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Use of Fluorescent Antibody in Studies of Immunity to Cholera in Infant Mice

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Infant mice 8 days of age were infected orally with virulent, motile, classical or El Tor strains of *Vibrio cholerae* and with nonmotile mutants of low virulence derived from the same strains. At intervals of 8 and 12 h postinfection, frozen thin sections of the ileum were prepared, stained with fluorescein isothiocyanate-labeled rabbit anti-vibrio antibody, and examined with the fluorescence microscope. The motile organisms were present in larger numbers, especially at 12 h, and had penetrated the intervillous spaces and crypts of Lieberkuhn more completely than nonmotile vibrios. Dilution counts were made on various regions of the intestines of infant mice challenged orally 12 h previously with either motile or nonmotile strains of *V. cholerae*. Greater numbers of organisms were found, especially in the upper intestinal regions, when motile organisms were used. Low numbers of vibrios, limited mostly to the lumen, were seen in the ileum of infant mice infected with motile organisms when the infants were the offspring of mothers that had been immunized with crude flagellar vaccine or a vesicular preparation derived from the vibrio cell surface. The distribution of vibrios in this case was similar to that found in infected infants of unvaccinated mothers challenged with nonmotile organisms. Motility appears to enable the bacteria to better populate the upper regions of the intestinal tract and to avoid the washing effects of secretions and peristalsis. Antibacterial immunity may function, at least in part, by making it impossible for motile vibrios to accomplish this widespread distribution within the ileum.

The role of motility in the pathogenesis of *Vibrio cholerae* has been studied in our laboratory. Guentzel and Berry (10) examined toxinogenic and prototrophic nonmotile strains that retained normal surface structures as judged by phage susceptibility tests. The nonmotile strains, including both Ogawa and Inaba serotypes of classical and El Tor biotypes, were found to be less virulent for orally challenged mice than the wild types from which they were derived. Eubanks et al. (3) extended these findings by showing that nonmotile vibrios were less virulent for adult mice infected intraperitoneally with organisms suspended in hog gastric mucin. As we have described most recently (4), crude flagellar vaccines or preparations of vesicular material, believed to be composed of flagellar sheaths of material from the vibrio cell surface, confer high levels of passive immunity on infant mice of mothers that had been vaccinated with either preparation at the time of mating. By implication, the virulent motile vibrios are immobilized by the antibodies in the milk and thereby permit the baby mice to survive an otherwise lethal challenge.

The importance of motility in other animal models also has been reported. Williams et al. (22) used isolated ileal loops of the rabbit to show that motile vibrios penetrated into the crypts of Lieberkuhn in larger numbers than a nonmotile mutant. Jones (14) has made similar observations.

On the basis of these findings, it is logical to assume that an intimate association of a toxinogenic strain of *V. cholerae* with the intestinal mucosa is an essential condition for the onset of the disease state. Since motility seems to be necessary for this association, the experiments reported here were undertaken with this in mind. The localization of the bacteria within the intestine was determined with the use of fluorescein-labeled anti-vibrio antibody. In the absence of a widespread distribution of vibrios between the villi and deep within the crypts of Lieberkuhn, no serious illness was observed.

MATERIALS AND METHODS

Mice. CFW mice were purchased from Carworth Division, Charles Rivers Farms, and raised in de-

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partmental animal facilities. The mice were housed and fed as described previously by Guentzel and Berry (9).

Bacterial strains. The highly virulent Inaba strain CA401 and El Tor Inaba strain 8233, and a weakly virulent nonmotile strain of each (CA401 M-1 and 82336B3), have been described previously (10). The nonmotile strains were prototrophic and toxinogenic in vivo and in vitro and manifested the same phage susceptibility as the parental strains. All cultures were maintained in the lyophilized state and restored as needed.

Oral challenge. Seven-day-old mice (eight to ten mice per litter) were fasted overnight and then challenged by the oral route as described by Guentzel and Berry (9). In each case, the challenge dose was approximately 10^8 colony-forming units representing approximately 1,000 50% lethal doses for the motile strains. The suckling mice were returned to their mothers.

Vaccines. The methods of preparation of crude flagella (CF) from Inaba CA401 and vesicles from a nonmotile mutant of this strain have been described in a separate report (4). HS222, a gift of Paul Actor, Smith Kline and French Laboratories, is an Ogawa-derived subcellular vaccine, the properties of which have been described (13). Female CFW mice, 10 weeks of age, were administered a single subcutaneous dose of the respective test vaccines just prior to mating, as described previously (9). Vaccines were diluted in sterile nonpyrogenic saline (Travenol, Inc.) immediately before use. Control animals were injected with saline alone.

Anti-vibrio antiserum production. Adult rabbits were bled prior to immunization to obtain normal rabbit serum. Antiserum was produced against live *V. cholerae* strain CA401. The antigen was washed from an 18-h heart infusion agar culture with saline and adjusted to 100 Klett units. The rabbits were immunized according to the following protocol: day 1, 0.2 ml subcutaneously; day 5, 0.5 ml subcutaneously; days 9 and 14, 0.5 ml intravenously; days 14, 19, 27, and 35, 1.0 ml intravenously. The rabbits were bled seven days after the last injection and the titer was determined by agglutination tests. The titer against live CA401 was 1:2,560.

