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Motility as a Virulence Factor for *Vibrio cholerae*[1]

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The ability of motile strains of the Ogawa and Inaba serotypes of classical *Vibrio cholerae* and of the El Tor biotypes to kill suckling mice after oral challenge with 10^8 colony-forming units (representing at least 100 to 1,000 minimal lethal doses) was compared to that of nonmotile derivatives of the same strains. Loss of motility, in each case, resulted in a marked reduction in virulence. The mortality (at 36 h) caused by 10 of the 13 nonmotile strains was 32% or less, whereas the motile wild-type strains resulted in nearly 100% deaths. The reduced virulence of the nonmotile strains was associated with reduced capacity to adsorb to the surface of segments of mouse intestine. The mutants were tested for alterations in enterotoxin production and surface properties. The results suggest that motility may contribute to virulence by increasing the chance for association of the vibrios with the intestinal mucosa.

Lankford suggested in an early report (13) that cholera vibrios may possess a mechanism which confers upon the cells the capacity to attach to and readily proliferate on the surface of the intestinal epithelium of infected hosts. Evidence suggesting an adherence of *Vibrio cholerae* to the intestinal mucosa of guinea pigs, infected by stomach tube, was provided by LaBrec et al. (12). Fluorescent antibody and conventional histological examination revealed large numbers of vibrios covering an intact epithelium without apparent penetration of the lamina propria. Subsequently, Freter (6) observed that about one-half of the vibrio population in infected rabbit ileal loops was associated with the mucosa with a firmness sufficient to resist removal by washing.

The possible relationship between "adhesiveness" of vibrios to immunity against cholera, and by implication to the virulence of the organism, was suggested by the observation that antibody in either passively or actively immunized loops resulted in a marked reduction in adsorbed vibrios but did not reduce the number of vibrios present in the intestinal lumen (6). The ability of antiserum to prevent absorption of vibrios to intestinal mucosa was confirmed in an in vitro system by Freter (7), who demonstrated two protective mechanisms, a direct interference with absorption and an antibacterial process requiring the participation of antibody and viable mucosal cells. Freter also found that maximal adherence of bacteria to mucosa occurred only with the use of viable bacteria (8). This suggested that bacterial death resulted in the loss of a surface structure involved in absorption.

We have investigated the possiblility that one surface structure potentially involved in the association of vibrios with mucosa is the flagellum. Motility should increase the frequency of impact of vibrios upon mucosal cells and, with the assistance of the vibrios' mucinase, could drive the bacterium through a mucus barrier. Alternatively, the flagella themselves might serve to bind bacteria to mucosa. Our results show that flagella and/or motility contribute to the absorption of vibrios to mucosa and strongly suggest that they are involved in the process of intestinal parasitism by *V. cholerae*.

MATERIALS AND METHODS

Mice. Outbred ICR mice (Texas Inbred, Houston, Tex.), raised in departmental animal facilities, were used in most of the experiments described in this report. CFW mice (Carworth Farms, New City, N.Y.) were used in some studies. The mice were housed and fed as described in a previous paper by Guentzel and Berry (10).

Bacterial strains. Cultures of *V. cholerae* and the El Tor biotype in the lyophilized state were kindly provided by C. E. Lankford of our department. Inaba 569B was received as an unopened vial from R. Finkelstein (Southwestern Medical School, Dallas, Tex.). A series of nonmotile mutants was selected by Lankford and colleagues after treatment of motile

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strains with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) according to the general procedure of Adelberg et al. (1). Additional nonmotile mutants were selected from soft agar pour plates (1.5% Trypticase [BBL], 0.5% Phytone [BBL], 0.5% agar, pH 7.3) after treatment of stationary-phase motile cells with NTG. Cells were exposed to NTG for 30 min at 37 C at a concentration (30 μg/ml) yielding 10% survival. Before use in these studies, all mutants were tested for agglutination in polyvalent and adsorbed type-specific antisera. The mutants were tested for motility in semisolid media (soft agar and motility medium [Difco] with 0.2% beef extract and TTZ indicator [Difco]) and in hanging drop preparations. Flagella were examined by the method of Leifson (15). To minimize the possibility of working with strains with multiple defects, only mutants capable of growth in a glucose-glutamate minimal medium were selected for study. All cultures were maintained in the lyophilized state and restored as needed to minimize changes which frequently occur in cultures maintained by regular serial transfer.

