Possible Role of Colonic Content in the Mucosal Association of Pathogenic *Shigella*

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Association of *Shigella flexneri* to cecal membranes was studied by incubating the pathogen with cecal slices of germfree mice. The slices were first incubated with stool supernatants from germfree, shigella-monocontaminated, and conventional animals. Quantitation of shigellae in homogenates of treated slices revealed an increase of organisms only in those slices exposed to contaminated stool supernatants.

Bacillary dysentery is a colonic disease that occurs when virulent shigellae invade the mucosa (3). Before invading colonic mucosa, the pathogenic shigellae must first become closely associated with the lubricating mucus, cell surface membrane, and the cell itself (9). Factors which have been implicated in that association are either bacterial or mucosal. Both the bacteria and the cell lining, however, are surrounded and bathed by the colonic content. Stools or colonic content from humans and experimental animals have been shown to contain bacterial and host elements which might influence that association. For instance, human feces contain blood group-degrading enzymes and proteases of bacterial origin (8, 10) which potentially could alter the mucosal glycoprotein lining. Likewise, intestinal cells secrete immunoglobulins which could react with the bacteria and therefore have a role in the host's defenses (11, 12). In this preliminary study, we examine the influence of stool supernatants from a variety of experimental animals in the mucosal association of pathogenic shigellae.

Germfree albino mice (Charles River Breeding Laboratories, Inc.) were kept germfree in aseptic isolators and given sterile Lab Chow and water ad libitum. For the monocontamination experiments, mice were transferred to an ultraviolet laminar flow hood. Contamination was achieved by adding to the drinking water approximately 10^7 viable *Shigella flexneri* 4b per ml. Normal stools were obtained from conventional mice (Charles River Breeding Laboratories, Inc.) housed in metabolic cages and fed a similar diet. All stools were collected in sterile culture tubes, suspended in cold 0.9% NaCl, and homogenized on ice. Homogenates were centrifuged at low speed to remove debris. Supernatants were centrifuged at $25,000 \times g$ to sediment the bacteria. Clear supernatants were desiccated and recon-

stituted to a volume twice the stools' weight in sterile distilled water containing 0.01% sodium azide.

Ceca of germfree mice were opened, contents were washed gently with cold saline, and the walls were cut into pieces (1 cm²). Cecal slices were then incubated for 24 h at 30°C with 1 ml of germfree, shigella-monocontaminated (collected on days 3 to 6 after contamination), or normal stool supernatants. Slices were also incubated with a mixture of normal and shigella-monocontaminated stool supernatants to resemble as closely as possible in vivo cecal conditions that might exist during shigella infection. As a control, slices were added to 1 ml of sterile Krebs-Ringer bicarbonate buffer (KRB [pH 6.2]) and incubated for a similar interval of time. After incubation, slices were rinsed thoroughly in sterile KRB solution. Rinsed slices were placed in Erlenmyer flasks containing 25 ml of KRB solution (pH 6.5). To this preparation, 10^9 *S. flexneri* in KRB were added. All preparations were flushed with CO₂ and incubated at 30°C for 1 h in a shaking bath (5). Slices were removed, rinsed several times in KRB, suspended in the buffer, and homogenized. Colony-forming units of shigella associated with each slice were calculated. Incubated KRB solutions were also cultured quantitatively. Counts of the rinsed tissue slices were related to the counts of 1 ml of KRB and defined as the association index. Association index = $t/t + k \times 100$, where t is the number of shigellae remaining in tissue slices after rinsing and k is the number of bacteria per ml of KRB solution after incubation.

Shigellae associated with slices were also evaluated by fluorescent anti-shigella antibody (2).

As expected, histological examination of several pretreated slices revealed some features of autolysis. Histological features, however, were identical in all slices whether incubated with

stool supernatants or KRB. The membrane skeleton and lubricating mucus appeared intact, however, as evidenced by periodic acid-Schiff staining. This particular feature of cells is not unusual since small intestinal brush border membranes are recovered intact after disruption of cells with hypotonic solutions (4). Membrane and lubricating glycoproteins are known to be resistant to spontaneous hydrolysis and require harsh treatment, such as boiling in acid media, to be disrupted (13).

