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Intestinal Distribution of *Vibrio cholerae* in Orally Infected Infant Mice: Kinetics of Recovery of Radiolabel and Viable Cells

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Kinetics of distribution of *Vibrio cholerae* in the gastrointestinal tract of orally challenged infant mice were examined by determining recovery of input dose from the whole gut and from individual segments of stomach, upper bowel, and lower bowel. The strains studied were 569B, CA401, and VB12 (a rough CA401). Recovery was determined as a percentage of either input radiolabel using ^{35}S-labeled cells or input colony-forming units. We found clearance of radiolabel and viable cells from the stomach into the intestines by 2 h. Early whole-gut clearance of label was greater for 569B and heat-killed CA401 than for CA401, VB12, or Formalinized CA401. At early times postchallenge, significant differences occurred between strains in the upper bowel, with greater recovery of label and viable cells for CA401 than for 569B or VB12. Beginning at 8 h postchallenge, radiolabel accumulated in the lower bowel with all experimental groups except CA401-challenged mice, where diarrhea was noted and label disappeared from the intestines. In vitro evaluation of mucosal association of these strains with bowel sections was also done. CA401 and VB12 associated to a greater extent than 569B or heat-killed or Formalin-killed CA401.

Vibrio cholerae causes a disease in man characterized by a voluminous fluid loss from the small intestine. The diarrhea is known to be caused entirely by an enterotoxin (9), but the critical events which promote a successful infection by *V. cholerae* are relatively ill defined. Orally introduced organisms must circumvent defensive tactics of the host including the acid gastric environment (5, 21), inhibitory action of saliva (6), clearance by peristalsis and mucus flow (7, 10), and intestinal antibacterial mechanisms (11, 27). It is also likely that organisms must interact with elements of the host mucosa in penetrating the mucus barrier (12, 32) and adhering to mucosal surface receptors (12, 25, 29). Furthermore, the organisms must find the gut environment favorable for multiplication (13, 24). However, the nature of factors which contribute to these host-parasite interactions is largely unknown. Studies of the in vivo infective process are necessarily hampered by the complexity of the system. The bacterial population size present at any given time in an infection is influenced by rates both of killing and of multiplication. In infection of an open system, such as the intestinal tract, elimination from the environment also occurs (27). We have attempted to gain some insight into the nature of the infective process by performing kinetic studies using the infant mouse model (33) to describe how organisms behave in the gut. *V. cholerae* has been shown to be noninvasive in infact mice (M. N. Guentzel, personal communication), inducing a typical diarrheal response while confined to the lumen of the bowel (17).

The present communication is a comparative kinetic study of several *V. cholerae* strains which have previously been shown to differ in virulence in infant mice (1, 16). Strain CA401 is a highly mouse-virulent human isolate (14). VB12 is an avirulent rough derivative of CA401, isolated by selection for resistance to the smooth specific vibriophage VCII (28). Strain 569B is a highly toxigenic, smooth, laboratory strain which induces diarrhea in infant mice only at high doses (1). The goal of the study was to determine whether differences in virulence for infant mice reflect differences in distribution of infecting organisms in the intestinal tract of infant mice by use of a method we have termed "dose-chase." A ^{35}S-labeled "dose" of *V. cholerae* was administered orally to infant mice and followed by a "chase" to determine the location of radiolabel in the gut. In addition, the distribution of viable organisms was determined.

MATERIALS AND METHODS

Bacterial cultures. Bacterial strains have been described previously (1). The strains and their virulence for infant mice by two methods are shown in

Table 1. Stocks were maintained in lyophilized form or on meat extract agar at 4°C.

Reagents and materials. $^{35}SO_4$ as sulfuric acid and Aquasol scintillation fluid were obtained from New England Nuclear. Lyophilized cholera toxin was a gift from R. A. Finkelstein, Southwestern Medical School, Dallas, Texas. Anticholeragenoid prepared by Finkelstein (8) was obtained from Carl Miller, Geographic Medicine Branch, National Institutes of Health, Bethesda, Md.

Infant mice. Mice were obtained from our CFW breeding colony housed in the Animal Resources Center on the University of Texas campus. Breeders were fed Wayne Lab Blocks, and infants used were 6 to 7 days old (weight of 3.0 ± 0.5 g).

