Relationships between Rotavirus Diarrhea and Intestinal Microflora Establishment in Conventional and Gnotobiotic Mice

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Intestinal microflora did not play a role in the intensity or course of EDIM rotavirus-induced diarrhea, since similar results were observed in axenic and conventional mice. In conventional mice, rotavirus-induced diarrhea did not modify the establishment of Lactobacillus spp. and Escherichia coli before weaning. The consequences of diarrhea on the establishment of strictly anaerobic bacteria after weaning were studied through the measurement of two bacterial functions, the microbial barrier effect against E. coli and the development of the immunoglobulin A intestinal immune system. These two bacterial functions were expressed in a similar way in diarrheic and control mice. In young gnotobiotic mice inoculated with Clostridium perfringens or C. difficile, rotavirus infection led to an earlier development of both strains, as compared with controls. This effect was more pronounced with C. difficile. These results suggest that rotavirus infections might enhance opportunistic bacterial infections.

Rotavirus has been recognized as a major cause of diarrhea, primarily in the neonatal period (5, 11, 12). It has also been shown that the association of enterotoxigenic Escherichia coli strains with rotavirus enhances the pathogenicity of E. coli diarrhea in pigs (18), calves (14), lambs (30), infant mice (23) and, presumably, human newborns (11). However, the effects of rotavirus-induced diarrhea on bacterial colonization of the intestine remain unknown.

The purpose of this work was to investigate the consequences of diarrhea induced by EDIM rotavirus on the kinetics of digestive microflora establishment, which occurs in conventional mice according to a definite time sequence (9, 22, 27). The establishment of Lactobacillus spp. and E. coli was studied during the acute phase of diarrhea before weaning. The further consequences of diarrhea after weaning were studied through the expression of two bacterial functions of the autochthonous microflora, i.e., the resistance to intestinal colonization by E. coli (13) and the development of the immunoglobulin A (IgA) intestinal immune system (22). Gnotobiotic mice were also used to study the effect of rotavirus-induced diarrhea on the kinetics of establishment of nonautochthonous, potentially pathogenic bacteria, such as Clostridium perfringens and C. difficile.

MATERIAL AND METHODS

Animals. Female and male C3H mice were supplied by the Centre de Sélection et d’Elevage des Animaux de Laboratoire, Orléans la Source, France. Conventional and gnotobiotic mice were reared in Trexler-type isolators (29) fitted with a rapid transfer system (la Calhene, Bezons, France). Animals received a commercial diet (Usine d’Alimentation Rationnelle, Valmennon-sur-Orge, France) sterilized by γ irradiation (40 kGy). Blood samples were collected from the ophthalmic venous plexus of each pregnant mouse. The absence of preexisting rotavirus antibodies was proved by an enzyme-linked immunosorbent assay (6). Two-day-old pups were orally inoculated with 20 µl of a suspension containing 100 50% infective doses of EDIM rotavirus. In some experiments with conventional mice, the pups received 100 living E. coli cells orally at day 3 after birth. In experiments with gnotobiotic mice, pregnant mice received 0.5 ml of a 24-h culture of C. difficile or C. perfringens by stomach intubation at 1 week before delivery. The intestinal tracts of the pups were colonized by contact with their mothers. After the pups were weaned, feces were individually collected at the anus of each mouse. For 8- to 25-day-old mice, feces were collected individually on a weighed piece of paper. For mice 1 to 7 days old, feces cannot be collected. Therefore, these mice were sacrificed, and bacteriological analyses were done on the entire intestinal tract, except for the stomach.

Rotavirus. The Tyrell strain of EDIM was a kind gift of C. la Bonnariere (Institut National de la Recherche Agronomique, Grignon, France). The virus stock used in these assays was kept at −20°C. Diarrhea was characterized by droplet feces after gentle palpation of the abdomens of young mice.

Immunohistochemical techniques. IgA plasmocyte counting was previously described (21). Commercial goat antimouse α-chain serum (Meloy Laboratories) was used to count the IgA plasma cells in the duodenal villi. The tissues were examined with a Nikon epifluorescence microscope.

