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Infectious Diarrhea of Infant Rats Produced by a Rotavirus-Like Agent

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During the investigation of an outbreak of diarrhea in suckling rats, a virus morphologically identical to but antigenically distinct from rotaviruses was identified. The disease was characterized clinically by erythema and cracking and bleeding of the perianal skin associated with the excretion of poorly formed fecal pellets, liquid, and gas. Light microscopy-observable changes consisted of small intestinal villous atrophy, villous epithelial necrosis, and villous epithelial syncytial cell formation. The cytoplasm of the epithelial syncytial cells contained large numbers of 80-nm viral particles that were often associated with reticular aggregates of electron-dense material. Viral infection principally involved the luminal one-fourth to one-third of the intestinal villi as determined by indirect immunofluorescence. This rotavirus-like agent contained 11 double-stranded RNA segments; however, the migration pattern of these segments in polyacrylamide gels differed from the electrophoretic pattern which is characteristic of the typical rotaviruses. The agent had a buoyant density in CsCl of 1.36 to 1.4 g/cm^3 and was labile at pH 3 and at 56°C; however, infectivity of viral inocula was not altered by extensive treatment with ether or by pH 5 buffers. This disease, which we have named infectious diarrhea of infant rats, is the first recognized viral diarrhea of rats and appears to be a good model for the study of the recently recognized group of atypical rotaviruses.

Rotaviruses are segmented, double-stranded RNA viruses which have been shown to be an important cause of diarrhea in many animal species, including cows, pigs, horses, mice, and humans (4, 9). Most of the rotaviruses found to cause diarrhea share a common antigen which is located in the inner capsid layer (22) and is coded by genome segment 6 (5). Recently, several viruses morphologically identical to rotavirus but not containing the common antigen have been identified in diarrheic piglets (1, 16), chickens (10), and humans (2, 3, 12, 14, 20). These atypical rotaviruses have been referred to as pararotaviruses (1, 3, 12), rotavirus-like viruses (14, 16), or novel rotaviruses (20). These viruses, like the rotaviruses, have 11 double-stranded RNA segments; however, their RNA electrophoretic profiles in polyacrylamide gels differ from the patterns which are typical of the rotaviruses.

We report here the identification and characterization of a new disease, infectious diarrhea of infant rats (IDIR), produced by a rotavirus-like agent. The disease came to our attention after our observations of a spontaneous outbreak of diarrhea in suckling rats. While in the process of investigating this outbreak, we discovered a transmissible agent that was morphologically identical to rotavirus but did not contain the common rotavirus antigen as determined by enzyme immunoassays.

MATERIALS AND METHODS

Animals. Pregnant CD rats were purchased from a commercial supplier (Charles River Breeding Laboratories, Wilmington, Mass.). The females were housed individually, given food and water ad libitum, and allowed to give birth naturally. Suckling rats remained with their dams throughout the study. At the time of experimental inoculation, each suckling rat was inoculated orally with 10 μl of the appropriate test material. They were then examined daily for signs of diarrhea.

Intestinal filtrates. Intestines from diarrheic, neonatal CD rats were collected, pooled, ground in hand-held Tenbroeck tissue grinders, and suspended in sufficient RPMI 1640 medium to produce a 10% (wt/vol) suspension. The suspension was centrifuged at 13,000 × *g* for 5 min, and the supernatant was collected and passed through either a 0.22- or a 0.45-μm-pore-size membrane filter in preparation for further experimentation.

Acid treatment. To determine the acid sensitivity of the agent, intestinal filtrates were adjusted to either pH 3 or pH 5 with 0.1 N HCl for 30 min at 37°C and then readjusted to pH 7 by the addition of 0.1 N NaOH. The control inoculum was diluted to an equivalent final volume with distilled water at pH 7. The inocula, thus treated, were given to suckling rats.

Ether treatment. Ether sensitivity of the agent was assessed by adding 1 ml of diethyl ether to 1 ml of intestinal filtrate, followed by vigorous shaking. The mixture was incubated for 1 h at room temperature with vigorous shaking every 5 min. The aqueous phase was collected and administered to suckling rats as above.

