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Enterococcus hirae Implicated as a Cause of Diarrhea in Suckling Rats

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A Lancefield group D enteric streptococcus was isolated from diarrheic suckling rats that had been inoculated orally with stool from a diarrheic human. After oral administration of the organism to other suckling rats, diarrhea was reproduced, and the enteric streptococcus was reisolated. The brush border of small intestinal villi in affected animals was coated with numerous adherent gram-positive cocci. The organism was identified as *Enterococcus hirae* by a battery of biochemical tests. These and previous studies indicate that certain enterococci should be considered as etiologic agents of diarrheal disease in neonatal animals.

Streptococci of Lancefield group D can be divided into enterococcal and nonenterococcal species. The nonenterococcal species, *Streptococcus bovis* and *S. equinus*, are similar to the viridans group streptococci and are associated with bacterial endocarditis in humans (2). The enterococcal species, for example, *Enterococcus faecalis* and *E. faecium*, are generally considered to be nonpathogenic commensals of the gastrointestinal tracts of humans and other animals, but in humans they are occasionally associated with urinary tract and wound infections and with bacterial endocarditis (2). In mice, enterococci isolated from female breeders produced necrotic foci in the liver and intestines of animals receiving daily cortisone injections (3). *E. durans* has been reported to cause profuse diarrhea in foals and gnotobiotic piglets (5) and was also associated with a fatal outbreak of diarrhea in infant rats (4). Other enteric pathogens of laboratory rats, such as *Salmonella* sp. (7), *Bacillus piliformis* (7), and a group B rotavirus (6), are well documented in the literature. In this paper, we report studies implicating another enterococcal strain, *E. hirae*, as an etiologic agent of diarrhea in suckling rats.

MATERIALS AND METHODS

Animals. Virus- and mycoplasma-antibody-free pregnant rats [Crl:CD(SD)BR] were obtained from a commercial breeder on days 15 to 17 of gestation. Animals were housed in a conventional animal room (in which only virus-antibody-free animals are housed) and caged individually in polycarbonate cages with hardwood chip contact bedding and cage filter tops. Animals received commercial rodent feed and tap water ad libitum. Dams were allowed to deliver naturally.

Initially, stool from a diarrheic human served as the original inoculum for a group of 1-day-old suckling rats. This specimen was obtained from an adult male and was negative for known bacterial agents of diarrhea and negative for rotavirus. Isolation of enterococci from the initial stool was not attempted. Each rat was inoculated orally with 10 μl of a 2% stool suspension in phosphate-buffered saline (PBS). The suckling rats were examined daily for signs of diarrhea. Intestinal washings, intestinal homogenates, or enterococci obtained from diarrheic suckling rats were used as inocula for further passages. The appropriate inoculum (intestinal washing, homogenate, or enterococci) was administered to 1- to 5-day-old suckling rats via a 24-gauge gastric gavage needle. For each experimental trial, age-matched suckling rats from two or more dams were mixed and randomly redistributed to minimize possible effects of the dam or of genetic variation on susceptibility to infection and disease.

Inoculum preparation. Small intestinal washings were prepared by removing the small intestine of each rat and flushing the lumen with 0.2 ml of PBS containing 0.01% $CaCl_2$ and 0.01% $MgCl_2 \cdot 6H_2O$ (Ca^{2+}-Mg^{2+}-PBS). For preparation of intestinal homogenates, the small intestines of diarrheic rats were homogenized in hand-held Ten Broeck tissue grinders containing sufficient Ca^{2+}-Mg^{2+}-PBS to produce a 10% (wt/vol) final suspension. Homogenates and washings were stored at −70°C prior to being administered. Each rat received 100 μl of either homogenate or washing by gastric gavage.

