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In Vivo and In Vitro Characterization of Virulence-Deficient Mutants of *Vibrio cholerae*

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In vitro and in vivo interactions between *Vibrio cholerae* and the infant mouse intestinal environment were examined by using a number of virulence-deficient mutants of strain CA401 which are unable to induce a typical diarrheal response. In vitro interactions with upper bowel sections were evaluated by determining percent association of radiolabeled organisms with sections. In vivo behavior was evaluated in the upper bowel early in infection with radiolabeled inocula. The relative degree of mechanical clearance was indicated by the percent recovery of input label. The relative degree of multiplication and killing was determined by changes in the specific activities (counts per minute per colony-forming unit) of inocula compared with recovered viable organisms. The results indicated that, whereas some virulence-deficient mutant classes exhibit net multiplication in the upper bowel, other classes show net killing in and accelerated clearance from the upper bowel. The in vitro association patterns failed to correlate with in vivo upper bowel recovery.

In symptomatic human cholera infections, disease becomes apparent when organisms located in the upper intestinal regions elaborate an enterotoxin which induces an acute diarrheal response (2, 10, 12). A critical stage in cholera pathogenesis, therefore, is the establishment of a bacterial population in the upper intestinal tract which can synthesize and deliver an effective level of cholera toxin to target epithelial cells. In an open system like the intestinal tract, the bacterial population size is influenced by the rates of multiplication, killing, and mechanical clearance (21). A decrease in multiplication or an increase in killing or mechanical clearance would reduce the size of the bacterial population and lessen its effectiveness in inducing disease. Additionally, any barrier to delivery of toxin to epithelial cells would inhibit the development of disease.

These concepts are also applicable to analyses of pathogenesis by *Vibrio cholerae* in orally challenged infant mice, where the upper bowel similarly appears to be the critical colonization site (5, 7, 19, 20). In this model, the development of disease symptomology is related to the number of viable organisms located in the small intestine (20). Furthermore, differences in recovery from the proximal half of the intestines among strains of varying virulence are noted at early times after infection (3). The goal of the present study was to determine whether the decreased diarrhea-inducing ability of a number of isogenic virulence-deficient mutants of *V. cholerae* strain CA401 (6) could be related to alterations in the rates of processes affecting the population size in the upper bowel.

The relative rates of multiplication, killing, and mechanical clearance have been determined by means of a radioisotopic procedure which has been evaluated by several investigators using the infant mouse system. In this procedure, mice are challenged orally with radiolabeled inocula (4, 5, 7, 19). The relative degree of mechanical clearance can be determined from the percent recovery of input label. The relative rates of killing and multiplication can be determined from an analysis of changes in specific activity (counts per minute per colony-forming unit [CFU]) of inocula compared with values found for the recovered viable organisms. In addition, the in vitro association of the mutants with upper intestinal sections was determined.

(A preliminary presentation of these results was made at the 78th Annual Meeting of the American Society for Microbiology, Las Vegas, Nev., 14-19 May 1978.)

MATERIALS AND METHODS

Bacterial cultures. A listing of the mutants used with a brief description of their phenotypes is given in Table 1. A more complete description of the FA mutants has been given previously (6). The FA mutants are isogenic derivatives of a human isolate, strain

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CA401 (18). These mutants were isolated on the basis of reduced diarrhea-inducing ability in orally challenged infant mice. All cultures were maintained frozen at −70°C in brain heart infusion broth plus 15% glycerol.

Infant mice. The infant mice were obtained from a CFW breeding colony at the University of Texas Animal Resources Center. The breeders were fed a Wayne Lab Block diet. The infants used weighed 3.0 ± 0.5 g and were generally 6 to 7 days old. Litters were removed from their mothers 6 h before inoculation and pooled for experiments. They were not returned to the mothers during the experiments.

Preparation of inocula. The radiolabeling procedure has been described previously (4, 5). With the exception of FA-131, brain heart infusion agar-grown cells were labeled with [^{35}S]sulfate in a minimal medium, washed to remove excess label, and resuspended in brain heart infusion broth plus 0.01% Evans blue dye to a concentration of approximately 10^8 CFU/ml.

