Colonization of the Rabbit Small Intestine by Clinical and Environmental Isolates of Non-O1 Vibrio cholerae and Vibrio mimicus

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We examined the capability of 12 isolates of non-cholera toxin-producing O1 and non-O1 Vibrio cholerae to colonize the small intestine of adult rabbits and cause diarrhea. Using the removable intestinal tie-adult rabbit diarrhea model, we found that eight environmental isolates that showed no or marginal biological activity in other diarrhea models (rabbit ileal loop, infant rabbit, and suckling mouse) appeared to be incapable of attaching to and colonizing, even transiently, the small intestinal mucosa of animals with normal clearance mechanisms. In contrast, three clinical isolates attached, proliferated rapidly, and colonized mucosal surfaces of the entire small intestine within 8 h of challenge. This led to diarrhea with strikingly high rates of mortality compared with that of rabbits given similar challenge doses with strains of O1 V. cholerae that produce cholera toxin and Vibrio mimicus, which produces a toxin similar to cholera toxin. We have further demonstrated that multiple exposures to enteric infection by these strains elicited local and serum antibodies that reacted strongly with cell surface antigens of the homologous strain and showed a high degree of cross-reactivity against the cell surface antigens of the two heterologous strains. The enteric infections appeared to engender protection against subsequent infection as well, as evidenced by reduced incidence of diarrhea and duration of fecal shedding of the challenge organism upon subsequent challenges.

The number of reported cases of gastroenteritis in the United States caused by Vibrio cholerae of serogroups other than O1 has increased during the past decade (2). This has been the result of an enhanced awareness of the importance of these organisms as human pathogens (2) and of their ubiquity in estuarine environments in this country and elsewhere (2, 11). It has also come about through an increased sensitivity stemming, indirectly, from the discovery of an endemic focus of cholera in the Louisiana-Texas Gulf coastal region in 1978 (1, 13).

V. cholerae O1, the causative agent of cholera, and some members of the non-O1 V. cholerae and Vibrio mimicus, a closely allied species, produce diarrhea mediated by cholera toxin (CT), a well-characterized enterotoxin (8), or by one of the closely related toxins which we designate as "CT-like toxins" (5, 19, 24). Most of the non-O1 V. cholerae isolated from cases of diarrhea, however, appear to produce disease through some other mechanism than CT-like toxin (17, 19, 20). Indeed, the majority of non-O1 V. cholerae isolates from clinical and environmental sources do not seem to possess the structural gene for this enterotoxin (12).

An outbreak of non-O1 V. cholerae diarrhea occurred in November 1979 and was associated with the eating of oysters taken from waters around Florida (4). Three isolates from this outbreak (designated strains 2193c, 2194c, and 2227c) were subsequently demonstrated to be pathogenic in both the rabbit ligated ileal loop assay and the infant rabbit model, although none of the three appeared to produce CT-like toxin (15). These isolates did not appear to be the same on the basis of serotyping data. Strain 2227c was Smith serotype 17, whereas the other two were untypable.

Although the mechanism whereby "nontoxicogenic" clinical isolates mediate diarrhea is unknown, it is clear that, like toxigenic strains, they possess the ability to colonize the small intestine. Since mucus secretion and peristalsis make the small intestine a generally inhospitable environment for bacteria, enteropathogens must possess effective attachment factors to resist the action of these natural clearance mechanisms.
We have been especially interested in the early events of small intestinal infection, those which determine whether an enteropathogen will succeed in colonizing some portion of the mucosal surface with enough tenacity to enable it to go on to overwhelm the host's defenses and infect the entire intestine. When we studied toxigenic O1 V. cholerae in the recently described removable intestinal tie-adult rabbit diarrhea (RITARD) model (22), we found them to be able to colonize rapidly the mucosal surface of the entire small intestine in a distinctive and reproducible kinetic pattern.

We have used this model to examine the capacity of both clinical and environmental isolates of nontoxigenic, non-O1 V. cholerae to colonize the small intestine and to begin to study the host immune response to such infections. We report here that the RITARD model clearly distinguishes two categories, colonizing and noncolonizing strains, and that the local and serum immune response to infection with each of three strains isolated from the 1979 Florida outbreak suggests a high degree of antigenic cross-reactivity between them, even though they appear to be of at least two different serogroups.

**MATERIALS AND METHODS**

**Animals.** Outbred New Zealand White rabbits of both sexes were used throughout these experiments. Unless otherwise indicated, their weights ranged from 1.6 to 2.7 kg. Animals were allowed to acclimate for at least 1 week in our animal care facility and were used only if they showed no evidence of diarrheal disease during that time.