Oral challenge. Seven-day-old mice (eight to 10 mice/litter) were fasted overnight and then challenged by the oral route as described previously by Guentzel and Berry (10). The suckling mice were returned to their mothers and scored for mortality.

Adsorption of ^{14}C-labeled vibrios. Wild-type and respective nonmotile mutant strains were grown for 12 h at 37 C in the TRY (casein hydrolysate-yeast extract) medium of Richardson (4) with 0.05% glucose. The cells were re-inoculated to a constant optical density at 600 nm (0.3) in fresh TRY medium plus 1.0 μCi of [U-^{14}C]glucose per ml. After a period of 2 h at 37 C, the cells were harvested, washed twice to remove free label, and resuspended to a constant turbidity (equivalent to approximately 5×10^8 colony-forming units [CFU]/ml in a total volume of 5.0 ml) in sterile nonpyrogenic saline (Travenol, Inc.). Cross sections of mid-small intestine measuring 2.0 cm in length were prepared from 10-day-old, fasted ICR mice. Two to three sections were obtained from each mouse. The sections were transferred to petri dishes containing moistened filter paper and cut once lengthwise to expose the mucosal surface. Segments that were cut through both external surfaces and separated into two pieces were discarded. All manipulations were carried out as quickly as possible to prevent drying of the segments or loss of viability in the vibrio suspensions.

Segments were selected at random and placed in the suspensions of labeled vibrios for 15 min at 37 C. Segments were washed by dipping three times in saline and then suspending in an additional container of saline for 5 min. Separate Coplin jars, each containing 50 ml of nonpyrogenic saline, were used for washing segments for each cell suspension. Washed segments were transferred to scintillation vials containing 0.7 ml of Biosolv organic solubilizer (Beckman). After overnight incubation at 37 C with shaking, 10 ml of scintillation fluid [5 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis-(5-phenyloxazolyl)-benzene per liter of toluene] was added to each vial. The activity (counts per minute) was determined by counting each vial for 10 min in a Beckman model LS-250 liquid scintillation counter. Total radioactivity (counts per minute) associated with the cell suspensions was determined by counting duplicate 0.1-ml samples.

The adsorption of labeled nonmotile mutant vibrios to the mouse tissue was compared to the respective wild-type strains according to the following formula: relative adsorption = $(A_{nm}/B_{nm}) \div (A_m/B_m) \times 100$, where A = ^{14}C-labeled vibrios (counts per minute) adsorbed to tissue, B = total counts per minute in 0.1 ml of cell suspension, m = motile wild type, and nm = nonmotile mutant.

Phage susceptibility. The patterns of susceptibility of parental and respective nonmotile strains to a large number of cholera phages were determined by C. D. Parker of our department. Bacteriophage strains used included the nine typing phages of Mukerjee (3, 17), five lysogenic phages obtained from Frank Newman (18), and four Calcutta sewage phages obtained from S. Bhattacharya of the Cholera Research Center, Calcutta.

Assay for toxin. Tests for in vivo production of toxin were carried out by a modification of the adult rabbit ileal loop method of De and Chatterjee (5). Rabbits were fasted for 72 h with water available ad libitum to allow elimination of fecal pellets. One-milliliter amounts of young peptone broth cultures, diluted to contain approximately 10^8 CFU/ml, were injected into loops of ligated ileum approximately 8 cm in length separated by short uninoculated loops. Control loops received 1 ml of sterile broth. After 16 h, the rabbits were sacrificed and the loops were examined. All loops were cultured for vibrios on DHI agar (21).

Quantitative assays for toxin production were carried out by determination of skin reactivity on the shaved backs of rabbits. Cultures were grown at 30 C with shaking in TRY (casein hydrolysate-yeast extract) medium (4) in 100-ml volumes contained in 500-ml Erlenmeyer flasks with glass beakers as closures. All cultures were harvested by centrifugation when the turbidities reached a standard value (optical density at 600 nm, 1.5) to minimize variations in toxin production due to differences in growth phase. A 60-ml amount of the respective supernatant fluids was passed through separate 0.45-μm membrane filters (Millipore Corp.). The initial 30 ml of each filtrate was discarded to minimize toxin adsorption to the filters during the initial filtration. Bluing doses per milliliter were determined as described by Callahan et al. (4). Each dilution was tested in duplicate (on the same animal) and then repeated in duplicate on another animal.