Preparation of anti-mouse IgA. The immunoglobulin A (IgA) secreting MOPC 315 tumor line was obtained from M. Potter, National Cancer Institute, NIH. Ascites fluid was harvested from BALB/c mice (Jackson Laboratories, Health Research, Inc.) bearing MOPC 315 tumors. IgA was purified according to a modification (W. Mandy of this department) of the procedure of Hashimoto et al. (11). The ascites fluid was centrifuged for clarification and filtered to remove fibrous clots. In the first step of the isolation procedure, IgA was precipitated with sodium sulfate at a final concentration of 18%. The fraction was dissolved in saline and reprecipitated with sodium sulfate at a final concentration of 16%. After overnight dialysis against 0.9% saline, the proteins were precipitated again with sodium sulfate at 12.5%. The IgA contained in the supernatent fluid was precipitated by adjusting the solution to 16% sodium sulfate. The precipitated IgA was collected, dissolved, and dialyzed against 0.01 M sodium phosphate buffer, pH 7.5. The IgA was then subjected to diethylaminoethyl-cellulose chromatography, using a stepwise elution technique in which the molarity of the eluting sodium phosphate buffers (pH 7.5) were increased from 0.01 to 0.3 M. The IgA, which eluted with 0.3 M buffer, was dialyzed against distilled water and preserved by lyophilization.

Rabbit anti-mouse IgA was prepared according to the following protocol: day 1, 0.5 mg in each footpad; day 14, 0.5 mg in Freund complete adjuvant given subscapularly; days 28, 42, and 56, 0.5 mg subcutaneously. Since no immunoelectrophoretic precipitin bands appeared when purified IgM or IgG was reacted against the anti-IgA, it is assumed that the common light chains in the different classes of immunoglobulins were not antigenically active under these conditions. A single precipitin band did form when purified IgA or normal mouse serum was tested against the anti-IgA.

Preparation of FA conjugates. Fluorescent antibody (FA) conjugates were prepared from the normal rabbit serum and the antiserum as outlined by Herbert et al. (12). The sera were precipitated three times with 35% saturated ammonium sulfate at 25°C. The globulin fraction from each was dialyzed at 4°C against frequent changes of 0.85% NaCl solution (pH 8.0) until sulfate was no longer detected in the dialysate. The protein concentration of each globulin fraction (and the final conjugates) was determined using the method of Lowry et al. (16). The protein concentration was adjusted to 20 mg/ml prior to labeling.

Fluorescein isothiocyanate (FITC) (International Biological Supplies, Inc., Melbourne, Fla.) used in the labeling was certified as 99.9% pure by the Biological Stain Commission. The globulin fractions were labeled by the dialysis method. The labeling reaction was carried out for 18 h at 25°C, pH 9.5, in 0.05 M Na_2HPO_4. Unreacted fluorescent material was removed by extensive dialysis against 0.01 M phosphate-buffered saline, pH 9.0, at 4°C. Merthiolate at a final concentration of 1:10,000 was added to the conjugates, and they were stored at 4°C.

FITC was determined as protein-bound FITC by absorbance at 490 nm in 0.1 N NaOH and related to a pure fluorescein diacetate reference standard (17). The fluorescein-to-protein ratios of the conjugates were calculated from the FITC and protein measurements and expressed as micrograms of protein-bound FITC per milligram of protein. The fluorescein-to-protein ratios of the normal rabbit serum conjugate and the anti-CA401 conjugate were 12.8 and 11.8, respectively. The conjugates were diluted 1:16 in 0.01 M phosphate-buffered saline, pH 9.0, for use in FA staining of intestinal sections.

Preparation and staining of sections. The intestines were removed from infant mice and immediately placed in a 0.9% NaCl solution. A 2-cm ileal segment (2 cm proximal to the cecum) was ligated and excised from the intestine. The excised ileal segment was quick-frozen in O.C.T. compound (Lab. Tech. Products) in a dry-ice and acetone bath. Approximately 30 sections (10 to 12 μm thick) were cut from each ileal segment using an International

cryostat. Sections were allowed to air dry on glass slides and then were fixed for 90 s in absolute methanol.

Several sections in each group were randomly chosen as controls and stained with the normal rabbit serum conjugate. All sections were stained for 30 min at 25°C in a moist chamber. Slides were rinsed in phosphate-buffered saline (pH 9.0) and allowed to air dry and were mounted in buffered glycerol saline, pH 9.0.

Slides were read with a Zeiss Universal fluorescence microscope equipped with an HBO 200 mercury arc lamp. Filters used were a BG 38 (red-absorbing filter), a BG 12 (primary filter), and Zeiss 43/56 (secondary filters). Kodak Tri X black-and-white film was used for photomicrography.

RESULTS

Distribution of vibrios in the ileum of mice 8 h postinfection. Infant mice, infected with the highly virulent, motile Inaba strain CA401, had a number of fluorescing bacteria in the ileal lumen 8 h postinfection. Some had penetrated into intervillous spaces, as can be seen in Fig. 1a. In Fig. 1b, a comparatively small number of vibrios located primarily in the lumen are visible at 8 h. This picture is typical of those seen with mice infected with a nonmotile mutant of strain CA401. This difference in the number and distribution of vibrios in intestines of mice infected with either motile or nonmotile organisms was typical of numerous sections.

