Demonstration of Invasiveness of *Vibrio parahaemolyticus* in Adult Rabbits by Immunofluorescence

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Received for publication 14 December 1978

To determine possible pathogenesis of *Vibrio parahaemolyticus*-host-organ system interactions, studies of invasiveness were made by a direct fluorescent-antibody method. Broth cultures of live cells isolated from seafish or symptomatic humans were inoculated separately into ligated ileal loops of young New Zealand white rabbits. After suitable incubation, rabbits were sacrificed, and ileal loops and tissue specimens were aseptically removed. Ileal loops were prepared and stained with specific fluorescein-tagged antibody, and organ specimens were cultured for isolation of the inoculated *Vibrio* strain. All strains tested penetrated into the lamina propria of the ileum and were isolated from the cultured tissue specimens, indicating that the organism is capable of more than a superficial colonization of the gut. The presence of *Vibrio* in cultured tissue specimens suggests invasion of deeper tissue by either the lymphatic or the circulatory system.

In 1967, Yahagi (20) reported the localization of a Kanagawa-positive strain of *Vibrio parahaemolyticus* in the epithelial tissue and lamina propria of ligated rabbit ileal loops stained by immunofluorescence. He found that the localized strain neither increased in number nor extended to deeper tissue of the intestine.

Caila and Johnson (2) recovered several Kanagawa-positive strains from blood, liver, and spleen of suckling rabbits 7 h after intragastric challenge. The Kanagawa-negative strain tested was not recovered under the same conditions.

In a study by Carruthers (3), Kanagawa-positive strains were found to adhere to human fetal intestinal cells at a greater rate than Kanagawa-negative strains.

The enteropathogenicity of *Vibrio* has not yet been conclusively tied to hemolytic activity; that is, Kanagawa-positive strains do not consistently cause a positive disease response in an appropriate animal test system (1, 17-19). Furthermore, Kanagawa-negative strains have been reported to comprise a small but significant proportion (3.5 to 11.6%) of the total *V. parahaemolyticus* strains isolated from patients' stools during outbreaks of gastroenteritis (10, 12). Often they are the only *V. parahaemolyticus* strains isolated from patients (16). Despite many attempts in our laboratory (1, 17-19) and by others (8), there is no satisfactory evidence to indicate that *V. parahaemolyticus* can produce any virulence factor other than hemolysin that might explain the presumed pathogenesis of Kanagawa-negative strains. Under these circumstances, it seemed reasonable to question whether an invasive mechanism, along with the unknown host response, may play a significant role in vibriosis.

Our objectives were (i) to determine if strains representative of both Kanagawa types are invasive and (ii) to compare the invasive capability and observe the extent of invasion of the two types.

**MATERIALS AND METHODS**

**Strains.** Five different *V. parahaemolyticus* strains were selected. Table 1 lists source, K type, and Kanagawa phenomenon for each. These strains were maintained at 27°C on slants of Trypticase soy agar + 2.5% NaCl. Biochemical and cultural characteristics, well established for identification of *V. parahaemolyticus*, were verified with each culture transfer (6).

**Fluorescein-tagged antibody preparation.** Whole cells of 18-h Trypticase soy broth + 2.5% NaCl cultures were collected, washed three times, and suspended in phosphate-buffered saline containing 0.4% Formalin to an optical density of 0.25 at 620 nm in a Coleman Jr. spectrophotometer. New Zealand white rabbits were injected twice weekly with increasing doses (0.5, 1.0, 2.0, 3.0 ml) of the standard cell suspension. When appropriately high agglutinin titers (1:2,560) were achieved, specific anti-K serum was prepared from blood drawn by a cardiac puncture procedure. Bacterial nonspecificity was removed from pooled, strain-specific serum by absorption of serum with packed, formalinized bacteria cultured from normal rabbit intestinal flora.

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An equal volume of 70% ammonium sulfate solution was added to specific serum, and the resulting precipitated globulin was resuspended in distilled water and dialyzed overnight at 4°C against distilled water. The amount of protein in the globulin was determined spectrophotometrically at wavelength 660 nm by the Folin-Ciocalteu method (15).