RESULTS

Virulence of motile V. cholerae strains. As a guideline for comparison of the virulence of nonmotile mutants, the susceptibility of 8-day-old mice to oral challenge with varying doses of the motile wild-type strains used in this study was determined. Litters of eight to 10 mice each were challenged with Ogawa and Inaba sero-

types of *V. cholerae* (Fig. 1) and the E1 Tor biotype (Fig. 2). In each case, an oral challenge of 10^8 CFU was found to be at least 100 to 1,000 times the minimal dose required for complete mortality in 36 h. Mice responded to challenges of less than 10^5 CFU in a dose-response fashion, with significant mortality at even the lower challenge doses after 1 week. There was some variation in the susceptibility of individual litters at the smaller test doses.

Virulence of nonmotile mutant strains. The role of motility in the virulence of *V. cholera* infection was investigated by oral challenge of 8-day-old mice. The virulence of motile Ogawa and Inaba strains of classical *V. cholerae* (Table 1) and the E1 Tor biotype (Table 2) was compared with that of respective nonmotile mutants at the large challenge dose of approximately 10^8 CFU/mouse (the challenge dose varied in individual experiments from 5×10^7 to 1×10^8 CFU/mouse).

The mortality (at 36 h) of the nonmotile strains used in this study ranged from no deaths (three strains) to 75% mortality (one strain). Ten of the 13 nonmotile strains tested caused a mortality rate (at 36 h) of 32% or less, whereas

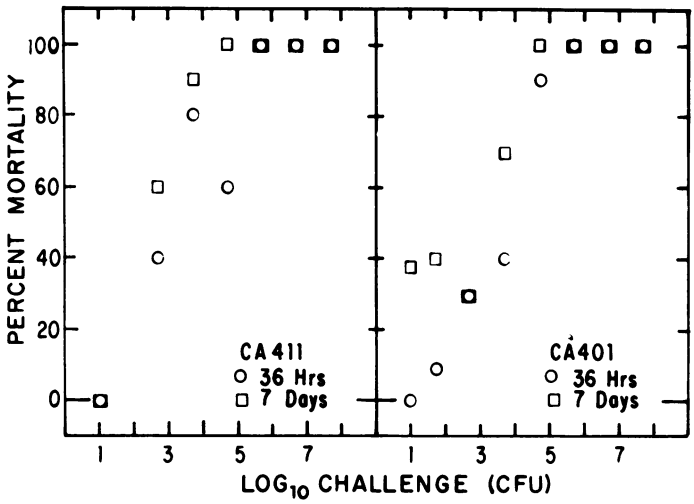

FIG. 1. *Susceptibility of 8-day-old suckling mice to oral challenge with V. cholerae Ogawa CA411 (left panel) and Inaba CA 401 (right panel). Symbols: ○, mortality at 36 h; □, mortality at 7 days. One litter of eight to 10 mice/challenge dose.*

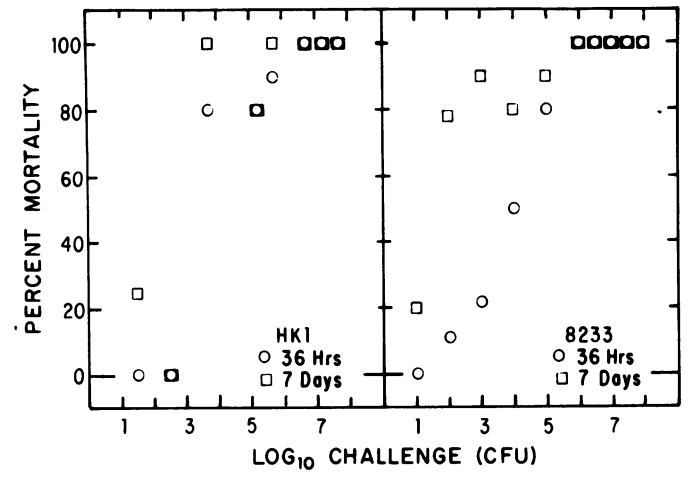

FIG. 2. *Susceptibility of 8-day-old suckling mice to oral challenge with El Tor Ogawa HKl (left panel) and Inaba 8233 (right panel). Symbols: ○, mortality at 36 h; □, mortality at 7 days. One litter of eight to 10 mice/challenge dose.*