Bacteriological techniques. Bacteria belonging to the genus Lactobacillus were enumerated on GAPTG10 medium (25) containing 10 g of autolyzed yeast (Difco Laboratories), 10 g of tryptone (Difco), 15 g of peptone (Difco), 10 g of glucose, and 1 g of Tween 80, pH 6.5. The inoculum strain, E. coli S4, was isolated from the intestinal tract of a 10-day-old mouse; it was a nonpathogenic indigenous strain. This strain and any spontaneously occurring E. coli strains were counted on deoxycholate agar medium (Difco) by the decimal dilution technique (24). C. difficile FD and C. perfringens type A JD were isolated from the feces of a young diarrheic hare (8). They were enumerated in medium B containing 0.09% streptomycin (Specia) and in medium B’, respectively (26). A 15-ml quantity of medium inoculated with 1 ml of an adequate decimal dilution was poured into tubes (8 by 400

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mm) (24) and immediately cooled to ensure prompt solidification.

Statistical analysis. The Student t test was used for a comparison of mean values of log counts, and the chi-square test was used for a comparison of the percentages of mortality.

RESULTS

Effect of the intestinal microflora on rotavirus-induced diarrhea. The course of diarrhea was the same in conventional and axenic mice (Fig. 1) inoculated with rotavirus when they were 2 days old. Diarrhea began 2 days postinoculation and ended 12 days later. In both groups, composed of 25 mice each, a high percentage of the mice had diarrhea from day 5 to day 8 postinoculation. At this time, the highest level of mortality was observed; 28 and 38% in conventional and axenic mice, respectively. This difference was not significant.

Establishment of autochthonous microflora during diarrhea. During the acute phase of diarrhea, no difference could be found in the kinetics and levels of establishment of Lactobacillus spp. (Fig. 2) and E. coli (Fig. 3) between diarrheic and control conventional mice. Lactobacillus implantation levels were high from the first days of life (Fig. 2), and individual variations were small in both groups, as shown by the standard error of the mean (SEM). The implantation levels of E. coli were low during the first days of life. After the first week of life, the counts reached 10⁷ viable cells per g of digestive content, but variations were large in both groups; however, the differences observed were not significant.

Effect of rotavirus-induced diarrhea on the resistance to colonization by E. coli and IgA plasmocyte intestinal stimulation in weaned conventional mice. The number of E. coli per gram of feces decreased from 10¹⁰ to 10⁶ at weaning (21 days) as a consequence of the microbial barrier effect (Fig. 4). However, no significant differences were observed in the expression of this function between rotavirus-infected mice and control mice on any day. Table 1 shows that similar numbers of duodenal IgA plasmocytes were reached in both control and rotavirus-infected conventional mice. Rotavirus did not stimulate the IgA intestinal immune system in axenic mice.

Effect of rotavirus-induced diarrhea on the establishment of nonautochthonous bacterial strains in gnotobiotic mice. C. perfringens JD (Fig. 5) and C. difficile FD (Fig. 6) became
established earlier in diarrheic than in control mice. The numbers of both strains were significantly higher in diarrheic than in control mice at days 14 ($P < 0.01$) and 12 ($P < 0.001$), respectively, whereas no significant differences were observed in the population levels of both strains after weaning. Moreover, individual variations were larger in the diarrheic group than in the control group until weaning.

**DISCUSSION**

The present studies were designed to develop a better understanding of the interactions between intestinal microflora establishment and rotavirus-induced diarrhea. The dependence of the susceptibility of mice to rotavirus-induced diarrhea on age was first reported by Wolf et al. (31). Several hypotheses were tested to elucidate the factors responsible for this susceptibility. However, the role of the intestinal microflora was not investigated. It has been demonstrated that the microflora increases the intestinal epithelial cell migration rate (1) and intestinal transit (2) and that EDIM multiplies in the epithelial cells lining the intestines of infant mice (20). However, our results show that the microflora has no effect on the age-dependent susceptibility to EDIM, since the kinetics of diarrhea were similar in conventional and axenic mice.

The consequences of rotavirus-induced diarrhea on the establishment of autochthonous intestinal microflora are not documented. *Lactobacillus* and *E. coli* strains are part of the autochthonous microflora as defined by Dubos et al. (10). *E. coli* is established at high levels 5 to 10 days after birth (9, 22), whereas anaerobic strains, such as *Bacteroides* spp., only became established 15 days after birth (22).