Distribution of vibrios in the ileum of mice 12 h postinfection. When infected infant mice were sacrificed 12 h after oral challenge with the motile CA401 strain of *V. cholerae*, observations similar to those in Fig. 2a and b were obtained. Numerous bacteria were found in the lumen, as the close-up shown in Fig. 2a makes evident. Many had penetrated into intervillous spaces and also deep into the crypts. The latter can be seen especially in Fig. 2b. Some distortion in the structure of the mucosa is visible in both plates, and this is believed to be due to the diarrhea that was present at the time. Figure 2c is a photograph of an ileal section from an infant mouse 12 h after infection with a nonmotile mutant of strain CA401. Bacteria were visible in the lumen and a few had penetrated into a crypt, but it was also true that many sections had no visible fluorescing organisms. Presumably the vibrios were unable to make close contact with the mucosal structures and were swept out of the intestine more rapidly and completely than the motile parent strain.

In infant mice infected orally with an El Tor Inaba strain, 8233, and a nonmotile mutant derived from it, observations similar to those just described were made. This can be seen in Fig. 3a and b. The motile form was present in very large numbers throughout the cross-sectional area 12 h after infection (Fig. 3a), and swollen and distorted villi were evident. No nonmotile organisms were visible at this time anywhere in the ileum, except for a few in the lumen (Fig. 3b). The difference in the appearance of the villi in this section compared to that in Fig. 3a is quite dramatic. Swollen and edematous structures were evident in the intestine containing the motile vibrios.

Effect of passive immunity on the number and distribution of motile vibrios in the ileum. Offspring nursed by mothers that had been vaccinated at the time of mating with 1 μg of CF or with 1 μg of vesicles were challenged orally with the virulent CA401 strain. This level of either vaccine was just sufficient to provide essentially complete protection against homologous challenge (4). The number of fluorescing vibrios seen in sections of ileum of infants sacrificed 12 h later was small and limited to the lumen, or in some cases no fluorescing vibrios were observed. The characteristic appearance in such animals can be seen in Fig. 4a and especially in the close-up photograph in Fig. 4b. The appearance in these sections is similar to that of Fig. 2c and 3b taken from mice challenged with nonmotile mutants.

An even higher level of protection was apparent in the intestines of infants suckled by mothers that had been vaccinated with 10 μg of flagella or vesicles. A typical section of ileum is shown in Fig. 4c, where no fluorescing organisms can be seen. In offspring of mice given a level of immunogen sufficient to prevent mortality in 50% of the animals, the appearance of vibrios in the gut ranged from that seen with motile organisms in nonimmune animals to that seen with nonmotile organisms in nonimmune animals. The presence of antibody in the milk is believed to immobilize the vibrios either through direct action on the flagella or else by clumping the organisms. The photographs, especially Fig. 4a, show the presence of clumps, but these were seen also in some sections from mice that were not passively immunized through vaccination of the mothers. Whatever the basis for the reduction in number of fluorescing bacteria and the prevention of their penetration into intervillous spaces and crypts, the antibody was clearly effective.

Reversal of passive immunity by prior treatment with anti-mouse IgA. One-tenth milliliter of rabbit anti-mouse IgA was given orally 30 min prior to challenge with 10^8 colony-forming units of Inaba CA401 to infant mice suckled by immunized mothers. A comparison was made between the percentage of survivors at 36 h in infants given anti-IgA serum and