TABLE 1. *Comparison of the virulence of motile and nonmotile strains of classical V. cholerae in mice*

Challenge organism	Serotype	Motility	No. of suckling mice[a]	% mortality[b]	
				36 h	2 Weeks
CA411	Ogawa				
WT		+	37	100	100
M-5		−	20	10	20
M-6		−	20	0	15
CA401	Inaba				
WT		+	32	97	100
M-1		−	28	32	43
M-4		−	30	0	17
M-5		−	30	63	73
M-6		−	20	30	45
569B	Inaba	Slight	25	40	40

[a] Eight-day-old ICR mice.
[b] Oral challenge dose per mouse, 5×10^7 to 1×10^8 CFU.

TABLE 2. *Comparison of the virulence of motile and nonmotile strains of El Tor V. cholerae in mice*

Challenge organism	Serotype	Motility	No. of suckling mice[a]	% Mortality[b]	
				36 h	2 Weeks
HK1	Ogawa				
WT		+	42	95	95
M-1		−	20	75	80
M-4		−	10	0	0
M-6		−	22	18	36
M-28		−	17	6	6
8233	Inaba				
WT		+	61	95	97
6B3		−	25	32	36
M-6		−	18	39	44
M-7		−	40	28	30
6B3R2	(Rough)	−	21	14	29

[a] Eight-day-old ICR mice.
[b] Oral challenge dose per mouse, 5×10^7 to 1×10^8 CFU.

the corresponding wild-type strains caused mortality rates ranging from 95 to 100%. Inaba 569B, known to be highly toxigenic but only slightly motile in our hands (a zone of growth of 7 mm or less compared to 14 mm for other wild-type strains through soft agar of 14-mm diameter), killed 40% of the mice at the test dose. The virulence of a nonmotile rough variant (8233-6B3R2) also is illustrated. The mortality at the end of a 2-week period is given since certain mutant strains caused additional deaths over an extended period. This may reflect a slower colonization and/or clearance from the gut. The data are in agreement with experiments cited in a preliminary report (M. N. Guentzel and L. J. Berry, Abstr. Annu. Meet. Am. Soc. Microbiol., 1973, m97, p. 89) using 8- to 11-day-old CFW mice.

Adsorption of labeled vibrios to mouse tissue. A close association of vibrios with the intestinal tissue of the host appears to be an important first step in cholera pathogenesis (6, 12). Motility might augment the chance for association. We have compared the in vitro adsorption to intestinal tissue of motile and nonmotile vibrios labeled with [U-^{14}C]glucose. Sections of mid-small intestine of fasted 10-day-old mice were exposed for 15 min at 37 C to washed suspensions of labeled motile vibrios and their corresponding nonmotile variants. Segments were washed by dipping and suspension in physiological saline for 5 min. The latter step was found to be essential in preliminary experiments to prevent the carryover of large numbers of apparently loosely bound vibrios (those not removed by the dipping process). Segments were incubated overnight in an organic solubilizer and then counted for radioactivity. An example of the data obtained in a typical experiment is shown in Table 3. Counts per minute associated with individual randomly selected segments exposed to the motile wild-type (HK1) and two nonmotile mutants (M-1, M-6) are arranged in increasing order. Although there was as much as a threefold difference in counts within an individual series, most of the counts were surprisingly similar, and there was no overlap between the motile and nonmotile strains. The nonmotile strains averaged between one-fourth and one-sixth as many counts as the motile strain. Major sources of variation within a series may be caused by differences in the individual mice, the amount of damage inflicted on the segment by cutting to expose the mucosal surface, or the time involved in the transfer of segments from the labeling suspension to the wash suspension.