Inoculation procedure. The inoculation procedure used has been previously described in detail (1). Essentially, after a 6-h fast, infants were inoculated orally with a blunted 21 gauge needle equipped with polyethylene tubing and attached to a tuberculin syringe. Evans blue dye (0.01%, wt/vol) was included in the dose as a tracer to insure proper delivery of the inoculum. Radiolabeled inocula were prepared as described below. Nonlabeled inocula were prepared from dilutions of an overnight, 37°C brain heart infusion slant. The inocula contained approximately 5×10^6 colony-forming units in 0.05 ml.

Preparation of ^{35}S-labeled inocula. The medium used was a sulfate-limiting modification of Richardson's minimal A-G medium (3), with $MgCl_2$ substituted for $MgSO_4$ in the trace salts solution to provide equivalent Mg^{2+} (designated MAG). $^{35}SO_4$ was added to 20 ml of MAG in a 125-ml flask at a concentration of approximately 10 μCi/ml. This was inoculated with 5×10^7 colony-forming units from an overnight brain heart infusion slant and grown for 4 h at 37°C with shaking. Approximately three doublings occurred in this time. The cells were removed by centrifugation at 7,000 rpm for 15 min, then washed three times with MAG buffer (MAG minus glucose and asparagine). Cell morphology and motility were unaffected by this process. Cells were suspended in brain heart infusion plus dye and diluted appropriately for the dose. Total counts per minute per dose were generally 100,000 to 200,000.

Treatment of radiolabeled cells. Formalinized cells were prepared by a 30-min exposure to 0.5% Formalin prior to centrifugation and washing. Heat-killed cells were prepared by exposure of the diluted inoculum sample to 65°C for 30 min.

Dose-chase procedure. At varying times postinoculation, animals were sacrificed, and the whole gut, i.e., stomach plus intestines, was removed. The gut was divided into three segments consisting of stomach and intestinal segments of equal length, termed upper bowel and lower bowel. For recovery of radiolabel, gut segments were placed in 10-ml scintillation vials. For viable counts, the tissue was homogenized in brain heart infusion, and suitable dilutions in brain heart infusion were plated on meat extract agar. Recovery was expressed as a percentage of the input dose (counts per minute or colony-forming units).

TABLE 1. *Infant-mouse virulence of cholera strains*

Strain	LD_{50} (CFU)[a]	FA ratio[b]
CA401	10^4	>0.075
569B	10^8	<0.065
VB12	$>10^8$	<0.065

[a] LD_{50}, 50% lethal dose in colony-forming units (CFU) at 48 h after oral challenge.

[b] FA ratio, Fluid accumulation ratio, calculated from: gut weight/(body weight − gut weight), at 16 h after oral challenge at a dose of 1×10^6 to 5×10^6 CFU.

In vitro association with intact mucosal surfaces. Our method is a modification of the method of Guentzel and Berry (16), using KRT buffer as described by Freter and Jones (12). Upper bowel segments from 6-h-starved infant mice were opened longitudinally and spread out on filter paper to prevent reclosure of the tissue. Three sections, each 2 cm in length, were cut from each animal, beginning 2 cm below the stomach. Sections were lifted from the paper and placed in 1 ml of a ^{35}S-labeled vibrio suspension (18^8 colony-forming units per ml) in KRT buffer. After 20 min of incubation at 37°C, tissue sections were washed by dipping in three individual KRT solutions and placed in individual 3-ml scintillation vials.

Solubilization of tissue for scintillation counting. The H_2O_2-perchloric acid procedure of Mahin and Lofberg was used (26). For gut segments, 0.25 ml of 70% perchloric acid and 0.5 ml of 30% H_2O_2 were used; for tissue sections, 0.1 ml of perchloric acid and 0.2 ml of H_2O_2 were used. Vials were sealed and placed in an 80°C water bath for 30 to 45 min. This protocol afforded complete solubilization of the tissue with minimal quenching.

Scintillation counting. Digests were mixed with Aquasol scintillation fluid (the amount dictated by the vial size) and allowed to sit overnight at room temperature to minimize chemoluminescence. Counting was done in a refrigerated Beckman counter for 1 min. Background determined on tissue digests was subtracted from total count values.