Globulin, specific for each strain, was individually tagged with fluorescein isothiocyanate (Calbiochem) by the direct method of Cherry et al. (4). Tagging was allowed to proceed under carefully controlled labeling conditions of 25°C and pH 9.5 to produce an estimated fluorescein-to-protein molar ratio of 15 or 16.

Unreacted dye was removed by passage of the tagged reaction mixture through a column of Sephadex G-25 (Pharmacia Fine Chemicals, Inc.) with the eluent 0.01 M phosphate buffer, pH 7 (5). Nonspecificity caused by overtagged globulin was removed by passage through a column of diethylaminoethyl-Sephadex A-50 (Pharmacia Fine Chemicals, Inc.) (7). Nonspecificity related to host cell constituents was removed by absorption with hydrated freeze-dried rabbit tissue (Pel-Freez Biologicals, Inc., Rogers, Ark.). Tagged globulin was preserved with Merthiolate at a final concentration of 1:10,000 and stored at 4°C.

Surgical inoculation. Six-week-old New Zealand white rabbits, acquired from a single reputable supplier, were quarantined for 2 weeks before surgery and observed for gross evidence of disease or debilitation. Healthy animals, free from symptoms of coccidial infestation, were inoculated by the modified ileal loop method of Twedt and Brown (18) with 1 ml of an overnight broth culture containing 10⁷ to 10⁹ cells per ml. Inoculum size was based on results from human volunteer studies conducted in several laboratories that required administration of 2 × 10⁷ to 3 × 10⁷ Kanagawa-positive V. parahaemolyticus cells to produce abdominal discomfort and diarrhea, whereas relatively large numbers (1 × 10⁶ to 2 × 10⁶) of Kanagawa-negative cells did not cause any symptoms of gastroenteritis (12-14). Recent studies (Twedt et al., unpublished data) indicate that the minimal ileal loop dose of Kanagawa-positive strains in rabbits ranged from 3.4 × 10⁵ to 2.4 × 10⁶ cells, whereas our previous investigations (1, 17, 18) had shown that ≥10⁶ Kanagawa-negative cells would, in virtually all cases, fail to produce loop dilatation. Each rabbit was challenged with a single Vibrio strain in several ileal loops alternating in sequence with control loops. The control inoculum was 1 ml of uninoculated Trypticase soy broth + 2.5% NaCl since previous data (1) show that ≥5% NaCl broth is required to produce a nonspecific loop dilatation. Ileal loops and visceral tissue specimens were aseptically removed 12 to 18 h later when control loops were normal in appearance, that is, neither dilated nor inflamed. When adjacent control loops were swollen or hemorrhagic, test loops were discarded.

Isolation of Vibrio. Specimens of blood, heart, liver, spleen, lung, and pancreas were placed in separate flasks of 50 ml of Trypticase soy broth + 2.5% NaCl. After 5 to 7 days of incubation at 35°C, V. parahaemolyticus was isolated and identified by the previously mentioned biochemical tests (6).

Ileal loop preparation. Ileal loops were placed immediately in a tray of chilled phosphate-buffered saline, cut longitudinally, and cleaned by agitation. The chilled tissue was cut into squares approximately 1 by 1 cm, curled over an acetone, dry-ice bath, and frozen. Freshly frozen sections of 4 μm were cut in a Harris Low-Temp cryostat. Tissue sections were placed on slides, fixed, and stained with tagged antibody by the direct method; consecutive sections for histological evaluation were stained by a hematoxylin and eosin method (11). Cover slips were mounted with Mayer's albumin fixative. The usual tissue and serum controls described by Kawamura et al. (9) were run simultaneously to assure that fluorescence observed in situ was due to homologous V. parahaemolyticus cells and not to host tissue components.

Microscopic observation and photography. Slides of stained sections were examined with a Zeiss photomicroscope with a 64 Plan lens (16/0.35 × 1.25) and an oil Planapo lens (40/1.0 × 1.25). The light source for the microscope was an Osram HBO-200 mercury light. Excitation filter no. 1 and a barrier filter were used to exclude light below 500 nm. Images were recorded on color film, Kodak high-speed Ektachrome (EHB) pushed to ASA 200 and DIN 24.