**Bacteria.** The sources and descriptions of the V. cholerae and Escherichia coli strains used in this study are shown in Table 1. Organisms were maintained at −60°C in brain heart infusion broth containing 15% glycerol. To prepare challenge doses, we added a loopful of thawed supernatant to 2 ml of 2% peptone water, which we incubated on a roller drum at 50 rpm for 16 h at 37°C. We used this culture to inoculate (0.5%, vol/vol) 10 ml of Casamino Acids-yeast extract (CAYE) broth (10) in a 50-ml Erlenmeyer flask, which was incubated in a 37°C shaker bath at about 200 cycles per min for 4 h. We prepared culture filtrates from supernatant fluids remaining after centrifugation at 6,000 × g for 20 min at 20°C. We then suspended the sedimented cells in an equivalent volume of 0.05 M phosphate-buffered saline (PBS), pH 7.4. We determined viable plate counts on gelatin agar. Serial dilutions were prepared in PBS.

**Assays for enterotoxigenicity and enteropathogenicity.** We screened for cholera toxin and CT-like toxin by using the ganglioside GM1-enzyme-linked immunosorbent assays (GM1-ELISA) described previously (18). We grew cultures in shake flasks (180 cycles per min) of CAYE medium (14) for 24 h at 32°C and tested supernatant fluids we obtained after centrifuging cultures at 6,000 × g for 20 min.

We assessed enteropathogenicity on the basis of the rabbit ileal loop assay (6). We challenged loops with cultures or sterile culture filtrates grown as described above for the toxin assay. We held the ileal loop animals for 18 h before sacrificing them and measuring the amount of fluid that had accumulated in each of the challenge loops. We used 15 loops in each animal and assigned challenge doses randomly to loop positions. The extreme loops were always given sham challenges of sterile medium, and two negative controls (a V. cholerae and an E. coli strain previously shown to be inactive in ileal loops) were randomly placed among the test cultures. We discarded the results from any animals in which any of the sham or negative control loops were positive.

**RITARD model procedure.** The RITARD procedure we used was described previously (22). Before challenge, we starved animals for 24 h, but gave them water ad libitum. Before surgery, we injected 0.05 ml of Innovar (a muscle relaxant) intramuscularly. We carried out the surgery under a local anesthetic (lidocaine hydrochloride), which was injected subcutaneously along the midline of the abdomen. We brought the cecum out through a midline incision and ligated it with no. 11 umbilical tape as close to the ileocecal junction as possible without compromising the blood supply to the area. We then brought out the small

| Table 1. V. cholerae and V. mimicus isolates used in this study |
|---------------|---------------|---------------|
| Isolates      | Source         | Received from  |
| N1, N4, N7, N8, N10 | Oysters, Florida, 1980 | J. M. Madden  |
| V37           | Water, Chesapeake Bay, 1977 | R. R. Colwell |
| West 2193c, 2194c, 2227c | Human diarrhea, Florida, 1977 | J. M. Madden  |
| 31IN, 47IN | Oysters, Florida | J. M. Madden  |
| 61882 | Human diarrhea, Bangladesh, 1978 | W. M. Spira  |
| Ogawa 395 | Human diarrhea, Calcutta | R. B. Sack  |

*Toxigenic means that the isolate produces CT or CT-like toxin; isolates listed are V. cholerae unless V. mimicus is specifically stated.*
intestine and tied a length of umbilical tape in a slip knot around it to close it in the vicinity of the mesoappendix.

We injected the challenge material into the lumen of the anterior jejunum. Usually, we administered the desired dose in an arbitrarily chosen volume of 10 ml of PBS. After injecting the challenge, we returned the intestine and cecum to the peritoneal cavity and closed the incision. We brought the loose ends of the slip of the slip knot through the incision in the muscle layers and sutured the incision. We left the loose ends in the space between the muscle and skin layers and clipped them to the skin by means of a surgical clip. We then sutured the rest of the skin and placed the animal in a holding box. We removed its temporary tie 2 h after the challenge had been introduced (after the surgical clip is removed, the slip knot can be opened easily and the umbilical tape can be pulled gently from the intestine of the animal). We sutured the incision, returned the animal to its cage, and provided it with food and water ad libitum.