Statistical analysis. *P* values were determined by the Wilcoxon rank test (34). Data are expressed as a mean or mean ± one standard error of the mean.

RESULTS

Kinetics of intestinal clearance of radiolabel. Figure 1 depicts the kinetics of clearance of radiolabel from the whole gut for the three strains. CA401 and its isogenic rough derivative, VB12, had identical kinetics initially, but diverged beginning at 8 h, with $P < 0.001$ at 16 h. Eight hours is the time when diarrhea first becomes detectable with CA401 by the fluid accumulation ratio model (1). Counts from CA401 continued to decrease, presumably due to the acute diarrheal response. In contrast, 569B and VB12, which do not promote detectable diarrhea at this dose level, exhibited plateaus.

Technical difficulties, including collection of total excreta, prohibited precise localization of the cleared label. Two points can be made, however. First, when diarrheal fluid was collected from CA401-infected mice, total counts recovered were similar to VB12 total counts. Second, counts above background level could be

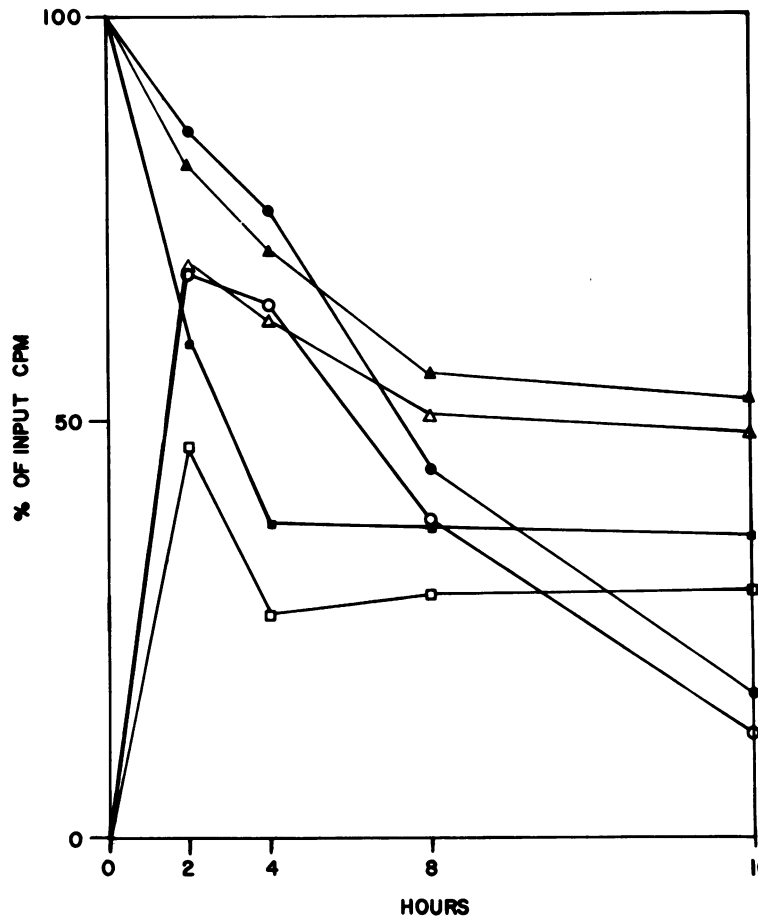

FIG. 1. *Kinetics of clearance of radiolabeled inocula from the whole gut and its lower bowel segment. The number of infant mice infected per strain was 9 to 10. Each point denotes the mean. Symbols:* (●) *whole gut, CA401;* (■) *whole gut, 569B;* (▲) *whole gut, VB12;* (○) *lower bowel, CA401;* (□) *lower bowel, 569B;* (△) *lower bowel, VB12.*

located in the liver and spleen of CA401-infected mice at 16 h. Since *V. cholerae* is a noninvasive pathogen, this suggests that noncellular radioactive material enters the circulation.