Bacterial suspensions for inoculation were prepared by adding Ca^{2+}-Mg^{2+}-PBS to a 5% sheep blood agar plate containing 24-h-old pure growth of *E. hirae*. The agar surface was gently swept with an inoculating loop to suspend the bacteria. Suspensions were transferred to a glass tube, diluted to a McFarland 10 standard (3×10^9 bacteria per ml), and used immediately for inoculation. In some instances, *E. hirae* grown in trypic soy broth for 24 h was used for inoculation. The organisms were centrifuged, washed three times in PBS, diluted to a McFarland 10 standard, and inoculated immediately. Each rat received 100 μl (3×10^8 bacteria) of the suspension. For storage, bacteria from blood agar plates were suspended in tryptic soy broth containing 15% glycerol and frozen at −70°C.

Depending upon the protocol used to prepare the inoculum for the principal animals in each experiment, age-matched control rats were inoculated with intestinal homogenate from normal suckling rats, with filtrate (0.2-μm pore) of intestinal homogenate from diarrheic *E. hirae*-infected rats, or with PBS. Additional control rats were inoculated with filtrate (0.2-μm pore) of tryptic soy broth *E. hirae* cultures that had been proven to cause diarrhea in suckling rats.

Bacterial isolation and characterization. The enteropathogenic enterococci were isolated from intestinal washings or homogenates of diarrheic rats by streaking 10 μl of either sample onto 5% sheep blood agar plates and incubating the plates aerobically at 35°C. Colonies with the characteristic

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appearance of streptococci were picked, subcultured, and then characterized biochemically.

Immunofluorescence. Segments of small intestine, cecum, and colon were processed for immunofluorescence by a previously described alcohol fixation-paraffin embedding technique (6). Deparaffinized tissue sections were first covered with sera obtained from uninoculated normal weanling rats, from weanling rats that had recovered from enterococcal diarrhea, or from suckling rats acutely ill with enterococcal diarrhea. The primary serum was followed by fluorescein-conjugated, affinity-purified goat anti-rat immunoglobulin G (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.).

Histopathology. Suckling rats were necropsied on the first day that signs of diarrhea occurred. Tissues were fixed in 10% neutral buffered Formalin, dehydrated in graded ethanol, embedded in paraffin, sectioned at 6 μm, and stained with Harris hematoxylin and eosin. A Brown-Brenn tissue Gram stain was applied to selected additional sections of small and large intestines.

RESULTS

Clinical observations and pathology. In all of the trials, the onset of diarrhea ranged from postinoculation day 2 to day 6, with day 4 being the most common. Diarrhea persisted for 7 to 10 days and was characterized by pasty, unformed, bright yellow stool that was easily expressed by gentle thumb pressure on the caudal abdomen. The stool of normal age-matched control animals was characterized by small, formed, brown pellets. Some animals showed significant weight loss during this time and remained stunted after recovery. Animals were kept for no more than 35 days postinoculation. There were no fatalities. None of the rats inoculated at 5 days of age developed diarrhea. Suckling rats were more susceptible to enterococcal infection and disease in the first 3 days of life and become resistant to disease by 5 days of age. Additionally, enterococcal infection was readily transmitted from inoculated suckling rats to their uninoculated litter mates. Cage-to-cage transmission was not observed. Diarrhea was not observed in control animals inoculated with intestinal homogenate obtained from normal suckling rats, with filtrate of intestinal homogenate from diarrheic *E. hirae*-infected rats, with filtrate of *E. hirae*-containing culture broth, or with PBS. Cultures of intestinal contents and intestinal homogenates taken from control animals were negative for *E. hirae*.

At necropsy, the distal small intestine and colon of the diarrheic rats were distended with gas and yellow fluid feces. Light microscopy revealed numerous gram-positive cocci covering the brush border of villi in all regions of the small intestine (Fig. 1). Aside from the bacteria coating the surface, small intestinal villi were morphologically normal. The cecum and colon were unaffected. There were no grossly observable changes and no light-microscopy-observable changes in other organs examined (brain, kidneys, liver, heart, lungs, thymus, spleen, and musculoskeletal system). Bacteria were not found coating the surface of small intestinal villi in control animals, and no lesions were observed grossly or by light microscopy.