Mutant FA-131 has a methionine requirement. It was therefore necessary to label this strain with [^{35}S]methionine instead of [^{35}S]sulfate in a labeling medium with 50 μCi of [^{35}S]methionine per ml and 0.01% unlabeled L-methionine. All other procedures were identical.

Inoculation procedure. The inoculation procedure has been described previously (3). A 0.05-ml dose of the labeled cell suspension was administered per os. This dose contained 4×10^6 to 7×10^6 CFU and 150,000 to 250,000 cpm. The specific activity of the inoculum was calculated from the counts per minute per dose divided by the CFU per dose. Each experimental group consisted of 7 to 10 animals.

Recovery of radiolabel and viable organisms. At 4 h post-inoculation the mice were sacrificed, and the proximal half of the small intestine (upper bowel) was removed and homogenized. The number of viable organisms, the amount of radioactivity, and the specific activity in the upper bowel were determined for each mouse as previously described (4). The results are expressed as a ratio of the value at 4 h in the homogenate to the value of the inoculum, i.e., ratio of counts per minute at 4 h to counts per minute of inoculum, ratio of CFU at 4 h to CFU of inoculum, or ratio of specific activity at 4 h to specific activity of inoculum.

Calculation of MIP. The mean infective potential (MIP) was determined from the following equation: $MIP = -\log [\Sigma SA_4/SA_0]/n$, where SA_4 is the specific activity in the upper bowel at 4 h post-inoculation, SA_0 is the specific activity of the inoculum, and n is the number of animals.

In vitro association with intact mucosal surfaces. The procedure has been described previously (5). Basically, the amount of radiolabel associating with an opened section of infant mouse upper bowel was determined after incubation of a tissue section in a ^{35}S-labeled cell suspension. Each experimental group consisted of six sections. The values for mutants are expressed as a percentage of the value found for strain CA401 run concurrently, and P values were determined by comparison of results in concurrent experiments.

Statistical analysis. P values were determined by the Wilcoxon rank test (23).

RESULTS

In vitro association of mutants with intestinal sections. The results of in vitro association experiments are given in Table 1. Most of the mutants associated with the tissue to the same degree as CA401. Some appeared to have slightly elevated (FA-9, FA-101) or decreased

TABLE 1. *In vitro association of mutants with infant mouse upper bowel sections*

Strain	Description	Designation	% of association[a]	P[b]
CA401	Parental			
FA-69	Rough	R	105 ± 8[c]	NS[d]
FA-28	Nonmotile (toxin deficient, cell surface altered)	Mot (Tox, Csa)	20.8 ± 1.5	<0.01
FA-54	Nonmotile	Mot	20.8 ± 1.9	<0.01
FA-129	Nonchemotactic	Che	15.5 ± 1.2	<0.01
FA-9	Protease deficient	Prt	137 ± 10	NS
FA-101	Protease deficient	Prt	146 ± 10	NS
FA-64	Toxin deficient	Tox	112 ± 3	NS
FA-131	Toxin deficient, methionine requiring	Tox	226 ± 18	<0.01
FA-18	Extracellular product deficient	Shr	58.9 ± 4.7	NS
FA-133	Purine requiring	Pur	ND[e]	
FA-4	Cell surface altered	Csa	98.6 ± 8.6	NS
FA-5	Cell surface altered	Csa	104 ± 12	NS
FA-79	Rapid death in broth	Rdb	90.6 ± 4.7	NS
FA-86	No defect identified	X	72.0 ± 4.7	NS

[a] Calculated from the following: (tissue section-associated label for mutant/mean tissue section-associated label for CA401) × 100%.

[b] P for mutant value compared with value for CA401 run concurrently.

[c] Mean ± standard error of the mean.

[d] NS, Not significant.

[e] ND, Not done.