The degree of adsorption to mouse intestine of motile Ogawa and Inaba serotypes of *V. cholerae* and E1 Tor Inaba are compared to respective nonmotile mutants in Fig. 3. Each wild-type value is adjusted to 100 for comparison with its nonmotile derivatives. Intestinal segments adsorbed 1/12 to 1/20 the number of nonmotile cells compared to the respective motile wild types. The capacity of the different motile strains to adsorb to the mouse tissue also was compared. Few differences were observed among the highly virulent strains CA411, CA401, and 8233, but the adsorption capacity of Inaba 569B was about 1/20 of that of Inaba CA401 (set arbitrarily at 100%).

The reduced virulence of nonmotile variants

TABLE 3. *In vitro adsorption of ^{14}C-labeled vibrios to intestinal tissue of mice*

Organism	Counts/min			% of control	Relative adsorption[c]
	Associated with cell suspension (control)[a]	Associated with tissue sections[b]	Avg of sections		
HK1 (wild type)	60,286	4,995 7,375 7,836 7,873 9,237 9,888 10,120 10,134 10,570 11,380 12,768	9,289	15.4	100
M-1 (nonmotile)	58,605	1,455 1,898 2,028 2,195 2,228 2,268 2,309 2,367 2,474 2,512 3,202	2,267	3.8	25
M-6 (nonmotile)	67,954	926 1,046 1,054 1,333 1,394 1,606 1,662 1,784 1,970 2,002 2,489 3,314	1,715	2.5	17

[a] Counts per minute of 0.1 ml of the washed cell suspension in a total volume of 5 ml of nonpyrogenic saline containing an average of 5×10^8 CFU/ml.

[b] Counts per minute associated with the 2-cm segments of intestine after exposure to the labeled vibrios and washing in saline.

[c] Determined by the formula given in Materials and Methods.

for suckling mice thus can be correlated with a reduced capacity to adsorb to mouse intestinal tissue. Motility of the vibrios apparently enhances the chance for this association.

Additional properties of motile and nonmotile strains. The reduced virulence observed with the mutant strains could have resulted from an alteration of other properties in addition to motility. This possibility was tested (Tables 4 and 5). Patterns of susceptibility of parental and mutant strains to 18 bacteriophages (see Materials and Methods) were compared to detect possible changes in the surface characteristics of the mutants. No changes were detected in the phage patterns of the mutants of the classical strains or of the E1 Tor Inaba strain. However, three of four mutants of the E1 Tor Ogawa strain (HK1) had altered phage patterns. As expected, the rough, nonmotile mutant of strain 8233 was altered.

Tests also were carried out to detect possible alterations in enterotoxin production. The rough strain and one of three nonmotile mutants with altered phage patterns were negative for in vivo toxin production in the adult rabbit ligated ileal loop model. The other mutants were equivalent to the respective parental strains at the challenge dose (10^8 CFU) tested. Quantitation, by means of skin reactivity, of toxin produced in vitro revealed that differences in quantities of toxin produced by parental and mutant strains could not explain the greater than 100-fold difference in virulence for the suckling mouse.

All nonmotile mutants used in this study were stable in vitro. However, the increased virulence of certain strains (e.g., HK1 M-1 and CA401 M-5), relative to the other nonmotile strains, suggested that reversion to motility in vivo might occur in the former at the high challenge doses used. This possibility was tested by examining the motility of randomly selected isolates from infected ligated loops and orally infected suckling mice. Motile revertants were isolated from loops infected with three mutant strains (CA401 M-1, CA401 M-5, and HK1 M-1) with increased relative virulence and one (CA411 M-6) with minimal virulence. It should be noted that the zone of motility produced by the latter was less than 4 nm and occurred maximally in a discrete zone near the surface. Zones produced by revertants of the other strains were greater than 5 mm along the entire stab. An even greater percentage of motile revertants of CA401 M-5 and KH1 M-1 was observed in randomly selected isolates from orally infected mice.