The initial, more rapid decline in counts for strain 569B (which is not isogenic with CA401) is unexplained ($P < 0.001$ at 4 h compared to CA401). To determine whether this difference was due to the high toxin production level of 569B (9), the experiment in Table 2 was performed. The loss of radiolabel from CA401-challenged mice at 16 h was significantly inhibited by antitoxoid due to inhibition of the toxin-induced diarrheal response. In contrast, the rapid clearance of 569B was unaffected by antitoxoid. Thus the reason for the difference in initial clearance rate between CA401 and 569B remains obscure.

TABLE 2. *Effect of antitoxoid on percentage of recovery of input ^{35}S-labeled inocula from the whole gut*

Strain	Time (h)	% Recovery of input label (mean ± 1 SEM)[a]		P
		+ AT	+ FCS	
CA401	16	37.6 ± 6.3 (9)	14.6 ± 2.7 (7)	<0.01
569B	4	45.5 ± 7.1 (9)	49.6 ± 6.2 (9)	NS[b]

[a] + AT, Plus heat-inactivated (56°C, 30 min) anticholeragenoid administered per os with challenge cells; dilution used will neutralize 5 to 14 μg of toxin. + FCS, Plus heat-inactivated fetal calf serum administered per os with challenge cells. Parentheses indicate number of animals. SEM, Standard error of the mean.

[b] NS, Not significant.

Effects of various treatments on whole-gut clearance. Table 3 provides evidence that cholera toxin facilitates clearance of label. Mice pretreated orally with 1 μg of toxin in tris(hydroxymethyl)aminomethane (pH 7.5) 8 h prior to CA401 challenge had a more rapid early clearance rate, although the final percentage cleared was the same as CA401 ($P < 0.001$ at 4 h, P not significant at 16 h). Also, Formalin-killed cells, which cannot make toxin, exhibited the high-level plateau previously noted to occur in the absence of diarrhea ($P < 0.001$ at 16 h compared to CA401).

Table 3 also presents data comparing the effect of heat and Formalin killing on clearance of smooth and rough CA401. It is interesting that although label from Formalinized inocula accumulated, heat-killed inocula displayed accelerated clearance. (For CA401 live versus Formalinized, P = not significant at 2 and 4 h; versus heated, $P = 0.001$ at the same times. For VB12 live versus Formalinized, P = not significant at 4 h; versus heated, $P < 0.01$.)

It should be noted that the apparent accumulation of radiolabel in the absence of diarrhea correlated with retention of Evans blue dye in the lower bowel. In contrast, where diarrhea was indicated by fluid distention of the intestines, only traces of dye remained.

Kinetics of distribution of radiolabel in the gut. Label disappeared rapidly from the stomach. At 2 h, less than 10% of input label remained; at 4 h, less than 5% of input label remained. At no time did any significant difference occur between strains. Figure 1 includes the percentage of input counts recovered from the lower bowel. After 4 h, the sets of curves for lower bowel and whole gut were essentially parallel, with the majority of the label recovered present in the lower bowel. Furthermore, by 8 h the difference between whole gut and lower bowel was similar for all three strains.

However, when we examined the radioactivity recovered from the upper bowel (Table 4), significant differences were found. At 2 h, CA401, 569B, and VB12 differed in percentage of input radiolabel associated with upper bowel. By 4 h, CA401 decreased to the same level as 569B, but remained higher than VB12. As expected, at 8 h or later, CA401 did not significantly differ from the other strains. The last column in Table 4 indicates upper-bowel association of Formalinized CA401. Note that, at 2 h, recovery was intermediate between CA401 and 569B, and not significantly different from either. Thus the radiolabel from Formalinized inocula appeared to associate with the upper bowel region to some extent. It was not possible to evaluate the pattern for heated CA401, because there was an apparent inhibition of gastric delivery of label into the intestines (ca. 15% in the stomach at 4 h).