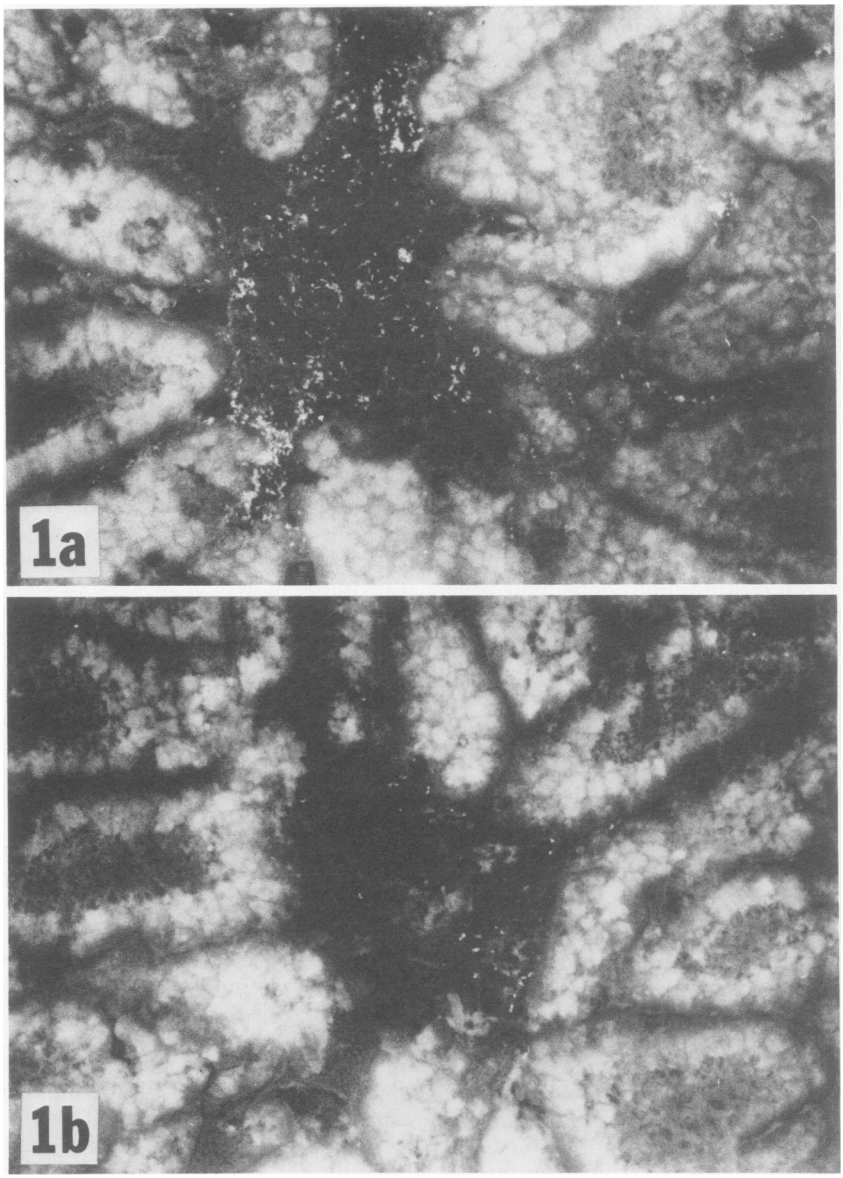
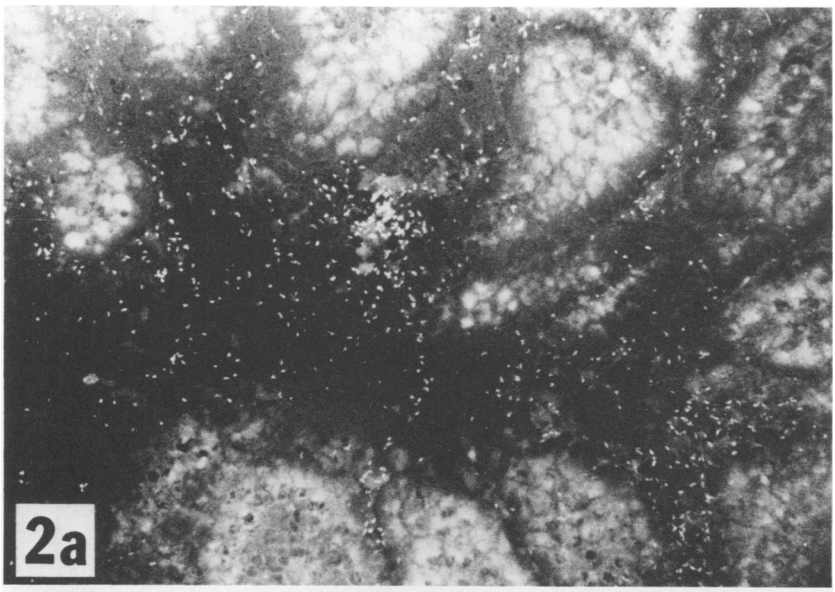


FIG. 1. *Distribution of V. cholerae in the ileum of infant mice at an early point (8 h) in the infection. (a) Motile classical strain. ×390. (b) Nonmotile classical strain. ×390. The organisms (Fig. 1–5) appear as bright spots, which may be located in the lumen, between the villi, or at the base of villi in the crypts of Lieberkuhn.*

those given control serum. The results are presented in Table 1. When mothers were immunized with 10 μg of CF, anti-IgA reduced survivors from 100 to 85%, a change that is not statistically significant. Vaccination of the mothers with 1 μg of CF protected 97% of the offspring, but this was reduced to 19% survivorship in those given anti-IgA. It seems obvious, therefore, that the former group of mice was protected by an excess of IgA in the intestine, even in the presence of the rabbit antiserum. This was not the case when mothers were vaccinated with the 1-μg dose of CF. Similar results were obtained when the HS222 vaccine was