Heat treatment. Heat sensitivity of the agent was assessed by placing a sample of intestinal filtrate into a 56°C water bath for 30 min. The filtrate was quickly returned to room temperature and administered to suckling rats.

Density gradients. The buoyant density of the agent in CsCl was determined after equilibrium centrifugation on four step gradients. The 10% (wt/vol) intestinal suspension was dissociated by sonication and extracted twice with one-half volume of Freon 113. The aqueous phase was collected and centrifuged at 12,000 × *g* for 20 min at 5°C to pellet cell debris and bacteria. The supernatant was centrifuged on a 20% (wt/wt) sucrose cushion at 100,000 × *g* for 2 h at 5°C to pellet the virus. Pellets were suspended, sonicated, and

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layered onto a four-step CsCl gradient. Steps in the gradient consisted of 2.5 ml of CsCl solution having a buoyant density of 1.2, 1.3, 1.4, or 1.5 g/cm^3. After centrifugation at 100,000 $\times$ g for 18 h at 5°C, 0.5-ml fractions were collected and dialyzed against phosphate-buffered saline. The dialyzed fractions were then administered to suckling rats.

Nucleic acid studies. Virus was isolated from infected rat intestinal homogenates, and double-stranded RNA was recovered from the extracted material by CF-11 cellulose chromatography (21). Human and murine rotavirus RNA were isolated from stool and intestinal homogenates, respectively, using previously described techniques (21). The RNA preparations were electrophoresed on 10% polyacrylamide gels (1.5 mm thickness) at 30 mA constant current for 15 h. The electrophoresis buffer was 0.04 M Tris base–0.002 M disodium EDTA (pH 7.8) with glacial acetic acid. Gels were stained for 2 h with 1 μg of ethidium bromide per ml and photographed under UV light.

Microscopy. Tissues for light microscopy were fixed in 10% neutral buffered Formalin, dehydrated in graded ethanols, and embedded in glycol methacrylate. Three-micrometer sections were cut on a JB4 microtome (Du Pont Instruments, Newton, Conn.) and stained with Harris hematoxylin and eosin. Tissues for electron microscopy were fixed in phosphate-buffered combined fixative containing 4% commercial formaldehyde and 1% glutaraldehyde (8), postfixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Fields for electron microscopy were selected from 1-μm sections stained with 1% toluidine blue. Sections 60 to 90 nm thick were stained with lead citrate and uranyl acetate and viewed with a JEOL 100S transmission electron microscope.

Immunofluorescence. Segments of distal small intestine were processed for immunofluorescence by a previously described alcohol-fixation, paraffin-embedding technique (18, 19). Deparaffinized tissue sections were stained by indirect immunofluorescence with either normal noninoculated rat or convalescent sera from diarrheic rats, followed by fluorescein-conjugated, affinity-purified goat anti-rat immunoglobulin G (IgG) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.).

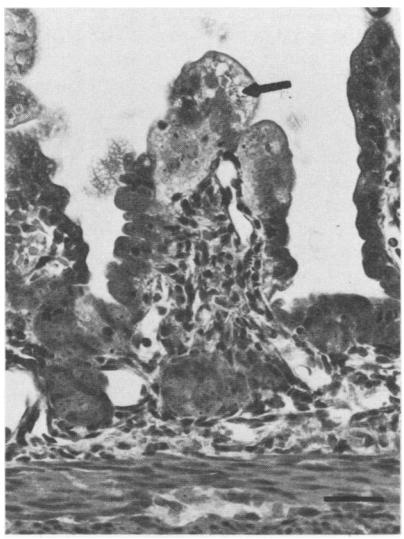

FIG. 1. Villous atrophy and epithelial syncytial cell formation in the distal small intestine of an IDIR virus-infected rat. Numerous cytoplasmic inclusions (arrow) are present in the syncytial cells. Hematoxylin and eosin stain was used. Bar, 25 μm.

Negative staining. Material for negative staining was obtained after centrifugation of the sonicated, Freon 113-extracted intestinal homogenate on a 20% (wt/wt) sucrose cushion as described above. The pelleted material was suspended in 2 drops of sterile, deionized water, placed on carbon-coated Formvar grids, and stained with 2% phosphotungstic acid (pH 6.6).