In vitro association with intact mucosal surfaces. In vitro evaluation of upper-bowel association was also done on the strains (Fig. 2). With CA401, for 18 sections tested, the percent-

TABLE 3. *Effect of various treatments on percentage of recovery of input ^{35}S-labeled inocula from the whole gut*

Strain	Time (h)	% Recovery of input label (mean ± 1 SEM) after treatment[a]:			
		Untreated	Toxin pretreatment	Formalin	Heat
CA401	2	86.0 ± 3.2 (9)	76.6 ± 5.5 (10)	86.0 ± 2.3 (5)	41.1 ± 3.6 (5)
	4	76.4 ± 3.8 (10)	37.3 ± 6.7 (10)	75.1 ± 1.3 (5)	36.0 ± 1.8 (5)
	8	45.1 ± 4.7 (10)	29.2 ± 6.3 (10)	65.6 ± 4.7 (5)	31.2 ± 2.4 (5)
	16	17.5 ± 3.4 (10)	20.8 ± 3.0 (10)	61.7 ± 2.8 (5)	27.0 ± 1.3 (5)
VB12	2	82.0 ± 4.0 (10)	ND	ND	ND
	4	72.3 ± 5.8 (10)	ND	67.0 ± 8.1 (5)	36.0 ± 3.8 (5)
	8	56.4 ± 5.4 (10)	ND	ND	ND
	16	53.1 ± 6.4 (10)	ND	57.4 ± 7.6 (5)	19.3 ± 0.7 (5)

[a] Untreated, Same animals seen in Fig. 1. Toxin pretreatment, 1 μg of toxin in tris(hydroxymethyl)-aminomethane buffer (pH 7.5) given orally 8 h before viable cell challenge. Formalin, cells were exposed to 0.5% Formalin for 30 min. Heat, cells were exposed to 65°C for 30 min. Parentheses indicate number of animals. SEM, Standard error of the mean; ND, not done.

TABLE 4. *Percentage of recovery of input ^{35}S-labeled inocula from upper bowel segments*[a]

Time (h)	% Recovery of input label (mean ± 1 SEM)[b]				P compared to CA401[c]		
	CA401	569B	VB12	Formalin[d] + CA401	569B	VB12	Formalin[d] + CA401
2	13.6 ± 2.2 (9)	7.4 ± 1.1 (10)	4.1 ± 1.6 (10)	9.3 ± 1.8 (5)	<0.01	<0.001	NS
4	9.6 ± 1.0 (10)	8.1 ± 0.5 (10)	4.7 ± 2.1 (10)	7.1 ± 0.9 (5)	NS	<0.01	NS
8	4.8 ± 0.4 (10)	6.4 ± 0.7 (10)	3.9 ± 0.2 (10)	6.8 ± 1.1 (5)	NS	NS	NS
16	4.4 ± 0.4 (10)	5.3 ± 0.6 (10)	3.6 ± 0.3 (10)	4.9 ± 0.4 (5)	NS	NS	NS

[a] Same animals as Fig. 1 and Table 3.
[b] SEM, Standard error of the mean. Parentheses indicate number of animals.
[c] NS, Not significant.
[d] Formalin (0.5%) was added to CA401 for 30 min.

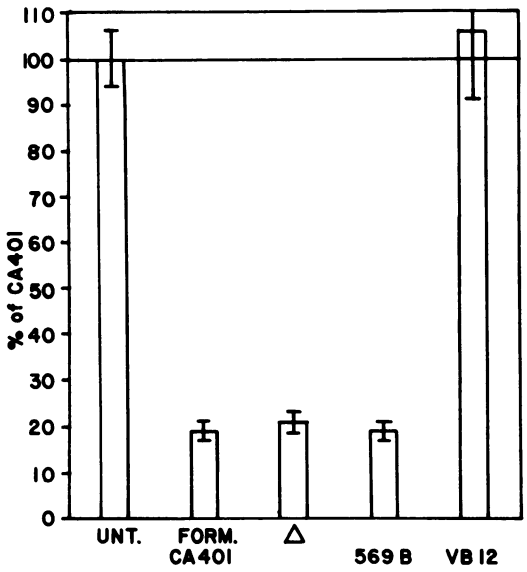

FIG. 2. *In vitro association of ^{35}S-labeled V. cholerae with longitudinal sections of infant mouse upper bowel. Labeled cells were incubated with sections for 20 min at 37°C. Each point represents the mean ± 1 standard error of the mean for six sections. All values are normalized to CA401 and expressed as a percentage. Symbols: UNT, untreated; FORM, Formalin killed; Δ, heat killed.*

age of counts associated with the tissue was 5.7 ± 0.3%. All other values were compared with CA401. Strain 569B, heated CA401, and Formalinized CA401 associated to a lesser extent than CA401; VB12 did not differ significantly from CA401. Thus roughness seems less important in vitro than other properties (12, 16).