FIG. 2. *Distribution of V. cholerae in the ileum of infant mice at a later point (12 h) in the infection. (a) Close-up of lumen, motile classical strain. ×390. (b) Photomicrograph showing extensive distribution of the motile strain. ×248. (c) Nonmotile classical strain. ×390.*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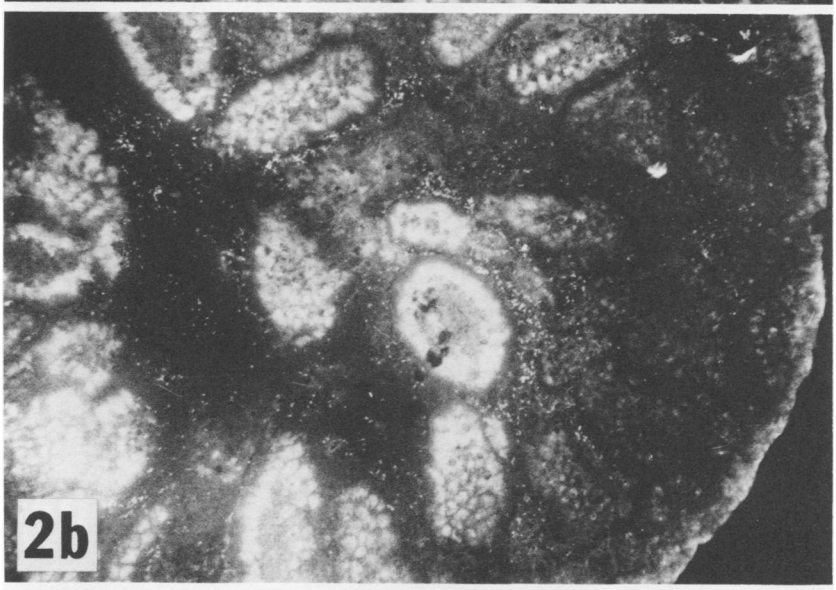
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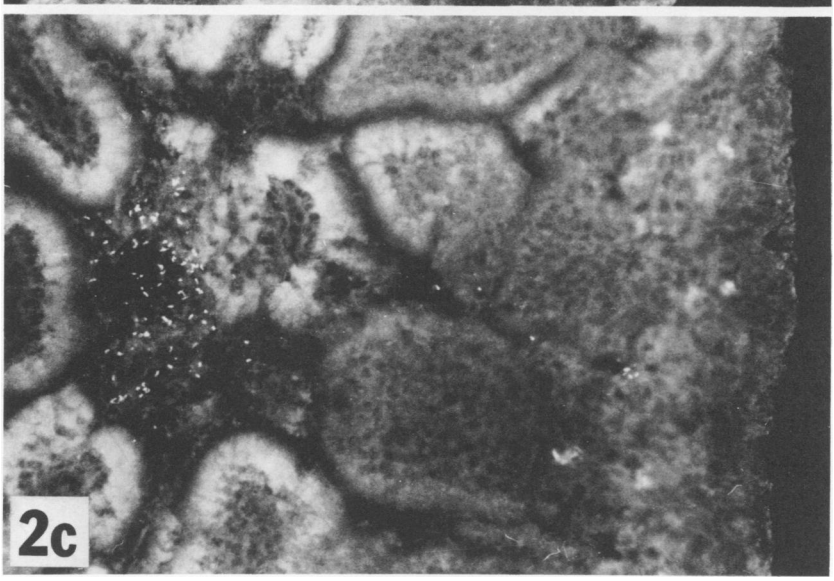
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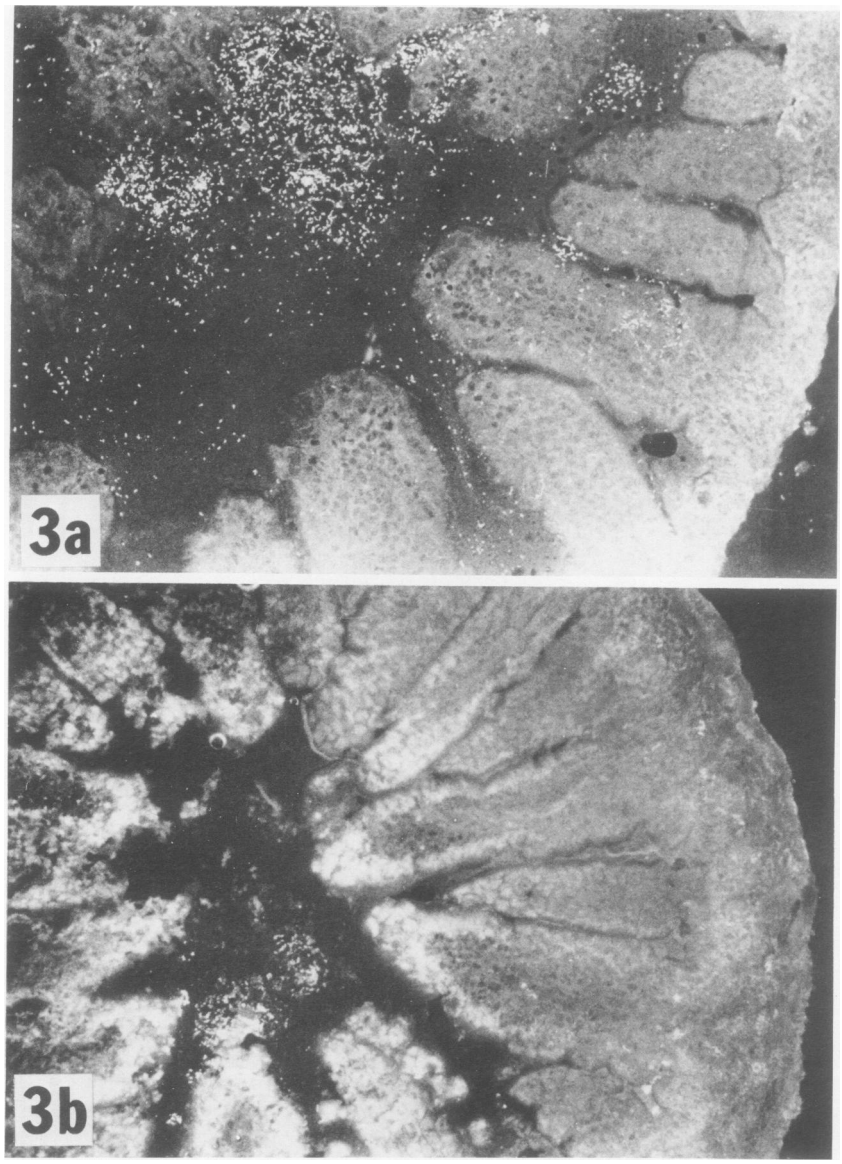
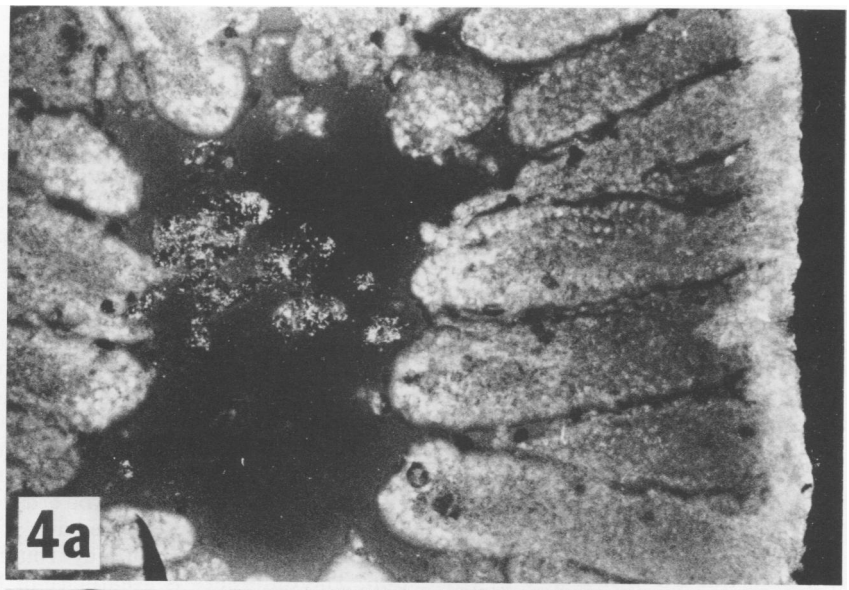