Monitoring the disease. For challenge experiments, we observed rabbits for overt diarrhea, signs of weakness, and death. We collected daily rectal swabs and plated them onto gelatin agar to identify shedding of the challenge organism. We considered animals that were still alive after 124 h to have survived the infection. Each dead animal was autopsied, and the appearance of the gut was recorded.

For colonization studies, we sacrificed animals at the indicated times with an intravenous overdose of pentabarbitol. We opened the abdominal cavity and ligated the small intestine at the site of the challenge injection and at the mesoappendix. We then removed the entire small intestine and tied it into five segments corresponding to the jejunal, midjejunum, jejunoileal junction, midileum, and terminal ileum.

We cut each segment away from the others and immediately chilled it. We then weighed each segment and slit, drained, everted, and washed it with two sequential 30-s rinses in 50 ml of PBS. We pooled the rinses from each section of the segment and thoroughly mixed the resulting suspension. We weighed the washed segments a second time and then homogenized them in Potter-Elvehjem tissue grinders in a volume of PBS equivalent to their washed weight. We determined viable plate counts on pooled intestinal contents plus washings and on homogenized tissue samples by using gelatin agar. We define adherent bacteria as cells remaining in tissue samples after washing and free bacteria as cells present in the material in the lumen of the intestine or washed from the tissue sample. These values are expressed as a function of unit weight of washed small intestine.

Local immunization of rabbits by intestinal infection. We fasted rabbits for oral challenge and prepared the challenge inoculum in the same manner as we did for RITARD challenge. The challenge organisms were suspended in 15 ml of CAYE broth to the appropriate concentration just before being used. Before administering the challenge with an orogastric tube, we gave each rabbit cimetidine (50 mg/kg) intravenously to suppress gastric acid secretion. We considered this time as time zero. At 15 min and 30 min, we administered 15 ml of a 5% NaHCO₃ solution. We administered the challenge immediately after the second bicarbonate treatment. At 60 min, we administered 2 ml of tincture of opium intraperitoneally to slow intestinal motility.

This regimen has been shown to support colonization and transient infection of the rabbit small intestine without, however, concomitant diarrhea (except an occasional mild syndrome) and death. Infections by CT-producing V. cholerai induced with this procedure have been shown to elicit local and serum antitoxin and antibacterial responses that are protective against rechallenge with the homologous organism by using the RITARD model (N. F. Pierce, personal communication).

ELISA assays of local and serum antibody. We monitored specific antibodies to vibrio cell surface antigens using a microtiter ELISA procedure in polyvinyl microtiter "U" plates (Dynatech Laboratories, Alexandria, Va.). The ELISA assay involved the consecutive addition of antigen preparation, rabbit samples to be tested for antibody, and peroxidase-labeled goat anti-rabbit immunoglobulin (IgG) or IgG. For the antigen, we used cells obtained from cultures grown as for RITARD model challenge. We washed cells three times in 0.05 M Tris-hydrochloride buffer (pH 7.8) and then suspended them in Tris buffer containing 1 mM EDTA, 0.1 mg of RNase per ml, and 0.5 mg of DNase per ml at a concentration of ca. 10¹⁴ cells per ml. We lysed the cells with ultrasound by using a Branson sonicator at 40 W for 60 s, centrifuged the lysate at 6,000 x g for 20 min, and discarded the pellet. We then ultracentrifuged the supernatant fluid at 41,000 x g for 60 min, recovered the pellet, washed it twice with Tris buffer containing 1 mM MgCl₂, and prepared final suspensions in Tris–MgCl₂ that contained 100 to 150 μg of protein per ml as measured by the Bradford assay (3). Working samples of this material were stored at −70°C.

The wells of polyvinyl microtiter plates were coated with antigen by adding 100 μl of 0.01 M PBS (pH 7.4) containing the selected antigen (10 μg/ml protein) to each and incubating overnight at 4°C. Only the inner 60 wells were used (13). Plates were washed three times after each step with PBS containing 0.1% Tween 20. Appropriate dilutions of sera or intestinal washings in 100 μl of PBS–Tween–1% bovine serum albumin were allowed to react with bound antigen for 1 hr at 37°C. Finally, peroxidase-conjugated goat antibody (Cappel Laboratories, Downingtown, Pa.), diluted 1:1,000 in PBS-Tween-bovine serum albumin, was added and incubated for 1 h at 37°C. Goat anti-rabbit IgG was used for serum samples, and anti-rabbit IgA was used for intestinal washings. The peroxidase substrate consisted of 0.004 M 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) in 0.05 M sodium citrate buffer (pH 4.0) plus 0.0015 M H₂O₂. We measured the optical density of the color reaction by using a fiberoptic ELISA reader (Dynatech) after the reaction had been allowed to proceed at 25°C for 60 min.