Kinetics of distribution of viable organisms. Radiolabel studies measure only recoverability of input counts and do not differentiate between viable organisms and nonviable labeled components. Therefore, we also examined the kinetics of distribution of colony-forming units in the intestines. As seen with radiolabeled inocula, organisms were rapidly removed from the stomach, with less than 1% of the input number remaining at 2 h. Figure 3 shows percentage of recovery of input organisms in the upper and lower bowel. This graph demonstrates the problems inherent in kinetic studies of recoverable viable organisms, since each point reflects only the net outcome of the concurrent processes of multiplication, killing, and clearance. Nevertheless, some observations can be made.

In all three strains the 8-h point appeared to reflect a purging of organisms from the lower bowel. However, between 8 and 16 h for CA401 and 12 and 16 h for the other strains, the organisms seemed to reaccumulate. This could reflect an inhibition of intestinal motility or of bactericidal mechanisms. The greater number of viable organisms recovered from the lower bowel at 8 h in CA401-challenged animals could reflect increased multiplicative potential in the upper bowel and clearance into lower bowel regions.

The pattern in the upper bowel also indicated a complex situation. We noted that all strains showed an accumulation of organisms at later time points. At early times, however, a rather interesting difference was seen. At 4 h (denoted by arrow in Fig. 3), 569B and VB12 demonstrated a dramatic decrease in recoverable viable organisms, whereas CA401 could be recovered at the input level. Thus CA401 could apparently better survive host clearance mechanisms in the upper bowel.

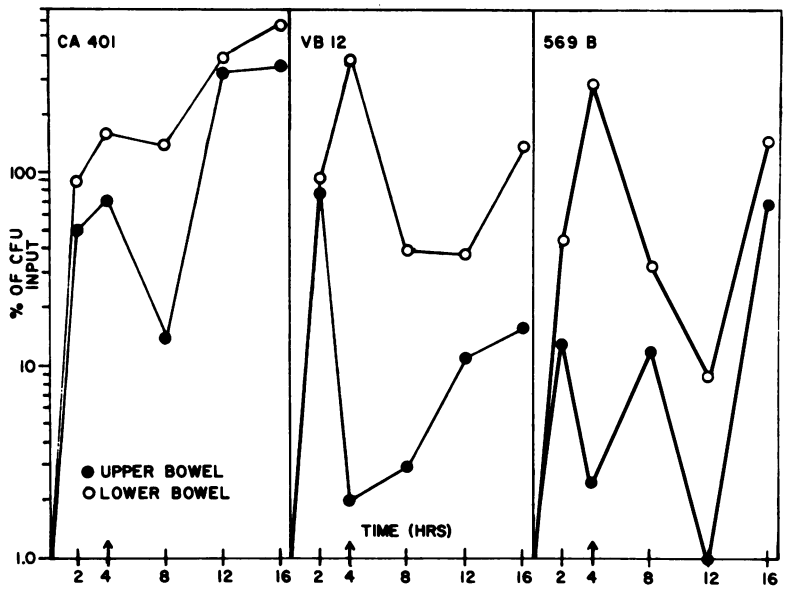

FIG. 3. *Kinetics of distribution of viable organisms in the intestines. The number of infant mice per point was three. Each point denotes the mean. Symbols: ○, lower bowel; ●, upper bowel.*

DISCUSSION

The use of radioactive inocula for studies of clearance of bacteria from and association of bacteria with mucosal surfaces has been examined by a number of researchers. In 1960, Dixon demonstrated the rapid clearance of several bacterial species from the small intestines of rats by including in the inocula a nonabsorbable ^{51}Cr marker to indicate location in the tract (7). More recent reports, however, utilize cells which have been radiolabeled. Green and Goldstein (15) and LaForce (23) examined mucociliary respiratory clearance in mice using ^{32}P-labeled bacteria. Rowley and co-workers have also used ^{32}P-labeled *V. cholerae* to compare clearance in the presence and absence of specific antibody from the intestinal tract of adult (2) and infant mice (2, 22). Perers et al. utilized ^{125}I- and ^{51}C-labeled, heat-killed cells in a comparison of smooth and rough *Salmonella* and *Escherichia coli* in perfused mouse small-intestinal segments (30). We have used ^{35}S-labeled *V. cholerae* for a comparison of intestinal distribution of strains of varying virulence in infant mice.

