera in these experimental animal models.

MATERIALS AND METHODS

Rabbits. Adult rabbits weighing 2 to 4 kg were obtained from local breeders.

Bacterial strains. The strains of *V. cholerae* and their nonmotile derivatives used in these studies have been described previously (10). All nonmotile strains were determined by transmission electron microscopy to be aflagellated. Cultures were maintained in the lyophilized state and restored as needed. Bacteria for ileal loop inoculation were grown at 37°C on brain heart infusion agar for 18 h. They were removed from the surface, washed once by centrifugation, and diluted in phosphate-buffered saline (pH 7.2) containing 0.1% gelatin (PBSG).

Preparation of ligated ileal loops. Ileal loops were prepared by a modification of the method of De

and Chatterjee (5). The animals were fasted for 48 h to allow elimination of fecal pellets. One-half milliliter of a vibrio suspension, appropriately diluted in phosphate-buffered saline containing 0.1% gelatin, was injected into segments of the ligated ileum, each approximately 6 to 8 cm in length and each separated by an uninoculated segment of about 2 cm. Control loops received 0.5 ml of phosphate-buffered saline containing 0.1% gelatin. After 18 h (or at shorter intervals, if required) the rabbits were sacrificed and the ileum was excised. Loops were carefully measured, and the ratio of the volume of fluid accumulated (in milliliters) to the length of the segment (in centimeters) was calculated and expressed as the fluid accumulation ratio.

Culture media. Fluid from the loops was cultured, when needed, on dextrin-heart infusion agar (17). For labeling the vibrios with ^{35}S, the AG medium of Callahan and Richardson (4) was modified (MAG medium) by replacing the $MgSO_4$ (in the trace metals solution) with 5.0% $MgCl_2$, rendering the medium sulfur deficient. MAG buffer was prepared by excluding the asparagine and glucose from MAG medium.

Adsorption of ^{35}S-labeled vibrios. Vibrios were labeled according to the procedure of Baselski and Parker (2). Vibrio strains were harvested from 18-h brain heart infusion slant cultures, diluted, and inoculated at a final density of 10^7 bacteria per ml into MAG medium containing 1 μCi of ^{35}S (as $H_2{}^{35}SO_4$) per ml. After a period of 4 h at 37°C, the cells were harvested, washed twice in MAG buffer to remove free label, and resuspended in the same buffer to an inoculum density of 2×10^8 colony-forming units (CFU)/ml. A 0.5-ml portion of this suspension was injected into each ligated segment.

After 3 h of incubation, the animal was sacrificed, and the ligated portion of the ileum was excised. The fluid was removed and measured. Each segment was then weighed, and 5 to 10 cross sections (less than 100 mg/section) were cut from the loop. The rings of tissue were dipped three times in saline and weighed. Tissue and fluid samples were digested for counting radioactivity in 7-ml scintillation vials by the perchloric acid-hydrogen peroxide method of Mahin and Lofberg (16). The counting cocktail (RPI, 3a70B) was added to the digested samples, and activity was determined by counting each vial for 1 min in a Packard Tri-Carb liquid scintillation spectrometer. Total radioactivity associated with the cell suspensions was determined by counting triplicate 0.1-ml samples and multiplying by a factor of 5 (0.5 ml injected). By this technique 70 to 98% of the injected counts were recoverable.

Assay for toxin. Quantitative assays for toxin production were carried out with Chinese hamster ovary cells by a procedure modified by Schneider and Parker (16a). Bacterial cultures were grown for 18 h at 30°C with shaking in 20 ml of syncase medium (7) contained in 125-ml Erlenmeyer flasks. Cultures were harvested by centrifugation, and the supernatant fluid was titrated for toxin. Toxin concentration was calculated per 10^{10} viable cells to minimize variations in toxin production due to differences in the growth phase of the cultures.

Statistics. Statistical significance was determined by the Wilcoxon rank sum test (22).

RESULTS

Enterotoxin production by wild-type and nonmotile strains. Guentzel and Berry (10) tested the strains used in these experiments for toxigenicity. The rabbit ligated ileal loop and bluing dose assays were used. With both tests, and particularly with the more quantitative bluing dose assay, all strains were comparably toxigenic. The more sensitive Chinese hamster ovary cell assay revealed that aflagellated mutants of strains C401 and CA411 were 61 to 116% as toxigenic as the respective parental strains (Table 1). These findings confirmed our previous observations.

Fluid accumulation by motile and nonmotile strains. Nonmotile mutants of *V. cholerae* derived from strains of classical and El Tor biotypes have reduced virulence for suckling mice (10). Their ability to stimulate fluid accumulation in the ligated ileal loops of adult rabbits was investigated.