RESULTS

Fluorescence, and hematoxylin and eosin studies. All strains, including 9020, penetrated into the lamina propria of the villi, but Vibrio strains 553, 554, 393, and 395 extended into the muscularis externa of the ileum. An invasive organism that can penetrate the lamina propria of a susceptible host may extend through the lymphatic or blood system to other organ sites. This possibility was investigated by attempting cultural isolation of Vibrio from the major organs of spleen, liver, pancreas, heart, lung, and heart blood of animals that had been surgically inoculated with a Vibrio strain. Table 2 compares observed invasion frequency, demonstrated by fluorescence, with the macroscopic appearance of the loop.

Kanagawa-negative strains 393 and 395 often extended beyond the lamina propria into the

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>K serotype</th>
<th>Kanagawa phenomenon</th>
<th>Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>393</td>
<td>Stool specimen</td>
<td>K29</td>
<td>-</td>
<td>R. Sakazaki*</td>
</tr>
<tr>
<td>395</td>
<td>Stool specimen</td>
<td>K13</td>
<td>-</td>
<td>R. Sakazaki</td>
</tr>
<tr>
<td>553</td>
<td>Stool specimen</td>
<td>K4</td>
<td>+</td>
<td>R. Sakazaki</td>
</tr>
<tr>
<td>554</td>
<td>Stool specimen</td>
<td>K4</td>
<td>+</td>
<td>R. Sakazaki</td>
</tr>
<tr>
<td>9020</td>
<td>Corbiciput</td>
<td>Untypeable</td>
<td>-</td>
<td>Y. Miyamoto*</td>
</tr>
</tbody>
</table>

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muscularis externa (Fig. 1). Macroscopically, loops inoculated with these strains usually appeared normal, with no accumulation of fluid and no inflammation. Microscopically, the ileal mucosa and muscularis externa were not structurally altered. The hematoxylin and eosin sections appeared normal except for foci of neutrophils, indicating that infection was scattered throughout the inoculated loops.

Fluorescent studies revealed the penetration of Kanagawa-positive strain 553 at least into the lamina propria of the villi in all rabbits challenged with this strain (Fig. 2). Viable cells of 553 were also isolated from all organs cultured from rabbits inoculated with strain 553 (Table 2). The hematoxylin and eosin sections, taken consecutively with the fluorescent sections from rabbit loops inoculated with strain 553, show lamina propria (Fig. 3) that is greatly altered in appearance compared with sections from loops inoculated with control media (Fig. 4).

Kanagawa-positive strain 554, which showed fluorescent evidence of invasion into the lamina propria of most rabbits tested, failed to be recovered from all organs cultured from all strain 554-challenged rabbits (Table 2). The presence of strain 554 is clearly visible in the fluorescent-antibody-stained section of villi (Fig. 5), in contrast to the absence of specific fluorescence in villi of loops inoculated with control media and stained with the same specific fluorescent antibody (Fig. 6).

Seafish isolate 9020 was found to penetrate into the lamina propria only once in five tests.

**Table 2. Comparison of strain invasion frequency, condition of ileal tissue, and organs of cultural isolation**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Times tested</th>
<th>Frequency of invasion</th>
<th>Condition of loop</th>
<th>Organs yielding cultural isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>393</td>
<td>15</td>
<td>3/15</td>
<td>Normal</td>
<td>Spleen</td>
</tr>
<tr>
<td>395</td>
<td>10</td>
<td>4/10</td>
<td>Normal</td>
<td>Spleen, liver, pancreas</td>
</tr>
<tr>
<td>553</td>
<td>10</td>
<td>10/10</td>
<td>Hemorrhagic</td>
<td>Heart, liver, spleen, blood, pancreas</td>
</tr>
<tr>
<td>554</td>
<td>10</td>
<td>9/10</td>
<td>Hemorrhagic</td>
<td>Spleen, pancreas</td>
</tr>
<tr>
<td>9020</td>
<td>5</td>
<td>1/5</td>
<td>Normal</td>
<td>Pancreas, spleen, liver</td>
</tr>
</tbody>
</table>

* Number of times invasion was detected microscopically/number of times tested.