Bacterial isolation and characterization. After 48 h on 5% sheep blood agar, streptococcal colonies were small (1 to 2 mm) and clear or milky, with alpha-hemolytic zones. The following biochemical reactions characterized the organisms as group D enterococci: catalase negative, bile esculin positive, and growth in 6.5% NaCl. Further analysis of the organism, performed by Richard H. Facklam (Reference Bacteriology Section, Respiratory Bacterial Reference Laboratory, Centers for Disease Control, Atlanta, Ga.), identified the species as *E. hirae* (Table 1).

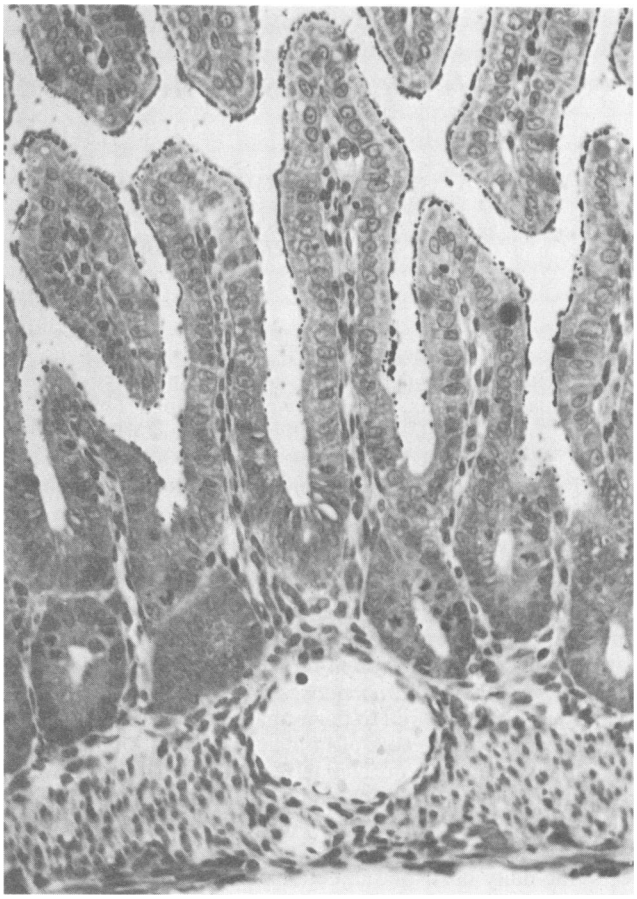

FIG. 1. Section of small intestine showing *E. hirae* organisms adhering to the brush border of villi. Brown-Brenn tissue Gram stain. Magnification, ×320.

Immunofluorescence. Adherent bacteria covering the villi of the proximal, middle, and distal small intestine stained strongly positive with convalescent-phase sera obtained from *E. hirae*-infected rats (Fig. 2). The cecum and colon were negative for fluorescent organisms. This pattern of immunofluorescence staining correlated with the distribution of gram-positive cocci on small intestinal microvilli observed by light microscopy. Sera obtained from either suckling rats that were acutely ill with *E. hirae*-induced diarrhea or uninoculated normal weanling rats did not stain the bacteria.

DISCUSSION

Enterococci are not generally considered to be gastrointestinal pathogens and, when isolated from the stool of a human or animal with diarrhea, are usually assumed to be normal intestinal flora unrelated to the diarrheal illness. This assumption is probably correct in most instances; however, in recent years, enterococci have been implicated as a cause of diarrhea in animals. *E. durans*, a species closely related to *E. hirae*, has been isolated from a diarrheic foal (5). This isolate also causes diarrhea when inoculated into gnotobiotic piglets (5). Additionally, a second isolate of *E. durans* has

TABLE 1. Profile of the enterococcal isolate (*E. hirae*)

Test	Result
Group D reaction	+
Catalase	−
Bile esculin	+
Arginine	+
Growth at 10°C	+
Growth in:	
6.5% NaCl	+
4.0% NaCl	+
2.0% NaCl	+
Starch hydrolysis	−
Growth at 45°C	+
Tellurite	−
Tetrazolium	−
Sodium hippurate	−
Pyruvate	−
Litmus milk acid clot	+
Trehalose	+
Sorbitol	−
Lactose	+
Mannitol	−
Sucrose	+
Inulin	−
Esculin	+
Raffinose	+
Glycerol	−
Arabinose	−
Salicin	+
Alpha-hemolysis	+
Hydrolysis of L-pyrrolidonyl-β-naphthylamide	+
Melibiose	+
Maltose	+
Sorbose	−

been associated with diarrhea in suckling rats (4). In all of these instances, the enterococci coated the brush border of the small intestinal villi. Another common feature of these reported spontaneous enterococcal diarrheas was that clinical disease occurred in young animals. This is consistent with our observation that rats 3 days of age or less were the most susceptible to enterococcal diarrhea.

