Enzyme Immunoassay Inhibition Assay for the Detection of Rat Rotavirus-Like Agent in Intestinal and Fecal Specimens Obtained from Diarrheic Rats and Humans

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An enzyme immunoassay inhibition assay was developed to detect rat rotavirus-like agent (RVLA) or antigenically related viruses in intestinal washings and homogenates obtained from diarrheic humans. In this assay, RVLA antigens in a test sample inhibited the binding of a biotin-labeled anti-rat RVLA antibody preparation to rat RVLA antigens bound to the solid phase. Intestinal washings and homogenates obtained 1 day after RVLA infection of suckling rats inhibited the binding of the biotin-labeled antibody to the solid-phase rat RVLA antigens by 76 to 100%. The inhibition was blocked by RVLA immune rat serum but not by nonimmune rat serum. Of 27 fecal specimens obtained from diarrheic humans, 6 produced disease characteristic of rat RVLA infection when inoculated into suckling rats. Four of these six specimens produced greater than 50% inhibition in the enzyme immunoassay. Fecal specimens obtained from diarrheic humans that were determined to be negative for RVLA produced an average inhibition of 9.2%. This enzyme immunoassay appears to be a useful diagnostic and research tool for the study of infections with at least one of the antigenically distinct rotaviruses.

MATERIALS AND METHODS

Animals. Pregnant CD rats were purchased from a commercial supplier (Charles River Breeding Laboratories, Inc., 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 material.

Antigen preparation. Intestinal contents and washings obtained from rat RVLA-infected suckling rats were orally administered to 5- to 7-day-old rats. One day later, small-intestinal and colonic contents were collected, and the small intestine of each rat was flushed with 0.2 ml of phosphate-buffered saline containing 0.01% CaCl2 and 0.01% MgCl2·6H2O (Ca,Mg-PBS). Intestinal contents and washings were pooled, briefly sonicated, and extracted twice with one-half volume of Freon 113 (J. T. Baker Chemical Co., Phillipsburg, N.J.). The aqueous phase was collected and centrifuged at 12,000 × g for 20 min at 5°C to pelleted debris and bacteria. The supernatant fluid was centrifuged on a 20/60% (wt/wt) discontinuous sucrose cushion at 100,000 × g for 2 h at 5°C. The material at the interface of these two layers was collected, sonicated, divided into small aliquots, and frozen at −70°C. This material served as the rat RVLA antigen stock. Intestinal contents and washings obtained from normal suckling rats were processed in an identical manner and served as control antigen.

Biotin-labeled anti-rat RVLA immunoglobulin. A White Leghorn laying hen was intramuscularly inoculated with 1 ml of RVLA antigen emulsified with an equal volume of Freund complete adjuvant. At 14 and 35 days postinoculation, the hen was again inoculated intramuscularly with 1 ml of RVLA antigen emulsified with an equal volume of Freund incomplete adjuvant. Serum obtained from the chicken 2 weeks....

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of RVLA-inoculated or control suckling rats were dissected from the mesentery. The intestine was cut at the ileocecal junction, and the small-intestinal and colonic contents were collected in a tube. The small intestine was flushed with 0.2 ml of either Ca,Mg-PBS or minimum essential medium and cut into three equal segments, proximal, middle, and distal. Each segment, as well as the colon, was wrapped individually and frozen at −70°C. Before analysis for rat RVLA antigen, each segment was ground in a hand-held Tenbroeck tissue grinder, suspended in sufficient Ca,Mg-PBS to produce a 10% (wt/vol) suspension, and centrifuged at 12,000 × g for 5 min. The supernatant fluid was collected and tested for viral antigens. The pooled intestinal contents and washings from each rat were also centrifuged to pellet solid material before analysis for rat RVLA antigen.

**Fecal specimens.** Nineteen fecal samples or rectal swabs were obtained from 17 hospitalized children, aged 0 to 2 years, with diarrheal disease that could not be ascribed to known viral or bacterial agents and occurring within a 3.5-month period. Additionally, fecal specimens were collected from diarrheic adult staff caring for these children. The clinical characterization of six of these cases is the subject of another report (4a). Fecal samples or rectal swabs were also obtained from random diarrheic or nondiarrheic children and adults. Liquid fecal samples were centrifuged at 12,000 × g for 5 min to pellet solid material. Solid samples were diluted 1:10 in Ca,Mg-PBS before centrifugation. Rectal swabs were eluted in 1 ml of Ca,Mg-PBS or minimal essential medium. All specimens were tested for rat RVLA by enzyme immunoassay. Specimens from diarrheic humans were tested for RVLA by rat inoculation.