RESULTS

Biological activity of isolates in standard assays. We tested all isolates listed in Table 1 for their ability to produce CT or CT-like toxin by using the ganglioside GM₁–ELISA (18). To screen rigorously, we grew these isolates in a variety of media (CAYE, trypticase soy broth, and brain heart infusion), temperatures (32 and 37°C), and
agitation (still flask culture, flasks shaken at 200 cycles per min, and roller drum culture). Our results were consistent regardless of condition: isolates N1, N4, N7, N8, N10, West 42, V37, 31IN, 47IN, 2193c, 2194c, and 2227c showed no evidence of producing CT or anything that cross-reacted antigenically with it. Isolates 61892 and Ogawa 395 produced detectable quantities of CT under virtually all conditions.

These isolates were also tested with a DNA probe consisting of the sequence coding for E. coli heat-labile toxin, which cross-reacts with CT (12). This was kindly done for us by James Kaper (Center for Vaccine Development, University of Maryland School of Medicine, Baltimore), who found that none of the isolates with the nontoxicigenic phenotype reacted to the probe, whereas all of the others were positive.

We then tested the isolates for their ability to elicit fluid accumulation in the rabbit ileal loop. We challenged loops either with 1.0 ml of fresh 18-h cultures containing between $2.1 \times 10^9$ to $6.3 \times 10^9$ viable cells or with 1.0 ml of filter-sterilized medium from the same cultures. Isolates N1 through N10, V37, West 42, 31 IN, and 47IN produced no significant fluid accumulation when either whole cultures or filtrates were used.

The isolates producing CT or CT-like toxin, 61892 and Ogawa 395 O2, elicited large amounts of fluid when loops were inoculated with either whole cultures or culture filtrates. Filtrates of nontoxicigenic strains 2193c, 2194c, and 2227c did not induce fluid accumulation, but challenge with viable cells resulted in substantial positive ileal loops (fluid/loop length ratios >1.0 ml/cm). This confirms the recent work of Madden et al. (15) on these isolates. We have previously reported that this pattern of bioactivity is one most commonly seen among clinical isolates of non-O1 V. cholerae (19).

**Virulence of isolates in the RITARD model.** Table 2 shows the results of our challenges of RITARD model animals with selected isolates. Strains of nontoxicigenic O1 or non-O1 V. cholerae that possessed marginal or no ileal loop activity failed to elicit overt diarrhea in the RITARD model. This includes N1, N10, V37, and 31IN. Rectal swabs taken at daily intervals from challenge animals revealed that shedding of the challenge organisms was brief (1 day maximum) or undetectable (three of four isolates). We found this to be the case even when challenge doses as high as ca. $10^{11}$ were used.

The three nontoxicigenic clinical non-O1 isolates that gave strong positive results in the ileal loop assay were equally effective in causing diarrhea and death in the RITARD model animals. Even at lower challenge doses than we used for the previous organisms (ca. $10^9$), strains 2193c, 2194c, and 2227c caused from 75 to 100% of animals challenged to produce profuse diarrhea, which was accompanied by an overall mortality rate of from 50 to 75%. These strains appeared to be more virulent in this model than either the toxicigenic V. mimicus or O1 V. cholerae that we included for comparison. Both of these strains needed doses about 10-fold higher than the above to elicit the same rate of diarrhea and death.

**Kinetics of small intestine colonization by various isolates.** Figure 1 presents the two patterns we have seen thus far in the colonization of the rabbit small intestine by V. cholerae or V. mimicus. We described the pattern of strain Ogawa 395 previously (21). It colonizes rapidly with dramatic increases in both free and adherent vibrios in the small intestine. The concentration of adherent vibrios appears to reach a plateau beginning at about 8 h that remains for the duration of the observation period (12 h). In contrast, the weakly virulent or nonviral strain N1 proliferates slightly during the first 2 to 4 h after challenge, but then its concentration in the intestine decreases rapidly for the rest of the observation period.

Figure 2 demonstrates the difference between the two organisms more clearly. It shows the distribution of adherent cells along the small intestine at each of the time periods studied. Both strains show similar distributions after 2 and 4 h. The jejunum, the site of injection, had a higher concentration of organisms than did the ileum. By 6 h, however, there was a significant reduction of N1 cells in the jejunum and a reversal of the slope of the distribution curve. This was followed by a generalized reduction in cell numbers in all parts of the small intestine. Strain Ogawa 395, on the other hand, quickly colonized the ileum with no reduction in jejunal concentration. By 6 h, the entire intestine was colonized; this was followed by a generalized increase in the number of adherent cells.