The relative advantages of the various labels have not been systematically examined. However, we chose to use ^{35}S for reasons of safety and economy. The chief advantage in using ^{32}P is probably its primary localization internally with little external loss. However, this advantage might be negated for *V. cholerae,* since exponentially growing cells excrete lipopolysaccharide (19, 31).

The results presented here indicate that the fate of radiolabeled *V. cholerae* in the intestinal tract of infant mice varies with respect to two parameters. The first parameter is the pattern of clearance of label from the whole gut. Two observations can be made from our data. First, in animals exhibiting a diarrheal response, more of the label is eliminated than in asymptomatic animals, where label accumulates in the lower bowel. Second, the initial rate of clearance of label varies among the strains. Reasons for this variation are not readily apparent, but the more rapid clearance of 569B is not preventable with antitoxoid. The overall trends noted here are essentially compatible with reports by Rowley and co-workers (2, 22) using ^{32}P-labeled 569B in orally challenged infant mice. They also noted that specific antibody effects a more rapid removal of label from the intestines.

The second distinguishing parameter is the degree of upper-bowel association which occurs early after inoculation. Radiolabeled virulent strains associated to a greater extent than less virulent strains. The viable count data also indicated that significant differences in recovery of viable organisms occurred in the upper bowel early in infection, with 569B and VB12 having lower recoveries than CA401. Reduced upper-bowel recovery has also been noted for another class of virulence-deficient mutants, nonmotiles (16). In addition, Bloom and Rowley have demonstrated that small-intestinal mucosal association is significantly lowered by antibody pretreatment (2). In view of the rapid clearance of

most of the label from the upper bowel, probably primarily by normal peristalsis (7) and mucus flow (10), it is possible that this early upper-bowel association phenomenon is essential for establishment of a successful infection. Reduced ability to associate with the upper bowel may contribute to reduced virulence. However, other concurrent processes such as resistance to host bactericidal mechanisms and multiplication must also be considered.

Attempts to correlate in vivo upper-bowel association of radiolabel with in vitro interaction with intact mucosal surfaces were unsuccessful. Thus, although 569B had reduced abilities in both systems, VB12, which was reduced in vivo, had equivalent ability in vitro compared to the smooth, parental CA401. These results are in contrast to interactions observed for *Salmonella,* in which rough mutants associate to a greater extent than smooth strains with HeLa cells (20) and with the intact mucosal surface of perfused mouse small-intestinal segments (30). Further, although radiolabel from Formalinized CA401 tends to remain in the upper bowel, Formalin or heat killing reduces in vitro association. Apparently, the bacterial processes involved in the two model systems are emphasized to varying degrees. Thus, although multiplication and survival are essential in vivo, other functions of viable cells such as motility (12, 16) may assume primary importance in vitro. However, the possibility that heat or Formalin destroys essential surface components should also be considered. It is known that Formalin decreases in vitro association of gonococci (18) and *Vibrio parahemolyticus* (4) with eucaryotic cells. In addition, *Salmonella typhimurium,* killed by heat or UV, attaches to HeLa cells to a lesser extent than viable cells do (20).

These studies emphasize the problems inherent in examining infection in an open system where several processes operate simultaneously to elicit a net outcome, i.e., disease versus nondisease. Nevertheless, our results point out important features of *V. cholerae* infections in infant mice. First, the upper bowel is the gut location where intrastrain differences are most significant. Second, differences can be detected only during the first few hours postchallenge.

Additional studies examining survival and multiplication of *V. cholerae* in the upper bowel early in infection will be described elsewhere (V. S. Baselski, R. A. Medina, and C. D. Parker, submitted for publication).

We are also using these procedures to investigate the infective capability of a series of diarrhea-deficient mutants of CA401.

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