**Rat inoculation.** Specimens obtained from diarrheic humans were administered orally to suckling rats as described above. Each rat received 10 μl of inoculum and was examined daily for signs of diarrhea. If diarrhea was observed, the intestinal tracts were removed and processed for electron microscopy as described previously (16). Infection with rat RVLA was confirmed by the identification of the characteristic viral particles and reticular arrays of viral precursor material in small intestinal sections (16).

**Enzyme immunoassay.** The principle of the immunoassay is illustrated in Fig. 1. Fecal specimens obtained from diarrheic humans and intestinal washings or homogenates obtained from rats were placed in borosilicate glass tubes and diluted 1:5 with fetal bovine serum (6). The diluted test material was mixed with an equal volume of biotin-conjugated chicken anti-RVLA antibody diluted in PBS containing 0.05% Tween 20 and 0.5% gelatin. The mixture was incubated for 1 h at 37°C and then placed into the wells of polystyrene microtiter plates (Immunulon 2; Dynatech Laboratories, Inc., Alexandria, Va.) that had been coated overnight at 4°C with either control or rat RVLA antigen diluted in carbonate buffer (pH 9.6). The optimal concentrations of viral antigen and biotin-conjugated chicken anti-RVLA immunoglobulin were determined by checkerboard titration (15). After incubation for 1 h at 37°C, the wells were washed with PBS−0.05% Tween 20, covered with preformed peroxidase–avidin-biotin complex (Vector Laboratories, Burlingame, Calif.), and again incubated for 1 h at 37°C. The wells were washed with PBS−0.05% Tween 20, and substrate consisting of 0.4 mg of o-phenylene-diamine and 0.4 μl of 30% H₂O₂ per ml of 0.01 M citrate buffer (pH 5) was added to each well. The reaction of enzyme with the substrate was quantitated at a wavelength of 450 nm in a spectrophotometer (Biotek Instruments Inc., Burlington, Vt.). The optical density in wells coated with control antigen was subtracted.

**Collection of animal specimens.** The entire intestinal tracts after the last injection was found to have antibodies to rat RVLA when evaluated in an enzyme immunoassay similar to that described below. For the next 3 weeks, eggs from the hen were collected and the yolks were separated. The immunoglobulin in the yolks was isolated by a previously described method (7) and conjugated to biotin (17).

**Rat sera.** Suckling rats were inoculated orally at 5, 11, and 18 days of age with 10 μl of intestinal homogenate obtained from RVLA-infected rats. At 7 days after the last inoculation, the rats were exsanguinated and the sera were collected and stored. The sera contained antibodies to rat RVLA when evaluated by indirect immunofluorescence (16). Control rat sera were collected from normal RVLA-free weanling rats. Antibodies to rat RVLA were not found in these sera when they were evaluated by indirect immunofluorescence.

**FIG. 1.** Diagrammatic representation of the enzyme immunoassay inhibition assay developed to detect rat RVLA antigens. In step 1, test specimen is incubated with biotin-labeled chicken anti-RVLA antibody. Antibody will bind to RVLA antigens in the specimen. In step 2, the mixture is transferred to a well coated with RVLA antigens. Antibody not bound to viral antigens in step 1 will bind to the solide phase, while antigen-bound antibodies will be washed off. In step 3, the antibody bound to the solid phase is assayed by means of peroxidase–avidin-biotin complex. The specificity of the inhibition reaction could be demonstrated by blocking the reaction with specific immune sera. This was done by first incubating the test specimen with sera obtained from either normal RVLA-free or from convalescent RVLA-immune rats. The inhibition assay was then completed on the test specimen as above.
from the optical density in RVLA antigen-coated wells to yield a net specific activity. In this inhibition immunoassay, the presence of RVLA antigen in the test specimen was manifested by the inhibition of the binding of the biotin-labeled chicken anti-RVLA antibody to microtiter plate wells coated with rat RVLA antigen. The degree to which the test sample inhibited the binding was determined by the formula 100 \times (1 - \text{net specific activity/negative control}) = \text{percent inhibition}. Intestinal washings collected from RVLA-free suckling rats were used as negative controls for the intestinal washings collected from the test rats and for the specimens obtained from diarrheic humans. Intestinal homogenates obtained from RVLA-free suckling rats served as negative controls for the intestinal homogenates obtained from test rats.