Preliminary experiments showed that there were great variations in the delay of *E. coli* establishment in 1-week-old mice. That is why we chose to inoculate 3-day-old mice with the indigenous strain, *E. coli* S4, to obtain a more regular implantation of *E. coli*. However, there were still great individual variations in the fecal *E. coli* counts, as previously observed (13). Rotavirus-induced diarrhea did not disturb the kinetics of *E. coli* and *Lactobacillus* establishment during the acute phase of diarrhea. In a previous work, Lee et al. (19) showed that a spontaneously occurring enterovirus infection delayed *E. coli* establishment, whereas *Lactobacillus* implantation was not modified. However, the viral agent of infection was not identified, and the duration of diarrhea and the proportion of diarrheic pups were not reported.

Davis et al. (9) showed that marked changes in the intestinal microflora occurred in 14- to 25-day-old mice, and they demonstrated that strictly anaerobic bacteria became established in the distal part of the intestine after weaning. At this time, the obligate anaerobes exerted a strong barrier effect against *E. coli*; e.g., *E. coli* disappeared from the intestine or remained at a low population level (9, 13). It could be assumed that rotavirus-induced diarrhea affects the further establishment of bacterial strains responsible for the

**TABLE 1. Effect of rotavirus-induced diarrhea on the development of the IgA intestinal immune system in 7-week-old conventional and axenic mice**

<table>
<thead>
<tr>
<th>Mice</th>
<th>No. tested</th>
<th>No. of IgA plasmocytes per duodenal villus (mean ± SEM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diarrheic</td>
<td>12</td>
<td>39.5 ± 3.7</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>40.5 ± 1.07</td>
</tr>
<tr>
<td>Axenic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diarrheic</td>
<td>5</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>3.6 ± 0.4</td>
</tr>
</tbody>
</table>

* Differences within the groups were not significant.
barrier effect against *E. coli*. This hypothesis was not supported by our results.

Another function exerted by the intestinal microflora of conventional mice is the stimulation of the IgA intestinal immune system (21). The maximal number of intestinal IgA plasmocytes is reached in 7-week-old conventional mice (7), and bacterial strains becoming established just after weaning are needed for a full stimulation of the IgA intestinal immune system (22). Thus, we tried to determine if rotavirus-induced diarrhea could prevent the establishment of such bacteria. Our results show that diarrhea did not produce any perturbation in the establishment of the bacteria responsible for the development of the IgA intestinal immune system.

The effect of rotavirus-induced diarrhea on the establish-
ment of bacterial strains which did not belong to the autochthonous microflora was also studied. *C. difficile* and *C. perfringens* were chosen because they are known to be potentially pathogenic for humans and animals (16, 17). Gnotobiotic mice were used to avoid the barrier effect exerted by the intestinal microflora of conventional mice on the establishment of these strains (15). Our results show that rotavirus-induced diarrhea enhanced the development of *C. perfringens* JD and *C. difficile* FD in the intestinal tracts of young mice. This effect was more pronounced for *C. difficile*. However, the mechanism by which rotavirus-induced diarrhea promoted *C. difficile* development in the feces of gnotobiotic mice is still unknown. It has been shown that there is a proximal to distal progression of rotavirus infection with time (20, 28). At the end of the course of diarrhea, rotavirus is located in the ileum and colon. It is known that strictly anaerobic strains colonize the mucin associated with the mucosal epithelium in the cecum and colon (9). One can suppose that a modification of mucin affected by rotavirus might promote the earlier development of strictly anaerobic strains. These observations could be of clinical importance. Antibiotic therapy could lead to a complete destruction of the intestinal microflora, as observed in babies kept in a special-care unit (4). Rotavirus-induced diarrhea occurring in such conditions could promote the development of potentially pathogenic bacteria present at low levels, thus allowing pathological events caused by these bacteria. Bishop et al. (3) noted that babies kept in a special-care unit were much more likely to develop symptomatic and severe illnesses after rotavirus infection than were full-term babies. The relationships between rotavirus and enterotoxigenic *E. coli* strains have also been described in different animal species (14, 18, 30). Thus, a synergistic effect between rotavirus infection and bacteria which are responsible for digestive disorders might exist in babies. Our results suggest that, even though rotavirus-induced diarrhea did not affect the establishment of autochthonous intestinal microflora in healthy individuals, the development of potentially pathogenic bacterial strains could occasionally be promoted by rotavirus infections.

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