As indicated by the results shown in Fig. 1, parental strain CA401 (classical, Inaba) produced a maximal fluid response at an inoculum of 10^6 CFU or greater per loop. A nonmotile derivative (CA401 M-4) required 100 times more cells (10^8 CFU) to elicit the same maximal response.

Using this type of dose-response technique, only one bacterial strain can be tested per rabbit, and at least four such tests are necessary for statistical comparison. Consequently, two doses (10^6 and 10^8 CFU) were selected for subsequent comparisons of available wild-type strains with their nomotile mutants. All nonmotile derivatives of CA401 (classical, Inaba) produced significantly lower fluid accumulation ratios ($P < 0.005$) than the appropriate wild type at a smaller dose level, whereas at the larger dose no significant differences in fluid response were noted ($P \geq 0.05$). The results are summarized in

TABLE 1. *Enterotoxin production by V. cholerae wild-type strains and their nonmotile derivatives as assayed with Chinese hamster ovary cells*

Strain	Motility	Toxin concn	
		$\mu g/10^{10}$ viable cells	% Wild type
CA401	+	1.85	100
CA401 M-1	−	1.12	60.5
CA401 M-5	−	2.15	116.2
CA401 M-6	−	1.91	103.2
CA411	+	2.09	100
CA411 M-5	−	1.83	87.6
CA411 M-6	−	1.42	67.9
Inaba 569B	±	10.70	100

Fig. 2.

The fluid accumulation ratio comparison of CA411, a classical Ogawa strain, and its nonmotile derivatives yielded similar results (Fig. 3). The nonmotile derivatives produced a significantly lower ($P < 0.005$) fluid response at the lower dose than the wild type. This relationship was true also for the wild-type El Tor strains HK-1 and 8233 and their respective nonflagellated mutants (Table 2).

Growth of wild-type and nonmotile strains in vivo. The inability of nonmotile strains to stimulate fluid accumulation is not due to their failure to multiply in vivo. Figure 4 shows the growth rate in the lumen of the rabbit ileal loops of mutant strain M-4 and its parent CA401. There is no significant difference between strains in cell numbers per unit volume of gut fluid at any of the times plotted during a growth period of 18 h. Other nonmotile strains grew equally as well as CA401 within the ileal lumen. Because the concentration of toxin in the fluid should be approximately the same with motile and nonmotile strains, whether or not fluid accumulates in the ileum is assumed, therefore, to depend on the locus of delivery of the toxin. This assumption is supported by the data presented below.

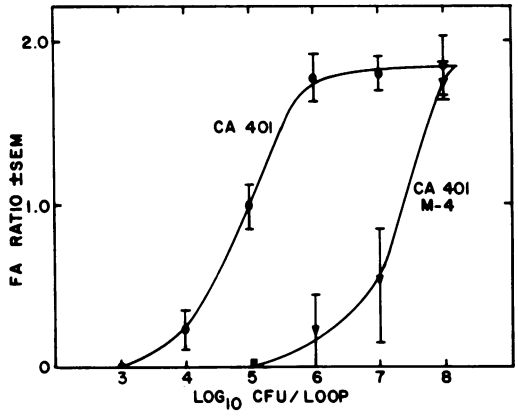

FIG. 1. *Fluid accumulation (FA) dose response. Ligated ileal loops were injected with various inoculum doses with either CA401 (●) or its nonmotile derivative, M-4 (▼). Each point represents the mean fluid accumulation ratio of four rabbits. The bars indicate the standard error of the mean (SEM) of this response.*

Association of labeled vibrios with the ileal mucosa. An intimate association between the vibrio and the host seems to be an important step in the pathogenesis of cholera (8). Motility of the bacterium probably augments its changes for association, as Guentzel and Berry (10) made evident in their studies with slices of mouse intestine. Since it has been demonstrated that, under certain conditions, vibrios adhere to the serosal side of intestinal slices as well as to the mucosal surface (E. T. Nelson and R. A. Finkelstein, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B25, p. 19), to insure the proper orientation potentially related to the pathogenesis of disease, the ^{35}S-labeled vibrios were injected into the lumen of ligated segments and then assayed, as described above, for their association with tissue and for those remaining unattached in the gut fluid. Figure 5 shows that after 3 h of incubation, 45 to 53% of the motile bacterial strains CA401, CA411, 8233, and HK-1 were associated