DISCUSSION

The experiments described in this report (Tables 1 and 2) suggest that motility is a factor in the virulence of *V. cholerae*, as measured by oral infection of suckling mice. The marked differences in mortality of the nonmotile mu-

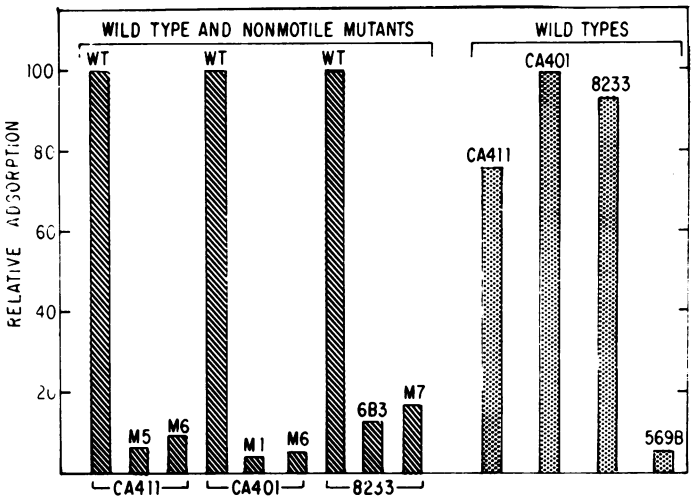

FIG. 3. *Comparison of the in vitro adsorption of ^{14}C-labeled vibrios. Relative adsorption is expressed according to the formula given in Materials and Methods. Each value represents the average counts adsorbed to at least six randomly selected segments per test variable as compared to the total counts in the respective uptake suspension.*

TABLE 4. *Properties of motile and nonmotile strains of classical V. cholerae*

Organism[a]	Phage sensitivity altered[b]	Toxigenicity		Reversion to motile	
		Ileal loop	BD[c]/ml $\times 10^{-2}$	Rabbit[d]	Mouse[e]
CA411					
WT	/	Pos[g]	100	/	/
M-5	No	Pos	30	0/10	0/10
M-6	No	Pos	20	3/8	ND
CA401					
WT	/	Pos	30	/	/
M-1	No	Pos	30	3/8	ND
M-4[h]	No	Pos	ND	0/10	ND
M-5	No	Pos	300	3/10	8/14
M-6	No	Pos	ND	0/10	ND
569B	/	Pos	1,000	/	/

[a] All cultures gave the "typical" reaction (gelatin positive, dextrin positive) on DHI agar.

[b] Compared to respective wild-type strains.

[c] BD, Blueing doses. ND, Not determined.

[d] Number motile/number tested from randomly selected isolates from ileal loops.

[e] Number motile/number tested from randomly selected isolates from mouse small intestine 12 h postinfection with 10^8 CFU.

[f] Wild type.

[g] Ileal loops infected with approximately 10^8 CFU; all positives (pos) (8 ml or greater of gross fluid accumulation) were equal to the respective parental strains.

[h] Small colony former at 24 h.

tants compared to the respective motile strains is more significant considering the fact that the challenge dose used was 100- to 1,000-fold greater than the minimal dose of wild-type vibrios required for complete mortality in 8-day-old mice (Fig. 1 and 2). A 10- to 100-fold reduction in the challenge size would have resulted in increased survival with the nonmotile strains without a corresponding reduction in the mortality of the motile controls. The occasional mouse, out of the large numbers tested, that survived the wild-type challenge (Tables 1 and 2) may have been the result of an improperly placed inoculum.

Differences in the virulence of nonmotile mutants of different strains were expected; however, differences also were observed with individual mutants of the same strain. Some strains had increased virulence relative to the other mutant strains, but (as discussed above) a reduction in the challenge dose of any of the wild-type strains to 10^6 CFU (Fig. 1 and 2) would have resulted in greater mortality than any of the nonmotile strains at 10^8 CFU. The large challenge size may permit in vivo selection of motile revertants of some nonmotile strains, although all were stable in vitro. This possibility is suggested by the results in Tables 4 and 5. Changes in serotype, as well as rough-to-smooth reversions, have been observed in orally infected gnotobiotic mice (16, 20). Differences in the virulence of nonmotile mutants may also reflect differences in additional factors required for

TABLE 5. *Properties of motile and nonmotile strains of El Tor V. cholerae*

Organism[a]	Phage sensitivity altered[b]	Toxigenicity		Reversion to motile	
		Ileal loop	BD[c]/ml × 10^{-2}	Rabbit[d]	Mouse[e]
HK1					
WT	/[f]	Pos[g]	ND	/	/
M-1	No	Pos	ND	1/10	8/9
M-4	Yes	Neg/slightly pos[h]	ND	0/10	ND
M-6	Yes	Pos	ND	0/11	ND
M-28	Yes	Pos	ND	0/6	ND
8233					
WT	/	Pos	3	/	/
6B3	No	Pos	10	0/8	ND
M-6	No	Pos	3	0/8	ND
M-7	No	Pos	3	0/8	ND
6B3R2 (rough)	Yes	Neg	ND	0/8	ND