**Fig. 1. Frozen section of muscularis externa from rabbit ileal loop injected with Kanagawa-negative V. parahaemolyticus strain 395. Clumps of Vibrio are present within the muscle layers (arrows). Fluorescence, ×192.**
Fig. 2. Transverse frozen section of villus from rabbit ileal loop injected with Kanagawa-positive V. parahaemolyticus strain 553. Vibrio cells (arrows) are seen within the lamina propria. Fluorescence, ×192.

Fig. 3. Longitudinal frozen section of villi from rabbit ileal loop injected with Kanagawa-positive V. parahaemolyticus strain 553 taken consecutively with that shown in Fig. 2. The lamina propria (L) is greatly altered. Epithelial cells (E) remain. Hematoxylin and eosin, ×192.
FIG. 4. Longitudinal frozen control section of rabbit ileal loop injected with sterile broth. The lamina propria (L) remains intact covered by the epithelial cells (E).

FIG. 5. Frozen section of rabbit ileal loop inoculated with Kanagawa-positive V. parahaemolyticus strain 554. The sites of infection are the large clumps of Vibrio (arrows). Fluorescence, ×480.
Control loops that were run simultaneously with test loops indicated no fluorescence from bacteria or host cell constituents with tagged globulin specific for any of the five tested strains.

**DISCUSSION**

All strains of *V. parahaemolyticus* tested with the rabbit ileal loop model penetrated with varying frequency into the lamina propria of the ileal villi. Although this system cannot be considered a natural situation, it is an in vivo mammalian system that biologically approximates the human gut.

When we initiated our study, we expected, on the basis of past experience with the rabbit ileal loop model (1, 17-19), that the consistently negative loops produced by the vast majority of Kanagawa-negative strains would invade less often or not at all. That speculation proved to be the case (Table 2), and in this respect those less-invasive Kanagawa-negative strains served as internal controls for the highly invasive Kanagawa-positive strains. We did not select another species (*Vibrio cholerae*) with dissimilar pathogenic mechanisms as the noninvasive control in order to avoid the resulting limitations to meaningful comparison. Furthermore, sterile broth control loops must be considered to be loaded with a natural flora of noninvasive bacteria. Their presence was not detected in sections (Fig. 6) even though unabsorbed tagged antibody was used in preliminary screening. For these reasons we feel that the presence of *V. parahaemolyticus* test strains in lamina propria and other organs resulted from tissue invasion by this species.

Alterations in tissue structure observed with strain 553 that might result from ischemia produced by the surgical procedure or swollen loop fluid pressure appear less likely when compared with test loops from noninvasive strains and control loops not exhibiting this damage. In most cases, Kanagawa-negative strains did not produce loop dilatation. However, when they occasionally did induce swollen loops, dilatation was unaccompanied by destructive tissue changes. So, alteration of tissue structure cannot be attributed only to obstruction by fluid pressure, and some action by *V. parahaemolyticus* present within tissue must be considered.

All strains of *Vibrio* were isolated from the spleen (Table 2), which is a sequestering organ for diseased macrophages, suggesting that the spread of *Vibrio* may be by the circulatory system. The exact route of spread from the intestinal tract remains to be determined and is under investigation. Although contamination of the
peritoneum by surgical error cannot be entirely dismissed, invasive strains might, as a result of a surgical "seeding," infect all of the abdominal viscera. Such a pattern of infection was not found. Furthermore, no strains of V. parahaemolyticus were isolated from organs of rabbits that did not show evidence of invasion by fluorescent microscopy.

The bloody, swollen loops inoculated with Kanagawa-positive strains of Vibrio are comparable to severe gastroenteritis in humans. Invasion by that organism, however, may result in the invasion of the liver, spleen, pancreas, and other vital organs. Such a pattern of infection must be considered in the pathogenesis of vibriosis.

ACKNOWLEDGMENTS

We thank R. Sakazaki and Y. Miyamoto for providing cultures and D. F. Brown and P. L. Spaulding for excellent surgical assistance. We also thank J. C. Williams for editorial and typing assistance.

LITERATURE CITED