FIG. 3. *Comparison of the distribution of El Tor vibrios in the ileum of infant mice at 12 h. (a) Motile strain. ×248. (b) Nonmotile. ×248.*

administered at a level of 1 μg to the mothers at the time of mating. The use of anti-IgA decreased survivorship from 52 to 0%. On the basis of these data, it seems clear that the passive immunity conferred by immunized mothers on their offspring can be reversed at least in part by anti-mouse IgA. The contribution of other immunoglobulins in this situation is not known.

Figure 5 shows that the reversal of protection by anti-IgA in passively immunized infant mice is accompanied by a distribution of vibrios in the ileum similar to that seen in nonimmune animals. The photomicrograph shows the pres-

FIG. 4. *Effect of passive immunization on the distribution of motile classical V. cholerae at 12 h postinfection. Ileal sections of offspring of mothers immunized with: (a) 1 μg of CF, ×248; (b) 1 μg of CF, ×390; (c) 10 μg of vesicles, ×248.*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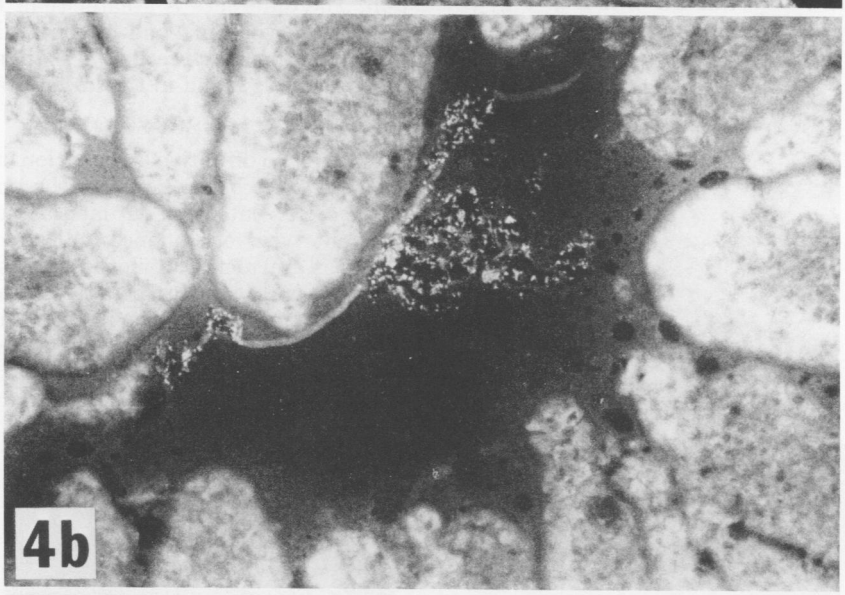
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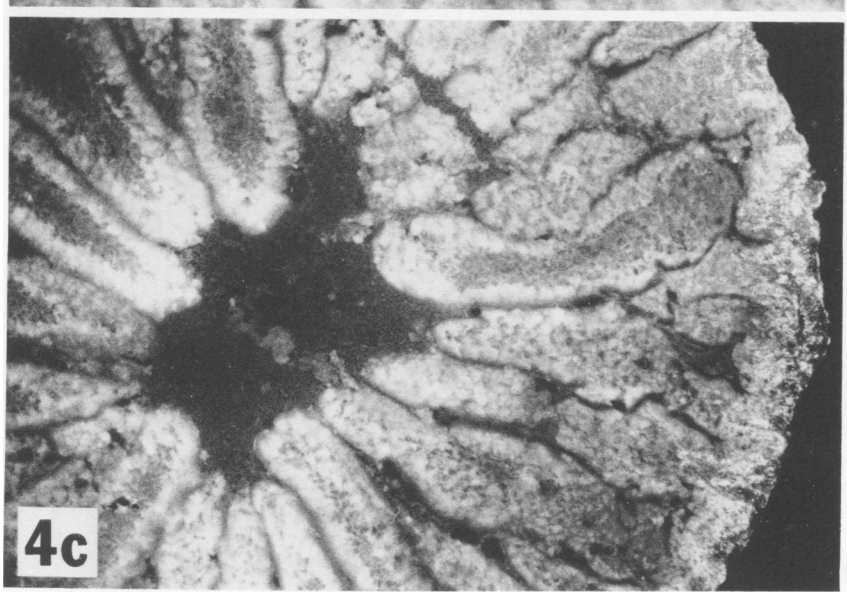
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ence of a large number of fluorescing bacteria extensively distributed throughout the edematous ileum.