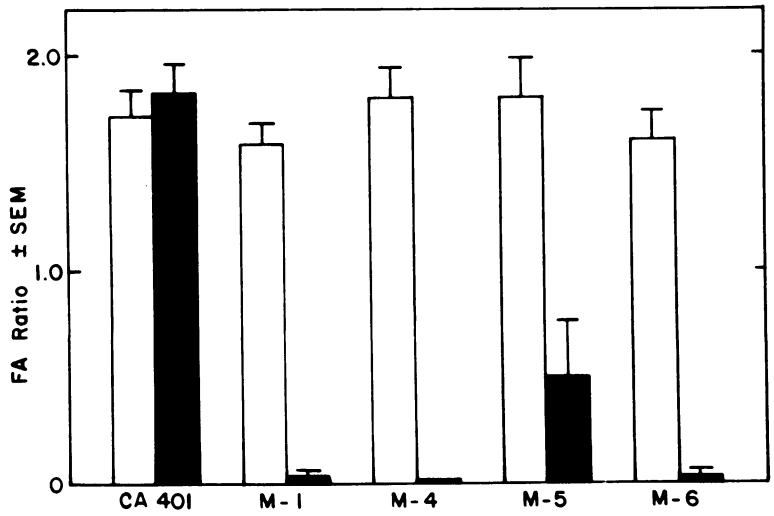

FIG. 2. *Fluid accumulation (FA) by rabbits inoculated with 10^8 (open bar) or 10^6 (cross-hatched bar) CFU of strain CA401 (classical, Inaba) and its aflagellated, nonmotile derivatives.*

with the intestinal wall. Nonmotile strains, however, associated with the intestinal surface at a lower frequency. Only 3 to 15% (depending upon the strain) of the nonmotile derivatives of CA401 were associated with the tissue sections. The remaining bacteria were located in the lumen fluid. Aflagellated mutants of CA411 showed a similar association pattern (data not shown).

DISCUSSION

These results strengthen the evidence that motility plays a critical role in the pathogenicity of *V. cholerae*. Compared with their parental types, nonmotile, aflagellated strains required a 100- to 1,000-fold larger inoculum to produce fluid accumulation response in the ileal loops of adult rabbits. The derivatives were fully toxigenic and prototrophic (10) and multiplied as well as the wild-type strains within the rabbit ileum (Fig. 4). Williams et al. (21) reported that the nonmotile mutant 8233 6B3, one of the strains used in this study, required a 100-fold-larger inoculum to produce postive ileal loops. Also, with the same inoculum dose, approximately 5 h longer was required for fluid accumulation in the loops with the mutant than with the wild-type strain. Our results confirm and extend their studies. We have noted similar rates of fluid accumulation for larger inoculum sizes of nonmotile derivatives and their parents.

Motility is required for in vivo and in vitro association of vibrios with the intestinal mucosa of infant mice. Guentzel and Berry (10) found that radiolabeled nonmotile organisms showed a reduced capacity to adsorb to the surface of mouse intestinal segments in vitro. Similarly, Guentzel et al. (11) found that the motile organisms penetrated the intervillous spaces and crypts of Lieberkühn of the intestines of orally challenged infant mice more completely than

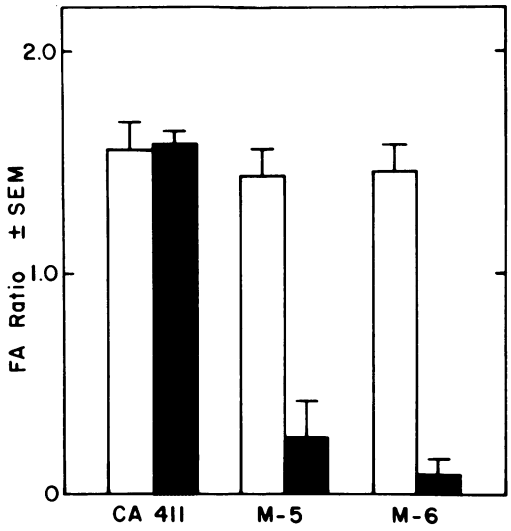

FIG. 3. *Fluid accumulation (FA) by rabbits inoculated with 10^8 (open bar) or 10^6 (cross-hatched bar) CFU of strain CA411 (classical, Ogawa) and its nonmotile derivatives.*

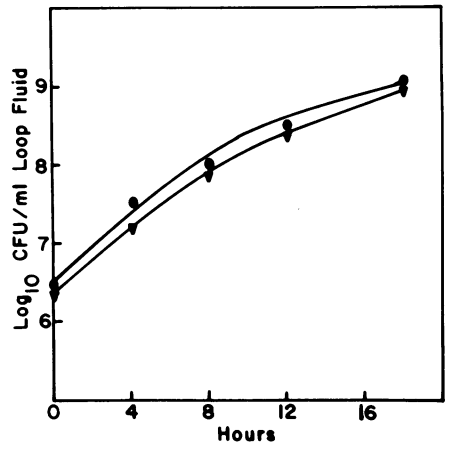

FIG. 4. *Growth of strain CA401 (●) and its nonmotile derivative M-4 (▼) within the lumen of rabbit ileal loops. Each point represents the average count from two different rabbits.*