[a] All cultures gave the "typical" reaction (gelatin positive, dextrin positive) on DHI agar.
[b] Compared to respective wild-type strains.
[c] BD, Blueing doses. ND, Not determined.
[d] Number motile/number tested from randomly selected isolates from ileal loops.
[e] Number motile/number tested from randomly selected isolates from mouse small intestine 12 h postinfection with 10^8 CFU.
[f] Wild type.
[g] Ileal loops infected with approximately 10^8 CFU; all positives (pos) (8 ml or greater of gross fluid accumulation) were equal to the respective parental strains; slight positive gave approximately 3 ml of fluid.
[h] Negative (neg) in one trial and slightly positive in the second trial.

virulence such as "adsorption factors" (as discussed below) not assayed in this study.

Adherence of cholera vibrios to the intestinal mucosa of infected animal hosts has been demonstrated in vivo and in vitro (6, 12). The importance of adherence in the production of cholera and in cholera immunity in man was suggested by reduction of adsorption by specific antibody (6–8). Fubara and Freter (9) recently have shown that purified secretory immunoglobulin A preparations, obtained from orally vaccinated mice, protect isolated mouse intestinal loops against challenge with the homologous organism. Protection was associated with a reduction in adsorption of vibrios to the mucosa. Peterson et al. (19) observed that cholera toxin introduced intraluminally into adult mice was specifically and selectively adsorbed to the mucosa. This led these authors to suggest that adsorption of toxin was an initial step in cholera pathogenesis. By implication, a close association of vibrios and mucosa would permit more efficient delivery of the toxin. A direct relation of adherence with the virulence of vibrios is indicated by the data in Table 3 and Fig. 3. Reduced virulence of nonmotile vibrios was correlated with a reduced capacity of the cells to adsorb to intestinal tissue. Williams et al. (23) have reported that one of the nonmotile mutants (82336B3) used in this study required a 100-fold greater inoculum to produce positive ileal loops or, with the same large inoculum, about 5 h more time to become positive than the motile wild-type strain.

The residual virulence and adsorption capacity of nonmotile strains suggest that motility functions only to increase the chance for association with the mucosa, although an actual role for the flagellum in adsorption is not ruled out by this study. The possibility that flagella provide the binding between the mucosal cell and the bacterium will be examined when sufficient flagella are purified to test for binding or for inhibition of binding by intact vibrios. Freter (8) observed that loss of viability (due to heating or neomycin treatment) resulted in a decreased capacity of vibrios to adsorb to slices of rabbit ileum. This effect may be ascribed to the loss of motility by the nonviable cells. Passive protection of offspring of survivors of oral infection with nonmotile strains was observed in the present study, suggesting that some colonization occurs even in the absence of mortality.

Other surface structures of the cholera vibrio may be involved in the actual adsorption process. Studies with K88-negative mutants of *Escherichia coli* demonstrated that the antigen

was responsible for attachment of K88-positive cells to intestinal mucosa, and that attachment was essential for virulence in conventionally reared piglets (11). A slime envelope could be responsible for the adhesive property of vibrio cells. Lankford and Legsomburana (14) reviewed evidence from their laboratory for a slime envelope and its probable relation to hemagglutinin activity of cholera vibrios. The thin slime layer could be observed around cells of *V. cholerae* and the E1 Tor biotype under all cultural conditions (even when the hemagglutinating activity failed to develop). Cultural conditions for the latter were exacting. Extraction of cell-bound hemagglutinin from E1 Tor cells without cell injury but with reduction in the slime envelope suggested their association (M. Chulasamaya and C. E. Lankford, Bacteriol. Proc., p. 90, 1970). Cholera vibrios also have been shown to possess pilus-like filamentous projections (2) which could be involved in the adsorption process. Isolation of mutants defective in these additional surface components could resolve their relative roles (and importance) in the adsorption process.

Identification and isolation of virulence factors such as flagella and those factors involved in adsorption will permit a better understanding of cholera pathogenesis and will perhaps aid in the development of a more effective cholera vaccine.

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