We found that the remaining isolates appeared
to colonize in one of these two patterns (Table 3). None of the strains that failed to elicit diarrhea in earlier challenges of RITARD model animals was able to colonize the small intestine. In all cases, concentrations in both the jejunum and ileum were similar or somewhat less than those of strain N1. For strains 2193c, 2194c, 2227c, and 61892, however, concentrations of adherent cells were as high or higher than the values obtained for strain Ogawa 395.

Immune response to infection with nontoxigenic non-O1 V. cholerae clinical isolates. The high level of colonization potential and virulence evidenced by strains 2193c and 2227c and, particularly, 2194c in rabbits became even more apparent when we attempted to immunize adult rabbits locally by intestinal infection. Using the rabbit colonization model, we challenged three rabbits for each strain with oral doses of $1 \times 10^{10}$ to $2 \times 10^{10}$ cells. Although this model is designed to minimize diarrhea and death while promoting a relatively brief and immunogenic infection, we found that all three rabbits challenged with strains 2194c and 2227c developed profuse diarrhea and died within 24 h of challenge. We have never observed this degree of virulence with any of the CT-producing strains we have used thus far. Only one of the three animals challenged with strain 2193c died.

We began challenging a second set of nine rabbits with an oral dose of $10^7$ cells. This time, all but two animals developed diarrhea, two died, both of them challenged with strain 2194c. All animals shed the challenge organism for at least 4 days. In contrast, when we rechallenged these rabbits after 14 days with a dose of $10^8$ cells, none died, none developed diarrhea, and none shed the challenge organism for more than 1 day. When we challenged for the third time, again after 14 days, we used a dose of $10^{10}$ cells. This time there was again no diarrhea or death, and we detected shedding in only two of the rabbits and for only 1 day.

Figure 3 shows the titration of specific IgA in intestinal washings taken from these rabbits after the last immunizing infection. We titrated each specimen against its homologous cell antigens and the antigens of the two heterologous strains to determine whether these isolates expressed antigenically similar immunogens while colonizing the rabbit intestine. A parallel set of titrations of specific IgG in sera collected at the same time is shown in Fig. 4.
TABLE 3. Colonization of the small intestine after challenge by $10^9$ cells of *V. cholerae* isolates with different biological activities*

| Isolate | Produces CT | Ileal loop activity | Log

| Isolate | Produces CT | Ileal loop activity | Log

| Isolate | Produces CT | Ileal loop activity | Log

| Isolate | Produces CT | Ileal loop activity | Log

| Isolate | Produces CT | Ileal loop activity | Log

#### a Cultures grown in CAYE shaker flasks (180 cycles per min) at 32°C to the experimental phase (ca. 4h). Cells were recovered by centrifugation, washed with 0.05 M PBS (pH 7.2), and suspended in PBS to give $10^9$ cells per ml. The range of the challenge was $1 \times 10^9$ to $5 \times 10^9$.

#### b All values are means of three determinations $\pm 1$ standard deviation.

#### c —, Not measured.

The titers in both serum and intestinal washings from animals infected with strain 2193c appear to be substantially lower than those in specimens from animals infected with the other two strains. This particularly evident in Fig. 3B and 4B, in which the apparent antibody titers against 2193c antigens in animals challenged with either 2194c or 2227c are higher than those in the animals challenged with the homologous strain, 2193c.

The titration curves we obtained for specific IgA in intestinal washings paralleled those for specific IgG in sera. The titration patterns of sera and intestinal washings from 2194c- and 2227c-infected animals appeared almost identical when either 2193c or 2227c antigens were

![Microtiter IgA ELISA assays of local antibody against cell wall antigens of three clinical isolates of nontoxigenic, non-O1 *V. cholerae*. Antibody was measured in intestinal washings taken from rabbits immunized by three sequential enteric infections with one of the three strains. Intestinal fluids were from rabbits infected with strain 2193c (○), 2194c (●), or 2227c (△). Titration was against cellular antigens of strain 2194c (A), 2193c (B), or 2227c (C).](http://iai.asm.org/)
used. This suggests a close antigenic similarity between these two strains. However, when 2194c antigen was used, only the homologous sera and intestinal washings demonstrated any significant affinity. The sera and intestinal washings from 2193c-infected animals seemed to fit the same pattern, even though the differences were smaller due to the generally lower titers.