In some cases, to document specificity the inhibition reaction was blocked with specific immune serum. This was accomplished by first incubating the test sample with sera obtained either from normal RVLA-free weanling rats or from convalescent RVLA-immune rats for 1 h at 37°C. This mixture was then added to the biotin-labeled chicken anti-RVLA antibody, and the inhibition assay was completed as described above.

RESULTS

Rats. Intestinal washings collected from rats 1 day after RVLA inoculation inhibited the binding of the biotin-conjugated chicken anti-RVLA antibody to the solid-phase RVLA antigens by 76 to 100%. Intestinal washes collected from control animals inhibited the binding by an average of 3.2% (Fig. 2). Homogenized intestinal tissue obtained from the same animals was also assayed for RVLA antigen by this enzyme immunoassay. Homogenates obtained from virus-inoculated animals produced 82 to 100% inhibition, while those obtained from control animals resulted in no inhibition (Fig. 2).

The specificity of the inhibition enzyme immunoassay was further assessed by blocking the inhibition with specific immune sera. The inhibitory activity of a pooled sample of rat RVLA-containing intestinal washings was 70.5% in the presence of sera from RVLA-free weanling rats and 0 in the presence of sera from RVLA-free weanling rats.

![FIG. 2. Enzyme immunoassay inhibition assay for rat RVLA antigens in the intestinal washings (●) and homogenates (□) obtained from control or RVLA-inoculated suckling rats.](http://jcm.asm.org/)

The sensitivity of the assay was assessed by adding serial twofold dilutions of RVLA-free or RVLA-containing intestinal washings collected 1 day after inoculation to the biotin-labeled chicken anti-RVLA antibody. The 50% inhibition level was obtained with the intestinal washings diluted approximately 1:5,100 (Fig. 3).

Humans. Of the 27 specimens obtained from diarrheic humans, 6 produced signs typical of RVLA infection after inoculation into suckling rats. All of the specimens obtained from diarrheic and nondiarrheic humans were analyzed by the enzyme inhibition immunoassay developed in rats for RVLA antigen detection. Specimens that induced diarrhea in suckling rats inhibited the binding of biotin-labeled chicken anti-RVLA antibody to solid-phase RVLA antigens by 96, 89, 78, 66, 38, and 22% (Fig. 4).

Two fecal samples obtained from diarrheic humans produced greater than 50% inhibition in the enzyme immunoassay but did not cause diarrhea in suckling rats (Fig. 4). The rats were examined for clinical signs of disease only and not for RVLA infection. Incubation of these fecal samples with sera obtained from convalescent RVLA-immune rats before the addition of the biotin-labeled chicken anti-rat RVLA antibody completely blocked the inhibitory activity when compared with the samples incubated with sera obtained from nonimmune rats. The two rat inoculation-negative samples that produced the next highest degree (30 and 37%) of inhibition were also analyzed in this manner. Sera obtained from convalescent RVLA immune rats did not block the inhibition in either instance. It was thus determined that these and all of the other rat inoculation-negative samples producing lesser degrees of inhibition were negative for rat RVLA antigen by enzyme immunoassay. The average inhibition of these samples was 9.2%, with a standard deviation of 13.4%. Samples producing inhibition 3 standard deviations above the mean were declared to be positive for rat RVLA; thus, a value of 49.4% was the cutoff point in separating rat RVLA antigen-positive samples from RVLA antigen-negative samples.

Three samples were collected from one subject during the
course of illness. The sample collected on day 4 of illness produced diarrhea in suckling rats and resulted in 89% inhibition in the enzyme immunoassay. The other samples, collected on days 5 and 7, did not produce diarrhea in suckling rats and resulted in 1% and 0% inhibition, respectively.