Rotavirus enzyme immunoassays. Intestinal homogenates from diarrheic rats were assayed for rotavirus antigen by indirect enzyme immunoassay techniques (25). The first assay was performed with goat antibody to human rotavirus subgroup 2 and guinea pig antibody to human rotavirus subgroups 1 and 2. The reaction was quantitated by reaction with alkaline phosphatase-labeled anti-guinea pig IgG. The second rotavirus antigen immunoassay was performed with guinea pig antibody to murine rotavirus, mouse anti-murine rotavirus serum, and peroxidase-labeled anti-mouse IgG. Additionally, sera from convalescent rats were examined for antibodies to simian, murine, and bovine rotaviruses by an enzyme immunoassay blocking assay as previously described (23) and by an antibody binding assay (24). Guinea pig antibody to murine rotavirus, murine rotavirus extracted from mouse intestinal homogenate, and peroxidase-labeled anti-rat IgG were used in the binding assay.

RESULTS

Clinical observations and pathology. Diarrheal disease was induced numerous times in suckling rats after serial inoculation with bacteria-free intestinal filtrates obtained from diarrheic rats. Characteristic clinical and pathological features were consistently produced.

At 24 to 36 h after inoculation, suckling rats developed diarrhea that persisted for 5 to 6 days and was associated with erythema and cracking and bleeding of the perianal skin. The disease resulted in growth retardation and drying and flaking of the skin that were apparent for at least 12 days after inoculation. None of the animals died.

Rats, 1 to 11 days old, developed diarrhea after experimental inoculation; however, animals, 14 days of age or older, were resistant to disease. Since the agent was found to be acid labile, it was considered that this age-dependent disease susceptibility might be related to an increase in gastric acidity. Therefore, litters of 14-day-old rats were given either 1 ml of 7.5% $NaHCO_3$ orally immediately before inoculation or the inoculum was administered directly into the duodenum via a small abdominal incision. In neither instance did the animals develop diarrhea.

The colon and distal small intestine of diarrheic rats contained fluid, poorly formed fecal pellets, gas, and, occasionally, mucinous material. Contents of the proximal small intestine were watery and often had a tan to green discoloration. The stomach was always filled with milk. Other organs were not remarkable.

Light microscopy-observable changes consisted of small intestinal villous epithelial necrosis, villous epithelial syncytial cell formation, and villous atrophy. Syncytial cells were found at any point along the villus but were never found in the crypts. Numerous 1- to 2-μm eosinophilic inclusions were seen frequently in the cytoplasm of syncytial cells (Fig. 1). Epithelial necrosis was most prominent on the upper one-third of the villus.

The cyotplasm of epithelial syncytial cells contained large numbers of viral particles that were often associated with and appeared to form from reticular or amorphous aggre-

gates of electron-dense material (Fig. 2). The particles were sometimes enclosed within vesicles of the rough endoplasmic reticulum. Individual particles were circular in profile, ca. 80 nm in diameter, and had a thick, well-defined outer electron-dense zone surrounding a thin innerlucent zone that, in turn, contained an irregular electron-dense core ca. 18 nm in diameter.

Immunofluorescence. Viral replication in the gastrointestinal tract was documented by immunofluorescence with convalescent sera from IDIR virus-infected rats and fluorescein-conjugated anti-rat IgG. Bright, coarsely granular fluorescence was present in the epithelium of the luminal one-fourth to one-third of villi in the distal small intestine of experimentally infected rats (Fig. 3). Occasionally, fluorescent cells were observed far down the sides of villi adjacent to the crypts. The distribution and course pattern of fluorescence corresponded to the intestinal lesions and large viral aggregates observed by light and electron microscopy, respectively. Fluorescence was not seen in intestinal crypts or lamina propria, nor was it seen when sera from normal noninoculated suckling rats were used in the procedure.

Negative staining. Several viral particles with morphology similar to that of rotaviruses were found in sonicated Freon 113-extracted intestinal material obtained from infected rats and examined by negative-stain electron microscopy (Fig. 4). Viral particles were uniform in size and shape, round, ca. 65 nm in diameter, and composed of capsomeres with cubic symmetry. Particles were rarely penetrated by the phosphotungstic acid stain.