TABLE 2. *Fluid accumulation (FA) ratio comparison of the El Tor biotypes 8233 (Inaba) and HK-1 (Ogawa) and their respective nonmotile derivatives*

Strain	Motility	FA ratio ± standard error of the mean at dose			
		10^6 CFU	(*P* value)	10^8 CFU	(*P* value)[a]
8233	+	1.120 ± 0.185		1.259 ± 0.159	
8233 M-6	−	0.132 ± 0.132	(0.014)	1.305 ± 0.167	(NS)[b]
8233 6B3	−	0.623 ± 0.171	(0.014)	1.566 ± 0.053	(NS)
HK-1	+	1.566 ± 0.057		1.497 ± 0.127	
HK-1 M-1	−	0.165 ± 0.135	(0.004)	1.586 ± 0.164	(NS)
HK-1 M-6	−	0.321 ± 0.201	(0.004)	1.381 ± 0.150	(NS)

[a] Significance comparison to the wild-type strain at that dose.
[b] NS, Not significant at $P \geq 0.05$.

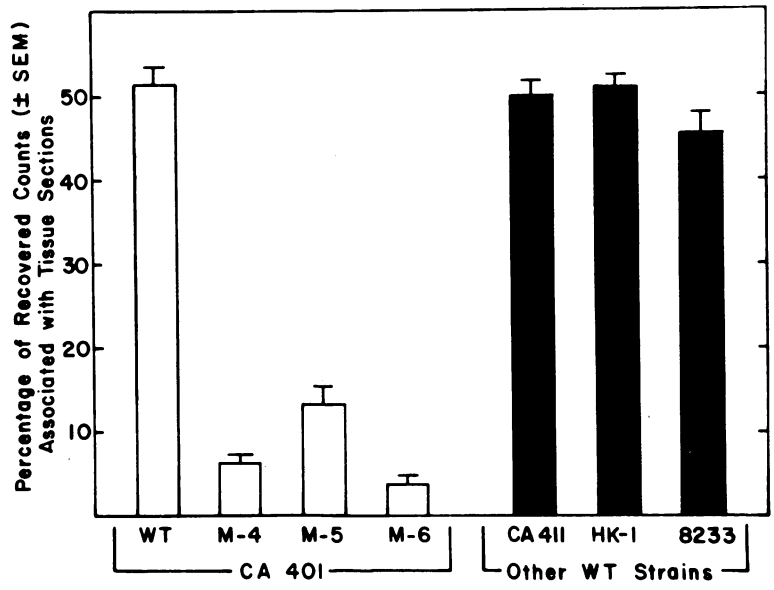

FIG. 5. *Association of ^{35}S-labeled vibrios with the rabbit ileal mucosa. This association was determined as described in the text. Each value represents the average counts adsorbed to at least five cross sections of the rabbit ileum.*

did nonmotile vibros. These studies demonstrated that motility, or, as was suggested by the studies of Jones and Freter (13), a motility-related factor, is required for association with the ileal mucosa of adult rabbits.

An intimate association between the cholera vibrios and the intestinal mucosa of the host seems to be necessary for efficient toxin delivery, especially when the inoculum is smaller than 10^8 CFU. Possibly, much of the toxin secreted by bacteria in the lumen is bound by a soluble agent (soluble ganglioside GM-1?) which competes with its binding to the fluid-secreting cells of the mucosa. However, bacteria closely associated with these mucosal cells might deposit the toxin molecules in contact with the proper receptors.

Adsorption of the bacterium to the intestinal mucosa may very well occur as a result of a positive chemotactic response. Allweiss et al. (1) found that a pepsin digest of rabbit intestinal mucosa (PMS) neutralized a positive chemotactic response of *V. cholerae* to slices of rabbit mucosa. Also, PMS and other gut-derived material produced a positive chemotactic response when incorporated into agar blocks or in standard capillary attraction assays. The factor in these preparations, or some other factor, might provide the positive stimulus directing the association attained by motile but not by nonmotile bacteria. In a human infection this chemotactic response and association with the tissues might also protect the organism from the usual flushing action of peristalsis.

ACKNOWLEDGMENT

This investigation was supported in part by Public Health Service grant AI 10466 from the National Institute of Allergy and Infectious Diseases.

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