Purification and Characterization of Adult Diarrhea Rotavirus: Identification of Viral Structural Proteins

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Adult diarrhea rotavirus (ADRV) is a newly identified strain of noncultivable human group B rotavirus that has been epidemic in the People's Republic of China since 1982. We have used sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western (immuno-) blot analysis to examine the viral proteins present in the outer and inner capsids of ADRV and compared these with the proteins of a group A rotavirus, SA11. EDTA treatment of double-shelled virions removed the outer capsid and resulted in the loss of three polypeptides of 64, 61, and 41, kilodaltons (kDa). Endo-β-N-acetylglucosaminidase H digestion of double-shelled virions identified the 41-kDa polypeptide as a glycoprotein. CaCl2 treatment of single-shelled particles removed the inner capsid and resulted in the loss of one polypeptide with a molecular mass of 47 kDa. The remaining core particle had two major structural proteins of 136 and 113 kDa. All of the proteins visualized on sodium dodecyl sulfate-polyacrylamide gel electrophoresis were antigenic by Western blot analysis when probed with convalescent-phase human and animal antisera. A 47-kDa polypeptide was most abundant and was strongly immunoreactive with human sera, animal sera raised against ADRV and against other group B animal rotaviruses (infectious diarrhea of infant rat virus, bovine and porcine group B rotavirus, and bovine enteric syncytial virus) and a monoclonal antibody prepared against infectious diarrhea of infant rat virus. This 47-kDa inner capsid polypeptide contains a common group B antigen and is similar to the VP6 of the group A rotaviruses. Human convalescent-phase sera also responded to a 41-kDa polypeptide of the outer capsid that seems similar to the VP7 of group A rotavirus. Other polypeptides have been given tentative designations on the basis of similarities to the control preparation of SA11, including a 136-kDa polypeptide designated VP1, a 113-kDa polypeptide designated VP2, 64- and 61-kDa polypeptides designated VP5 and VP5a, and several proteins in the 110- to 72-kDa range that may be VP3, VP4, or related proteins. The lack of cross-reactivity on Western blots between antisera to group A versus group B rotaviruses confirmed that these viruses are antigenically quite distinct.

The rotaviruses have recently been subdivided into groups that share the characteristic rotavirus morphology but can be distinguished by RNA electropherotype and by group-specific antigens (3). The group A rotaviruses are recognized as a major cause of diarrhea in children (12). The non-group A rotaviruses have been identified in a variety of animal species with diarrhea but have rarely been found in association with human disease (4, 6, 8, 19). In 1982 and 1983, epidemics of severe, dehydrating diarrhea affecting tens of thousands of adults were reported throughout the People's Republic of China and were traced to a group B rotavirus called adult diarrhea rotavirus (ADRV) (10, 11). These outbreaks have persisted in the People's Republic of China and have raised many questions concerning the origin of these strains, their relatedness to other rotaviruses, and the prospects that group B rotaviruses might become epidemic in other parts of the world. Work with human isolates of non-group A rotaviruses has been hampered by an inability to grow these viruses in cell culture (10a). Preliminary cDNA cloning experiments with group B rotaviruses were initiated, but neither the gene coding assignments nor the protein structures have been determined (16).

We have purified ADRV from human stool specimens collected during an outbreak in China and have characterized the virus, with specific emphasis on the viral proteins. Proteins present in the outer capsid, inner capsid, and core have been identified by disruption of double-shelled virus into single-shelled particles and cores. Viral proteins that react with human, animal, and monoclonal antisera have been characterized by using Western (immuno-) blot analysis, and the proteins of ADRV have been compared with those of group A rotaviruses to identify major similarities and differences.

MATERIALS AND METHODS

Rotaviruses and antisera. Fecal specimens containing ADRV were collected from patients in a single outbreak that occurred in Chengde, Hebei Province, the People's Republic of China, in 1987. Specimens that were positive by an enzyme-linked immunosorbent assay for ADRV (25) and confirmed by electron microscopy (EM) were pooled and frozen at −20°C until use. For comparison, the standard group A rotavirus SA11 (simian rotavirus, serotype 3) was
grown in MA104 cells in the presence of trypsin (1 μg/ml) by standard methods (9).

Paired serum specimens from patients with acute diarrhea caused by ADRV were collected during an outbreak in Qinhuangdao, Hebei Province, the People’s Republic of China, in 1987 (Z. Y. Fang, Q. Ye, S. Qing, M. S. Ho, T. Hung, and R. I. Glass, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 504, 1988). Paired sera to group A rotavirus were obtained from vaccinees inoculated with hensus rotavirus vaccine as part of a vaccine trial. Hyperimmune guinea pig antisera to SA11 and ADRV were prepared at the Centers for Disease Control. Hyperimmune rabbit antisera to ADRV were prepared at the Baylor College of Medicine, Houston, Tex. (17); pig antisera to porcine group B rotavirus (Ohio strain) and calf antisera to bovine group B rotavirus (NB strain) were prepared at The Ohio State University, Wooster, Ohio (21, 22); and rat antibody to the group B rotavirus associated with IDIR (7), guinea pig antisera to bovine enteric syncytial virus (24), and monoclonal antibodies (MAbs) to IDIR virus 1553 (26) were prepared at the Johns Hopkins Hospital, Baltimore, Md. MAbs to ADRV were provided by Wei-Wei Ye (Institute of Virology, Beijing, People’s Republic of China) and John Burns (5).