Viral physical characteristics. Fractions obtained from all portions of the CsCl gradients frequently produced diarrhea when administered to suckling rats; however, those having a buoyant density of 1.36 to 1.4 g/cm^3 most consistently produced disease. Additionally, intestinal filtrates were treated with heat, acid, or ether and assayed for the ability to produce disease in suckling rats. Diarrhea was not seen in suckling rats after the filtrates were adjusted to pH 3 or held at 56°C for 30 min. The ability to produce disease was not altered by extensive treatment with ether or by pH 5 buffers.

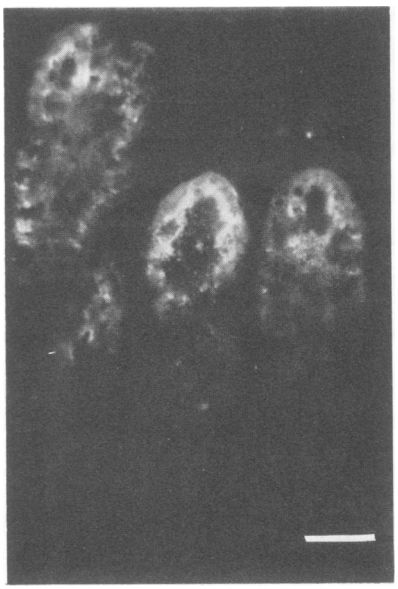

FIG. 3. Bright, granular fluorescence in the epithelium of the upper one-fourth of the villi from the distal small intestine of an IDIR virus-infected rat. Sections were covered with convalescent sera from IDIR virus-infected rats, followed by fluorescein-conjugated affinity-purified anti-rat IgG. Bar, 12 μm.

Nucleic acids. When CF-11-purified IDIR virus nucleic acid was subjected to polyacrylamide gel electrophoresis (PAGE), 11 distinct bands were resolved (Fig. 5A and B). The number of segments and their overall range of mobility were similar to that of human or murine rotavirus genomes coelectrophoresed with the IDIR virus. All of the preparations had a group of four slow-moving bands at the top of the

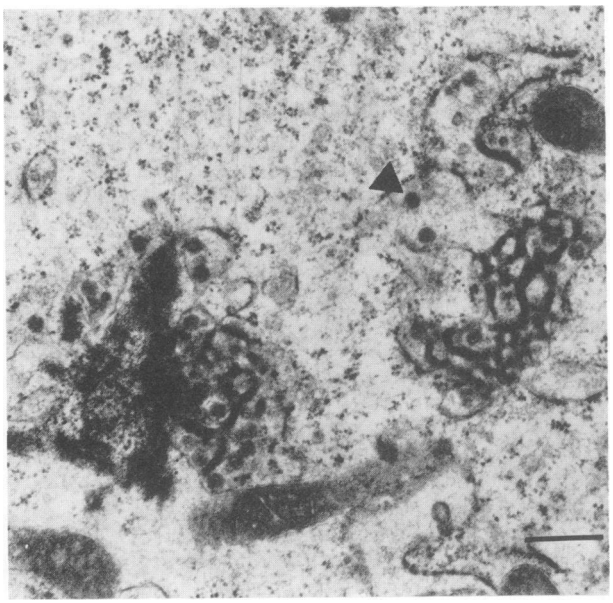

FIG. 2. Electron-dense reticular aggregates of viral precursor material and associated viral particles in the cytoplasm of an IDIR virus-infected epithelial syncytial cell. Note viral particles in cisternae of the rough endoplasmic reticulum (arrowhead). Bar, 380 nm.