Two samples containing rotavirus antigen as determined by solid-phase enzyme immunoassay and one sample containing both rotavirus and adenovirus were also tested in the enzyme immunoassay inhibition assay for rat RVLA. The former samples produced 0 and 10% inhibition, while the latter sample produced 28% inhibition. None of the three samples induced diarrhea in suckling rats. Specimens obtained from nondiarrheic humans produced no inhibition in the enzyme immunoassay (Fig. 4).

**DISCUSSION**

Four of the six human fecal specimens that induced diarrhea in suckling rats tested positive for rat RVLA antigen in the enzyme immunoassay inhibition assay developed in the present study. Virus particles were not seen in these specimens when examined by routine negative-stain electron microscopy, but were found in one of the specimens when examined by solid-phase immune electron microscopy (4a). That particular specimen produced the highest degree of inhibition in the enzyme immunoassay. It thus appears that this enzyme immunoassay is more sensitive as a diagnostic test than is routine electron microscopy. Limited studies to determine the prevalence of the antigenically distinct rotaviruses in stools obtained from diarrheic humans (4, 5, 9, 10) and calves (12) indicate that infection with these agents is uncommon. Our observations suggest that infections with RVLA can occur in humans and that a more sensitive assay, such as the enzyme immunoassay developed in the present study, might be used to more accurately determine the prevalence of the antigenically distinct rotaviruses.

Two human fecal specimens that produced greater than 50% inhibition in the enzyme immunoassay failed to induce diarrhea in suckling rats. The addition of rat RVLA immune serum reduced the inhibitory activity of these fecal specimens, while sera obtained from normal rats did not. At least three explanations could account for these observations. First, the individuals may have been infected with an atypical rotavirus or another virus that was serologically related to rat RVLA but was incapable of inducing disease in suckling rats. Antigenically distinct rotaviruses have been identified in feces obtained from diarrheic humans (4, 5, 9, 10, 13); however, the serologic relationships among these viruses have not been determined. Second, although rat RVLA antigens were present, the quantity of viable virus may have been insufficient to produce disease. A third, unlikely, possibility is that the results were false-positive reactions that for some reason were inhibited by immune but not by nonimmune rat sera.

Three fecal specimens were collected from one person during the course of illness. The first specimen, collected on day 4 of illness, showed a high level of inhibition in the enzyme immunoassay and induced diarrhea in suckling rats. The other two specimens, collected on days 5 and 7 of illness, showed essentially no inhibition and failed to cause diarrhea in suckling rats. This observation suggests that virus clearance may be very rapid; therefore, the time of sample collection may be critical for an accurate diagnosis. Delay in sample collection of just 1 to 2 days may cause negative test results in an individual with diarrhea that was, in fact, caused by rat RVLA or a related agent. Future studies should be directed at determining the kinetics of RVLA replication and clearance.

Although binding or sandwich solid-phase enzyme immunoassays involving the use of both capture and detecting antibodies may be sensitive systems for the detection of antigens (6), we have been unsuccessful in developing such assays for the detection of RVLA. This is at least partially due to the difficulty we have experienced in producing useful hyperimmune antiserum to this virus. This is in marked contrast to our experience with the typical (group A) rotaviruses, to which hyperimmune sera can be produced with relative ease. The reasons for this difficulty are unknown, but are perhaps related to a low concentration of cell-free antigen in the fecal extracts used to immunize animals for the production of hyperimmune sera. On the other hand, we were able to use available reagents to develop a sensitive assay involving the use of inhibition techniques. It is possible that the enzyme immunoassay inhibition methods outlined in this report will be useful in situations in which postinfection sera are used and highly specific hyperimmune sera are not available.

The enzyme immunoassay inhibition assay developed in the present study was effective in detecting rat RVLA or antigenically related viruses in intestinal washes, intestinal homogenates, and fecal preparations obtained from diarrheic rats and humans. The assay was easy to perform and was highly reproducible; it therefore has potential applications as both a diagnostic and a research tool. Further studies should include efforts to determine the incidence of infection with
rat RVLA or related agents in humans and animals and efforts to determine the kinetics of rat RVLA replication.

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LITERATURE CITED