Reaction of cecal slices that had been treated with KRB solution or germfree stool supernatants showed only a few bacteria when examined with fluorescent anti-shigella antibody. On the other hand, fluorescent bacteria were found in high numbers uniformly distributed throughout the tissue slice in those cecal slices that had been treated with shigella-monocontaminated or normal stool supernatants or a mixture of both. This increased number of organisms was estimated to be at least five times the number found in the control slices.

To estimate the numbers of shigellae associated with cecal slices after incubation in the KRB solutions, the slices and surrounding KRB solution were evaluated quantitatively for the enteropathogen. Between 10^6 and 10^7 shigellae were recovered per ml of surrounding KRB solution. Cecal slices that had been exposed to only KRB solution for 24 h had an association index of 9.7 ± 1.1 . Similarly, cecal slices that had been treated with germfree stool supernatants had an association index of 12.0 ± 0.9 (Fig. 1). Each reported number represents the mean $\pm$ standard error of 10 experiments. This difference was not significant by the Student's *t* test. Conversely, after exposing the slices to shigella-monocontaminated or normal stool supernatants, the association of shigellae increased 2.5 to 3 times. Thus, the association index was 35.4 ± 3.6 in slices that had been exposed to shigella-monocontaminated stool supernatants, 33.5 ± 2.1 in slices exposed to normal stool supernatants, and 34.5 ± 3.2 in slices exposed to a mixture of both stool supernatants. As compared with the slices treated with germfree stool supernatants, this difference was highly significant ($P < 0.005$). Although the shigella association index was somewhat higher in those cecal slices exposed to shigella-monocontaminated stool supernatants, the numbers were not significantly different from normal stool supernatant-treated slices.

In a recent article, Hale and Bonventre speculate that cell penetration of pathogenic shigellae could be multifactorial (6). The first step in the penetration of shigellae appears likely to be their association with the colonic cell surface. To overcome the cell barriers, the pathogenic

FIG. 1. Association of *S. flexneri* to homogenized cecal slices after incubation of intestinal tissue with KRB solution and various experimental stool supernatants. Bars represent mean $\pm$ standard error of 10 experiments. The percentage expressed on the ordinate represents the association index of the *S. flexneri*. Asterisks indicate statistical significance ($P < 0.005$) as compared with germfree stool supernatant experiments. Association of shigellae to KRB solution or germfree stool supernatant-treated slices was not statistically significant.

shigellae require complete metabolic integrity since ultraviolet radiation of the bacteria or inhibition by kanamycin abolishes the penetrative process. Our experiments suggest that exposure of germfree cecum to stool supernatants of shigella-monocontaminated mice alters the mucosal membranes in such a way as to increase the association with shigellae. A similar action upon the colonic mucosal membranes is exerted by conventional stool supernatants. Thus, even though conventional flora or their metabolic products antagonize the growth of shigellae in the colonic lumen (7), they appear to act synergistically upon the colonic mucosa to increase the bacterial association. This suggests that colonic content may favor the infectivity of pathogenic shigellae.

The possible mechanisms involved in the modification of cell membranes by contaminated colonic content were not determined in these preliminary experiments. In fact, attachment mechanisms have not been defined for shigellae. With certain other invasive pathogens, these mechanisms are better understood. For instance, in attaching to intestinal mucosa, salmonellae have been shown to interact with mannose residues of the glycoprotein in intestinal epithelial cell surfaces (1). It is reasonable to postulate

that colonic content increases the association of shigellae by somehow altering the mucosal membranes. In that respect, conventional flora are known to produce and secrete glycosidases into the colonic content which conceivably could modify terminal sugar residues of intestinal glycoprotein membranes (8). Alternatively, these glycosidases or proteases (10) could decrease host defenses by altering intestinal immunoglobulins which bathe the colonic mucosa (11).

In conclusion, these experiments suggest that in the multifactorial equation affecting the colonic association of shigella, the nature of the surrounding colonic content must also be considered. Further studies with purified glycosidases, proteases, inhibiting sugars, and other bacteria in intestinal cell culture could be of great interest in elucidating the mediator role of the intestinal content in the shigella-mucosa association.

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