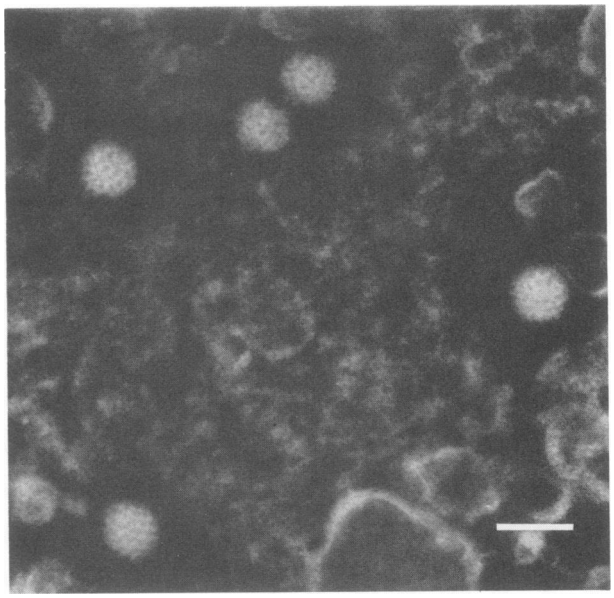

FIG. 4. Rotavirus-like particles observed in Freon 113-extracted, ultracentrifuged intestinal homogenates obtained from IDIR virus-infected rats. Bar, 100 nm.

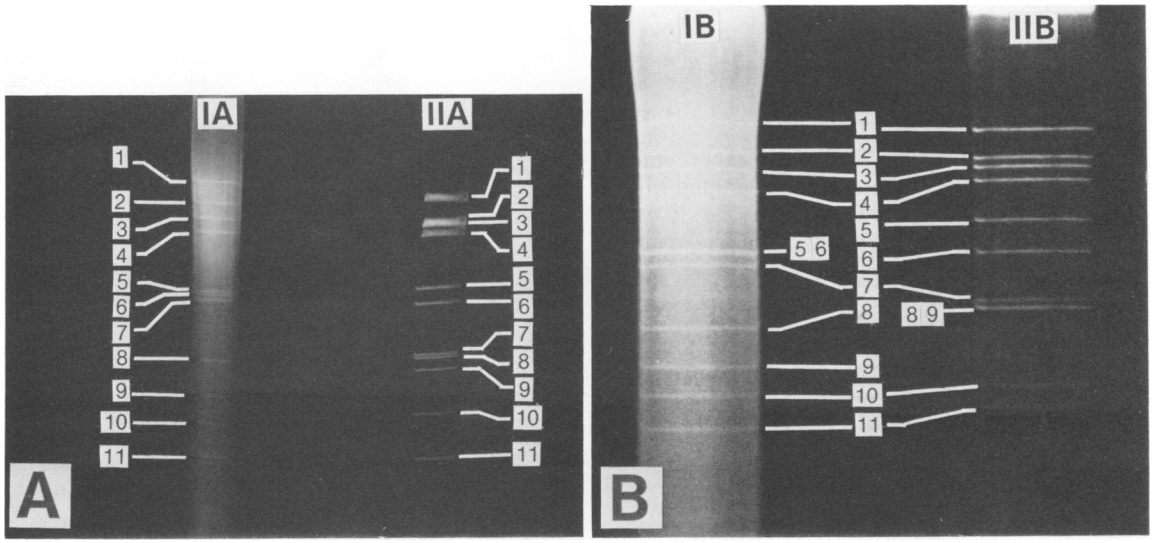

FIG. 5. PAGE of double-stranded RNA obtained from (A) IDIR virus (lane IA) and human rotavirus (lane IIA) and (B) IDIR virus (lane IB) and murine rotavirus (lane IIB).

gel; however, the migration of the remaining RNA segments was distinctly different for the viruses (Fig. 5A and B). Segments 7, 8, and 9 of the human and murine rotaviruses migrated as a triplet, whereas segments 5, 6, and 7 of IDIR virus nucleic acid migrated close together. The four smallest segments of the IDIR virus genome were about equidistant from each other on the gel. The pattern of the IDIR virus nucleic acid segments on PAGE was thus 4-3-1-1-1-1 (from largest to smallest segment), whereas that of the human and murine rotaviruses was 4-2-3-2.

Rotavirus immunoassays. Rotavirus antigen was not detected in intestinal homogenates from diarrheic rats by using hyperimmune sera directed at the common antigen. Assays were also negative when reagents directed at either subgroup 1 or subgroup 2 viruses were used. In addition, sera from convalescent rats obtained after IDIR virus infection did not contain antibody to simian, murine, or bovine rotaviruses as measured by enzyme immunoassay blocking assays and by an antibody-binding enzyme immunoassay.