Effect of motility on the number and distribution of vibrios in the intestinal tract. Infant mice, 8 days of age, were infected orally with 5 $\times 10^7$ colony-forming units of the motile El Tor Inaba strain 8233 and with the nonmotile mutant derived from it. Twelve hours later, the mice were sacrificed and the intestines were removed and divided into four sections. These were made up of: (i) the duodenum, jejunum, and proximal ileum; (ii) the 2-cm section of ileum used for the FA experiments; (iii) the remainder of the ileum plus the cecum; and (iv) the large intestine. Each segment was homogenized in 5 ml of sterile saline, and appropriate dilutions were plated on Verwey's dextrin heart infusion medium (19). The results obtained with seven mice in each group are shown in Fig. 6 and 7. Greater numbers of motile than nonmotile organisms were present in all segments of the intestine, but the smallest difference was seen in the large intestine. In the segment of ileum used for the photographs, there were nearly 100 times as many motile vibrios as nonmotile. This is the order of magnitude one might expect on the basis of what the photomicrographs indicate by rough estimation. The difference is greater than 100 in the duodenum, jejunum, and proximal ileum and less in the ileum and cecum. Less than a 10-fold difference was found in the large intestine.

TABLE 1. *Effect of anti-IgA on susceptibility of passively immunized mice to V. cholerae*

Immunogen	Dose (μg)	Anti-IgA[a]	% Survivors at 36 h	Survivors/ total
CF	10	−	100	34/34
	10	+	85	11/13
	1	−	97	32/33
	1	+	19	5/27
HS222	1	−	52	23/44
	1	+	0	0/10
Saline		−	0	0/20
		+	0	0/10
Unchallenged control		+	100	10/10

[a] Rabbit anti-mouse IgA (0.1 ml) was given orally 30 min prior to oral challenge.

DISCUSSION

There is no difinitive explanation of why motility seems to be essential for the virulence of *V. cholerae*. Data from our laboratory based on different types of evidence indicate, however, that it is. Virulence in motile strains seems to correlate with a deeper penetration of the bacteria into the crypts of the intestine and with a larger population of organisms present. There is the question, then, of whether the organisms reach the deeper recesses of the ileum because

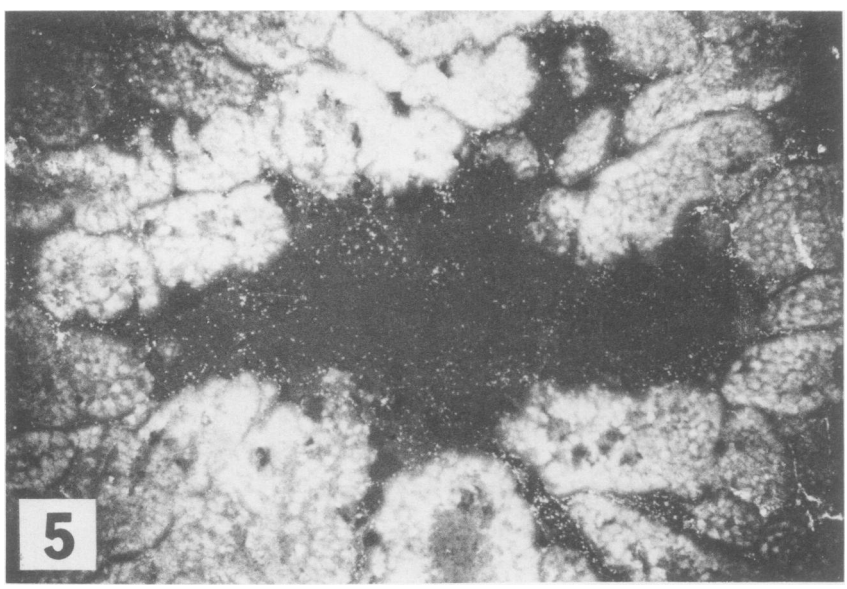

FIG. 5. *Effect of anti-IgA on the distribution of motile classical V. cholerae in passively immunized mice. Ileal section of offspring of mother immunized with 1 μg of CF.*

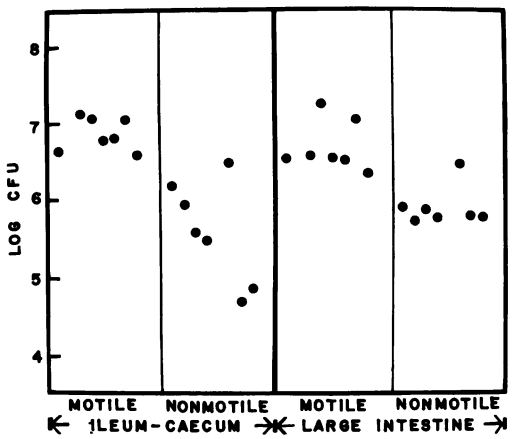

FIG. 6. *Comparison of the distribution of a motile and a nonmotile strain of V. cholerae in the upper regions of the intestinal tract of infant mice.*