(FA-18, FA-86) values, but did not show statistically significant differences from CA401. The nonmotile (FA-28, FA-54) and nonchemotactic (FA-129) mutants were significantly lower than CA401. One toxin-deficient mutant (FA-131) had a significantly elevated value.

Recovery of radiolabel from the upper bowel. Table 2 gives the percentage of input radioactivity remaining in the upper bowel at 4 h after challenge. The percent recovery of input label gives an indication of the relative degree of mechanical clearance; i.e., differences in recovery reflect differences in the rate of mechanical clearance (5, 7, 19). Radiolabel from Formalin-killed vibrio strain CA401 is cleared from the upper bowel with the same kinetics as is radiolabel from viable cells of strain CA401 (4). For most of the mutants, the percent recovery did not significantly differ from CA401. However, a significant reduction in recovery was noted for rough and toxin-deficient mutants (including FA-18, an extracellular product-deficient mutant). The difference was greater for the toxin-deficient types. It should be noted that there is no correlation between increased mechanical clearance from the upper bowel in vivo and reduced tissue association ability in vitro.

Changes in specific activity in the upper bowel. Four hours post-inoculation was chosen as the time for evaluation of changes in specific activity in the upper bowel. This was based on previous observations demonstrating significant differences between virulent and avirulent strains at this time (4, 5). Changes in the specific activity have been interpreted as relating to the degree of multiplication versus killing which occurs in the upper bowel (4). Table 3 outlines the results expected from either process. Thus, when the MIP is <0, killing exceeds multiplication; and when the MIP is >0, multiplication exceeds killing.

Table 4 gives the viable organism recoveries and specific activity changes for the mutants examined. In every case a decrease in viable organism recovery was noted, with a corresponding significant increase in the specific activity ratios. Table 4 also gives the MIPs for the mutants, which are decreased accordingly from the CA401 value, although the extent of the reductions vary. Thus, for some mutant types net multiplication was noted (but to a lesser extent than for CA401), whereas other types were subjected to net killing. In the case of one nonsurviving type, the purine auxotroph FA-133, reversion to prototrophy restored the MIP to a parental value. For another nonsurviving mutant, the toxin-deficient FA-131, addition of an exogenous toxin level of 2.5 μg to the dose did not significantly alter the net killing observed (specific activity at 4 h/specific activity of inoculum, 16.94; range, 3.29 to 36.21). Furthermore, the accelerated clearance of label was still noted (percent recovery of input label, 2.9 ± 0.2% [mean ± standard error of the mean]).

TABLE 2. *Recovery of input radiolabel from the upper bowel at 4 h after challenge*

Strain	Class[a]	No. of animals	% Recovery[b]	P[c]
CA401	Parental	11	7.7 ± 0.8[d]	NA[e]
FA-69	R	8	5.3 ± 0.9	0.05
FA-28	Mot	8	7.9 ± 0.7	NS[f]
FA-54	Mot	7	7.6 ± 0.7	NS
FA-129	Che	10	10.2 ± 1.1	NS
FA-9	Prt	9	8.5 ± 0.6	NS
FA-101	Prt	8	5.5 ± 1.0	NS
FA-64	Tox	7	2.9 ± 0.6	<0.001
FA-131	Tox	10	3.3 ± 0.2	<0.001
FA-18	Shr	9	4.7 ± 0.3	<0.01
FA-133	Pur	10	6.5 ± 0.3	NS
FA-4	Csa	7	12.0 ± 2.5	NS
FA-5	Csa	10	10.7 ± 1.2	NS
FA-79	Rdb	7	9.8 ± 1.4	NS
FA-86	X	9	6.0 ± 0.2	NS

[a] See Table 1.
[b] Calculated from the following: (counts per minute in upper bowel/counts per minute in dose) × 100%.
[c] *P* compared with CA401.
[d] Mean ± standard error of the mean.
[e] NA, Not applicable.
[f] NS, Not significant.