DISCUSSION

The rotavirus genome consists of 11 segments of double-stranded RNA that separate into four size classes by PAGE. Although variability in the RNA electrophoretic pattern is noted when different rotavirus strains are compared, the number of segments within each size class remains the same. The typical rotaviruses possessing the common group antigen have a characteristic PAGE pattern of segments (from longest to smallest) of 4-2-3-2 (6, 15).

Recently, viruses that are morphologically identical to but antigenically distinct from rotaviruses have been identified in diarrheic humans (2, 3, 12, 14, 20), piglets (1, 16), and chickens (10). These atypical rotaviruses, which have been called pararotaviruses (1, 3, 12), rotavirus-like viruses (14, 16), or novel rotaviruses (20) contain 11 double-stranded RNA segments but have PAGE patterns that do not fit the 4-2-3-2 pattern of the typical rotaviruses. The IDIR virus described here also resembles the rotaviruses morphologically, but like the atypical viruses, it is antigenically distinct from the rotaviruses and has a genome electrophoretic pattern distinct from the typical 4-2-3-2 rotavirus pattern.

On the basis of comparative serological and nucleic acid studies of the porcine viruses, Pedley et al. have proposed that the typical rotaviruses, the pararotaviruses, and the rotavirus-like viruses all be classified as rotaviruses and subclassified into groups A, B, and C (13). According to this scheme, the major difference in the RNA electrophoretic profiles of the three groups is that RNA species 7, 8, and 9 migrate as a tight triplet in the typical (group A) rotaviruses, whereas in both groups B and C, this triplet is replaced by a doublet. The displaced RNA segment migrates in the species 10 region for the group B rotaviruses and in the species 6 region for the group C viruses. The RNA electrophoretic profile of IDIR virus thus most closely resembles the group C rotaviruses in that segments 5, 6, and 7 migrate as a triplet in polyacrylamide gels. However, the 4-3-1-1-1-1 pattern seen for the IDIR virus apparently differs from the published PAGE patterns for groups B and C rotaviruses. Coelectrophoresis of these viral genomes will be needed to confirm the apparent differences and similarities.

Recently, an antigenically distinct rotavirus, referred to as adult diarrhea rotavirus, has been implicated as the cause of two large epidemics of acute diarrhea in Chinese adults (20). The RNA electrophoretic profile of this virus is remarkably similar to that of IDIR virus. Again, coelectrophoresis of the viral genomes will be necessary to confirm this apparent similarity.

Epithelial syncytial cell formation observed in the small intestine of IDIR virus-infected rats has not been described with other typical or atypical rotaviruses. Epithelial syncytial cells are seen in the intestine of mice infected with murine hepatitis virus, which is a coronavirus, and have also been described in diarrheic neonatal calves infected with an uncharacterized viral agent (11). Feces obtained from gnotobiotic calves inoculated with the latter agent failed to induce diarrhea when orally administered to suckling rats (S. L. Vonderfecht, unpublished data). The electron-dense reticular aggregates of viral precursor material that were so prominent in the intestinal epithelium of IDIR virus-infected rats have not been reported in other diarrhea-producing virus infections. Although viral particles were present in vesicles of the rough endoplasmic reticulum, we were not

able to identify particles budding into the vesicles. This has been reported with the typical rotaviruses (17). The morphology of the viral particles in tissue sections was similar to that described for the typical (17) and antigenically distinct (10) rotaviruses.

The IDIR virus is apparently similar to other enteric viruses in that it is fastidious and not readily adapted to cell culture. Attempts to propagate the virus in MA-104 and primary monkey kidney cells have not led to growth as determined by the development of cytopathic effects (R. H. Yolken, unpublished data).

This is the first report of virally induced diarrhea in the laboratory rat. Rats are small, economical, and easy to manipulate experimentally, thus IDIR virus infection may be a useful model in the study of enteric viral infections in general and of atypical rotavirus infections in particular. The significance of IDIR virus or related agents in diarrhea of humans and animals and the pathogenesis of and immune response to atypical rotavirus infections will be the subject of future investigations.

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