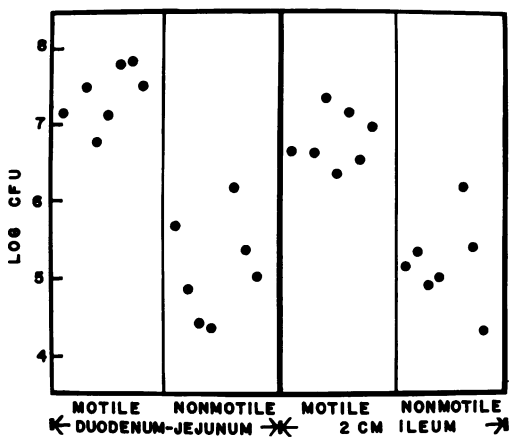

FIG. 7. *Comparison of the distribution of a motile and nonmotile strain of V. cholerae in the lower regions of the intestinal tract of infant mice.*

of their swimming power, their ability to respond positively to chemotactic stimuli, or because of an attachment site between mucosal cells and flagellar structures. From the scanning electron photomicrographs of Nelson et al. (18), the major site of attachment of vibrios to mucosal surfaces appears to be along the sides of the organism rather than at the end where the single polar flagellum emerges. Moreover, the lack of ability of isolated flagella given in advance of oral challenge to reduce mortality, as shown in the preceding paper (4), makes it unlikely that flagella serve as the major attachment site. There is always some uncertainty, when mutants of a given type are isolated, whether characters for which there has been no selection have also changed. It was with this in mind that Guentzel and Berry (10) subjected the nonmotile strains to a variety of tests designed to identify additional mutations, but none was detected. Moreover, the one common characteristic that resulted in loss of virulence in a number of mutants was the loss of their ability to swim. Were some other mutation responsible, it would not likely show up in all of the nonmotile strains, unless it were closely linked genetically.

The difference in the numer of colony-forming units obtained by culturing homogenized intestines of infant mice infected with motile vibrios versus the number obtained when challenge was with nonmotile strains is as great as anticipated from visualization with FA (see Fig. 6 and 7 versus the photomicrographs). The close association of vibrios seen in sections of intestine must have retarded the rate at which they were swept along the intestinal tract. A combination of greater retention plus time for multiplication would account for the numerical differences seen visually and by dilution counts.

A marked reduction in the number of motile vibrios and their presence in intervillous spaces and crypts was observed in baby mice passively immunized at a level sufficient to prevent mortality in these animals (Fig. 4). Antibody might function by directly preventing attachment of the organisms to the mucosal surface. Freter and his associates (5–7) observed that specific antibody reduced adherence of *V. cholerae* in vitro and in ligated ileal loops of rabbits.

Antibody could also prevent attachment indirectly, as suggested by Verwey (20, 22), by immobilizing the organisms and thus reducing their penetration of a mucous barrier and subsequent migration into the intervillous spaces. This immobilization of vibrios may depend on cross-linking and physical aggregation of the organisms, as the results of Verwey and co-workers (20, 22) and Rowley and co-workers (1, 21) suggest. We observed more extensive clumping of vibrios in sections from immunized baby mice than in control animals, although apparent clumping was sometimes observed even in the latter case.

The reduction in numbers of vibrios and their restriction to the lumen in ileal sections was similar in baby mice born to mothers immunized with CF or vesicular (predominately lipopolysaccharide) preparations. It is significant that antisomatic antibody can prevent migration of vibrios through soft agar and mucous gels (20, 22). Specific antiflagellar antibody is at least as protective, if not more protective, against oral challenge than antisomatic anti-

body in passively immunized baby mice (1, 21). Antibody of either class may inhibit mobility by immobilizing the flagellum directly. Benenson et al. (2) observed in 1964 that antisomatic antibody quickly rendered motile vibrios nonmotile in wet mounts of the stools of cholera patients. These results, coupled with the observations reported in the present study, suggest that prevention of attachment by antibody can occur by processes other than by directly blocking an adhesive substance on the vibrio surface.

The reversal of the passive immunity in infant mice with anti-mouse IgA is not unexpected. Fubara and Freter (8) have found that purified secretory IgA preparations derived from orally vaccinated animals, and free of demonstrable IgG or IgM, protected isolated ileal loops of rabbits against fluid accumulation after challenge with homologous organisms. Our observations do not exclude a contribution of other classes of immunoglobulins to the immunity we observe.

It is our hypothesis that the ability of motile strains to penetrate between villi and into crypts helps the organisms avoid removal by intestinal secretions and the peristaltic action of the digestive tract. The vibrio mucinase may assist the organism in the penetration of mucus barrier. Adsorption of the bacteria to the mucosal epithelium may be mediated by the slime envelope or hemagglutinin described by Lankford and Legsomburana (15). It has not been proven, but it can be suggested that the antibody that is passively acquired by infant mice from their mothers immobilizes the vibrios and also may agglutinate them and in one or both ways convert motile forms into functionally nonmotile ones. This is subject to experimental verification and will be investigated.

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