DISCUSSION

Table 5 summarizes the in vivo behavior patterns for the mutant types examined. Certain of the mutant classes exhibited net multiplication in the upper bowel early in infection, but the resultant viable cell population was smaller than that found with CA401. This may be due to an increased rate of killing or a decreased rate of multiplication. The decreased disease-inducing capacity of these mutants may be related to failure to attain the critical population size required for synthesis and delivery of an effective level of toxin. Alternatively, some of the mutants (particularly those with a slight decrease in pop-

TABLE 3. *Interpretation of mean infective potential*

Mechanism	Sp act at 4 h/sp act of inoculum[a]	MIP[b]
Killing	>1	<0
Multiplication	<1	>0

[a] Specific activity at 4 h in the upper bowel/specific activity of the inoculum.
[b] MIP = −log (specific activity at 4 h/specific activity of inoculum).

TABLE 4. *Recovery of viable organisms and changes in specific activities in the upper bowel at 4 h post-inoculation*[a]

Strain	Class[b]	No. of animals	Recovery[c]	Change in sp act		MIP
				Mean[d]	P[e]	
CA401	Parental	11	1.12 ± 0.19[f]	0.083 (0.036–0.153)	NA[g]	+1.1
FA-69	R	8	0.022 ± 0.005	2.9 (0.9–6.7)	<0.001	−0.5
FA-28	Mot	8	0.016 ± 0.008	20.3 (1.0–52.5)	<0.001	−1.3
FA-54	Mot	7	0.176 ± 0.041	0.68 (0.18–1.73)	<0.001	+0.2
FA-129	Che	10	0.78 ± 0.13	0.16 (0.08–0.28)	<0.05	+0.8
FA-9	Prt	9	0.36 ± 0.08	0.33 (0.13–0.61)	<0.001	+0.5
FA-101	Prt	8	0.37 ± 0.13	0.33 (0.07–0.94)	<0.01	+0.5
FA-64	Tox	7	0.0067 ± 0.0029	7.7 (3.2–16.8)	<0.001	−0.9
FA-131	Tox	10	0.0014 ± 0.0005	50.3 (8.3–148.8)	<0.001	−1.7
FA-18	Shr	9	0.0011 ± 0.0003	71.1 (13.0–139.6)	<0.001	−1.9
FA-133	Pur	10	0.011 ± 0.003	10.6 (5.1–40.6)	<0.001	−1.0
FA-133-3[h]	Pur$^+$	10	1.19 ± 0.17	0.085 (0.055–0.126)	NS[i]	+1.1
FA-4	Csa	7	0.31 ± 0.14	0.62 (0.21–0.80)	<0.001	+0.2
FA-5	Csa	10	0.42 ± 0.04	0.25 (0.18–0.32)	<0.001	+0.6
FA-79	Rdb	7	0.076 ± 0.020	2.06 (0.46–5.43)	<0.001	−0.3
FA-86	X	9	0.142 ± 0.056	0.75 (0.13–1.50)	<0.001	+0.1

[a] Same animals as in Table 2.
[b] See Table 1.
[c] CFU in upper bowel/CFU in dose.
[d] Mean specific activity at 4 h/specific activity of dose. Values in parentheses are ranges.
[e] *P* compared with CA401.
[f] Mean ± standard error of the mean.
[g] NA, Not applicable.
[h] Prototrophic revertant of FA-133.
[i] NS, Not significant.

TABLE 5. *Summary of in vivo behavior of virulence-deficient mutants*

Mutant class[a]	In vivo behavior		
	Net multiplication	Net killing	Increased mechanical clearance
Mot	+		
Che	+		
Prt	+		
Csa	+		
X	+		
Rdb		+	
Pur		+	
Mot (Tox, Csa)		+	
Tox		+	+
Shr		+	+
R		+	+

[a] See Table 1.

ulation size) may be unable to direct efficient delivery of toxin to epithelial cells. The mutant classes in this group are nonmotile, nonchemotactic, protease deficient, and cell surface altered. In addition, one mutant for which no defect has been identified is in this group. For these mutants exhibiting net multiplication, increased mechanical clearance was not noted. However, reduced in vitro tissue association ability was noted for nonmotile and nonchemotactic mutants, as has been reported previously by other workers (1, 11, 13, 14).