**DISCUSSION**

The strains of *V. cholerae* and *V. mimicus* that possess human enteropathogenic potential can be divided on the basis of whether or not they produce CT or CT-like toxin (2, 19, 20). The important virulence factors of nontoxigenic isolates have yet to be defined, as do the colonization factors of both classes. Toxigenic O1 *V. cholerae*, the etiological agent of cholera, has very rarely been isolated from waters that are uncontaminated with human sewage. Toxigenic non-O1 *V. cholerae* and *V. mimicus* can be isolated with slightly greater regularity (5, 10, 12), but production of CT-like toxin is rare among randomly selected isolates from water and seafood samples in the United States (23).

With the exception of the Louisiana-Texas cholera endemic focus (1, 13), nontoxigenic but enteropathogenic *V. cholerae* and *V. mimicus* are the organisms associated with gastroenteritis in the United States (2). Since there is no facile test for enteropathogenic potential in nontoxigenic isolates, the distribution of nontoxigenic enteropathogens in the general population has not been closely defined. The work of Twedd et al. (23) suggests that this figure might be about 5 to 10%, based on their screening of isolates for intestinal fluid accumulation in infant rabbits.

In evaluating the ability of nontoxigenic isolates to colonize RITARD model animals, we have found all of the environmental isolates to be incapable of establishing even a transient colonization of the small intestine. Even strains that demonstrated some slight activity in rabbit ileal loops were incapable of overcoming the normal clearance mechanisms of the unligated small intestine. This enhances the perceived differences between enteropathogens and non-enteropathogens. It suggests that, in addition to unidentified toxic factors, enteropathogens also possess unidentified colonization factors not shared by non-enteropathogens. It may, indeed, prove to be the case that the actual toxic factors may be widely expressed in vibrios, whereas the critical difference between pathogen and non-pathogen actually depends on colonization potential.

In examining the diarrheagenic capabilities of the clinical isolates 2193c, 2194c, and 2227c, we have found them (particularly the latter two) to be the most virulent in the RITARD model of any strains of *V. cholerae* so far tested, including those that produce CT or CT-like toxin. They colonize the small intestine as rapidly and produce fatal diarrhea as quickly as any of the toxigenic cholera vibrios and, for equivalent doses, elicit a diarrhea that is more likely to be fatal. This suggests that the reason(s) why strains such as these are not associated with epidemics, in contrast to cholera, may stem more from traits influencing transmissibility than from any fundamental difference in ability to proliferate in the small intestine.

The mechanism(s) underlying the observed differences in colonization potential in the
strains studied are not yet known, but may include differences in motility or chemotaxis as well as possession of specific adhesions (9). The availability of wild-type isolates with documented differences in their capacity to colonize the small intestine and cause diarrhea should prove valuable in studies to determine which mechanisms play an important role.

Being able to induce a series of nonfatal infections of the adult rabbit small intestine by the same strain has given us the opportunity to study the immune response to these infections. In theory, it also enables us to produce local and serum antibodies with specificities directed against vibrio products that were expressed during the actual infection. All critical virulence factors must be in this class of products, although whether all were immunogenic remains to be determined.

Our first results with local and serum antibodies directed against cell antigens of three clinical isolates from the same outbreak suggest that a significant degree of antigenic cross-reactivity may exist between these strains. Strain 2193c, which was demonstrably the least pathogenic of the three virulent strains studied, elicited the weakest intestinal and serum antibody response. This may indicate a more limited capacity to colonize than the other two strains, even though this is not apparent in the data of Table 3.

The fact that animals infected with strains 2194c and 2227c demonstrated similar local and serum antibody titers against the antigens of lysed cells of 2193c and 2227c is significant because these strains are of different serotypes. Strain 2227c is Smith 17, whereas 2194c and 2193c are untypable in the Smith serotyping scheme (15). This suggests that at least one nonlipopolysaccharide antigen is common to all three strains. It is thus intriguing that ELISA carried out with the lysed cell antigens of strain 2194c showed significant antibody titers in sera and intestinal washings from 2194c-infected animals, but not from those infected with 2227c. This would seem to suggest that strain 2194c may produce a number of antigens in vivo, including at least one that is common to the other two strains, that it does not produce in vitro. We anticipate that more detailed analyses with gel diffusion, immunoprecipitation, and immunoblotting of isolated cell proteins may reveal similar patterns in the other strains and suggest which antigens may be important in colonization.

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