Prior to 1985, several strains of enterococci were classified as atypical strains of *E. faecium*; however, studies of DNA base composition, lipid content, biochemical reactions, and DNA-DNA hybridization demonstrated that these atypical strains actually belong to a single species that is distinct from *E. faecium*. This new species is named *E. hirae* (1). In contrast to *E. faecium*, all *E. hirae* strains are L-arabinose and hippurate negative (1). This organism has thus far been implicated as a pathogen only in chickens, in which it was associated with growth depression (1). Our studies indicate that *E. hirae* should also be considered as an etiologic agent of diarrhea, at least in suckling rats. The pathogenicity of this organism appears to be dependent upon the specific isolate, since attempts to produce diarrhea in rats with an American Type Culture Collection (Rockville, Md.) strain of *E. hirae* (ATCC 8043 = NCDO 1258^T [National Collection of Dairy Organisms, Shinfield, Reading, United Kingdom]) were successful (unpublished observations). Factors such as adhesins or toxins that might be important in determining the pathogenicity of *E. hirae* and other enterococci should be the subject of future investigations.

It seems reasonable to suggest from observations in the present study and in previous studies that enterococci should be considered as potential etiologic agents of diarrheal disease in neonatal animals. Unfortunately, because of the small amount of stool available to us, we were unable to definitively determine whether the *E. hirae* isolate in our study came from the original human stool inoculum or from the rats that were inoculated with it. However, it is clear from our studies that oral inoculation of a pure culture of our strain of *E. hirae* could reproducibly cause diarrheal disease in infant rats. A goal of future studies will be to more carefully define enteric streptococci isolated from diarrheic laboratory animals and humans to determine the significance of this group of organisms in the etiology of acute enteritis.

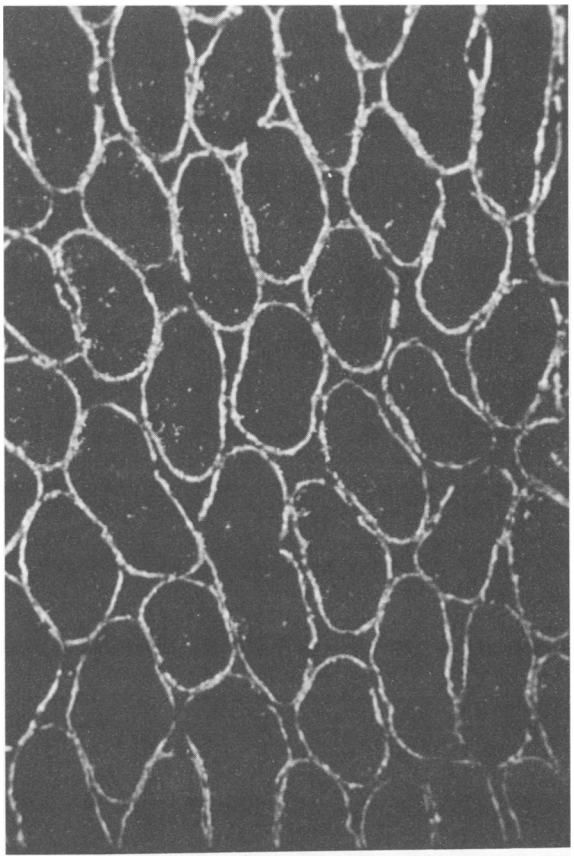

FIG. 2. Cross-section of small intestinal villi showing positive immunofluorescence staining of *E. hirae* organisms coating the epithelial cell surface. Magnification, ×160.

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