Reduced recovery of viable organisms from the upper intestinal regions has also been reported previously for nonmotiles (14). It has been suggested that nonmotile and nonchemotactic variants would be more efficiently mechanically removed due to their relative inability to associate with host tissues, as demonstrated by in vitro studies. Our results conflict with this interpretation. However, the point should be made that the nonmotiles examined in this study were flagellated. Thus, if a flagellum-associated "adhesin" is involved in mucosal association, these mutants may not be cleared as efficiently as a nonmotile, aflagellate strain. For the mutants in this study, however, lack of motility does not correlate with increased mechanical removal but rather with reduced multiplicative ability.

The other mutant classes examined demonstrate net killing in the upper bowel. This was expected for mutant FA-79 which also dies rapidly in vitro in broth (6). For the other mutants in this group, there appears to be increased susceptibility to bactericidal mechanisms in the gut. In the case of the purine auxotroph (FA-133), the net killing appears to be related to

metabolic restriction in the host, as reversion to prototrophy restores infective potential and virulence (Baselski and Parker, unpublished data) to the wild-type level. Thus, it appears that in vivo metabolism is essential for the pathogen to evade host defenses. A similar conclusion was reached in studies of the susceptibility of purine auxotrophs of *Pasteurella pestis* to host defenses (8).

Several mutant classes which demonstrate net killing are also susceptible to accelerated mechanical clearance. These include the rough and toxin-deficient phenotypes, including the Shr phenotype, which displays reduced activity of several extracellular products besides toxin. An accelerated clearance from the upper bowel has been noted previously for roughs (5). It is possible that decreased viability is a contributing factor toward the more rapid clearance. However, loss of viability alone cannot account for increased mechanical removal, as Formalin-killed CA401 cells do not show a similar enhanced clearance (4, 5). It should also be noted that again there is no correlation between the relative degree of in vivo and in vitro association. Indeed, one toxin-deficient mutant (FA-131) has an in vitro association potential greater than that of CA401. The reasons for this enhanced association are unknown, but may relate to some physiochemical cell surface alteration. Physiochemical properties of bacterial surfaces have been shown to play a role in the association of bacteria with eucaryotic cell surfaces (15).

The enhanced killing of rough and toxin-deficient mutants is consistent with several previous observations. Roughs are known to have increased susceptibility to a number of host antibacterial mechanisms (18, 22), and toxin-deficient mutants are known to show reduced recoverability from a gut environment (16, 17). The inability to reverse the increased killing and washout of a toxin-deficient mutant by addition of exogenous toxin to the dose is also consistent with previous observations (16). This might be due to use of an inadequate level of toxin, or the timing of exposure to toxin may not have coincided with the normal disease process. Alternatively, in the infection toxin may need to be concentrated at infective loci or even at the cell surface (toxin subunits appear to be membrane associated [9]). Exogenously supplied toxin would probably not localize in these ways.

The results with a pleiotropic nonmotile mutant (FA-28) are more difficult to interpret. Thus, although the mutant is toxin deficient, net killing but not accelerated clearance is noted. These observations serve to emphasize the complex nature of an infection which must certainly involve many concurrent host-parasite interactions.

In summary, the results of this study demonstrate that the various classes of virulence-deficient mutants of *V. cholerae* present characteristic altered patterns of in vivo behavior. Whereas some mutant types show net, but reduced, multiplication in the upper intestinal tract early in infection, other mutant classes are rapidly killed and may also be susceptible to more rapid mechanical clearance. Present experimentation is focused upon analyses of how bacterial functions serve to restrict elimination by killing or mechanical clearance and enhance colonization to result in apparent disease. In addition, the methods described are being applied in analysis of the infective potential of a number of clinical isolates of *V. cholerae*.

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