**Virus purification.** Group A rotavirus was purified from infected MA104 cells by previously described methods (14). In brief, rotavirus was released from the cells after three cycles of freezing and thawing followed by sonication for 1 min and then mixed with an equal volume of trichlorofluoroethane (Genetron). The cell lysate was homogenized by shaking, and the phases were separated by centrifugation at 1,100 × g for 10 min. The organic phase was reextracted with an equal volume of Tris-buffered saline (TBS), pH 7.2 (20 mM Tris hydrochloride, 140 mM NaCl, 5 mM KCl, 0.4 mM Na₂HPO₄, 6 mM glucose, 0.5 mM MgCl₂, and 5 mM CaCl₂). The aqueous phases were combined, layered onto 1 ml of 30% (wt/vol) sucrose in TBS, and centrifuged in an SW40 rotor (38,000 rpm, 2 h, 4°C). The virus pellet was suspended in TBS by sonication. CsCl was added to a density of 1.37 g/ml and the virus particles were centrifuged to isopycnic equilibrium in an SW50.1 rotor (35,000 rpm, 18 h, 4°C). Visible virus bands were collected by side puncture, deproteinized with 1% (wt/vol) sodium dodecyl sulfate (SDS), and examined in a Beckman DU-6 spectrophotometer.

ADRV was purified directly from infected stool specimens diluted 1:5 with TBS. After the addition of Zwittergent 3-14 (Calbiochem-Behring) to 0.5% and extraction with Genetron, virus was pelleted in a type 45Ti rotor (40,000 rpm, 60 min, 4°C). The virus was suspended and centrifuged through a 25% sucrose cushion at 35,000 rpm for 100 min at 4°C in an SW40 rotor. The suspended virus was layered on top of a preformed metrizamide gradient (Accurate Chemical and Scientific) (20 to 50% [wt/vol]) in TBS and centrifuged at 45,000 rpm for 7 h (4°C) in an SW50.1 rotor. Bands containing virus were collected and desalted with a Centricon 30. To obtain highly purified virions, the concentrated virus was centrifuged through a preformed sucrose gradient (20 to 40% [wt/wt]) in TBS at 20,000 rpm for 3.9 h (5°C) in an SW40 rotor. The virus band was collected as described above. Virus density was measured in CsCl, with suspended virus spun to equilibrium in a density gradient (1.37 g/ml) at 4°C as described for group A rotavirus.

**Treatment with EDTA and CaCl₂.** Intact, purified double-shelled virions were confirmed by EM. To remove the outer capsid, the virus was incubated for 20 min at 37°C with 10 mM EDTA. CsCl was added to a final density of 1.40 g/ml, and the sample was centrifuged to equilibrium at 35,000 rpm for 18 h (4°C) in an SW50.1 rotor to yield purified single-shelled virus particles. The successful isolation of single-shelled particles was confirmed by EM. Core particles of ADRV were obtained by treating single-shelled particles with 1 M CaCl₂ for 20 min at room temperature to remove the inner capsid (1).

**Endo-β-N-acetylgalactosaminidase H digestion.** Purified virus was denatured by adding 1.75% sodium dodecyl sulfate (SDS) and boiling it for 2 min. This was digested with endo-β-N-acetylgalactosaminidase H (Endoglycosidase H; final concentration, 0.25 U/ml; ICN Biochemicals, Cleveland, Ohio) for 1.5 h at 37°C. The reaction was stopped by adding an equal volume of 2× sample buffer and boiling it for 2 min before analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

**PAGE.** SDS-PAGE was performed using 12% polyacrylamide slab gels (0.75 mm thick) and Laemmli discontinuous buffer system (13, 14). Electrophoresis was performed for 5 h at 15 to 20 mA per gel. The resulting gels were stained with Coomassie brilliant blue (0.25%). Samples were suspended in sample buffer (5 mM Tris hydrochloride, pH 6.8, 8% glycerol, 1% SDS, 0.5 M urea, 1% β-mercaptoethanol, 0.01% phenol red) and boiled for 2 min before being loaded onto gels.

Molecular weights were estimated by comparing the viral polypeptides with the following standard reference proteins: *Escherichia coli* β-galactosidase (116,250), rabbit muscle phosphorylase B (94,400), bovine serum albumin (66,200), hen ovalbumin (42,699), bovine carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500) (Bio-Rad Laboratories).

**Western blotting.** Viral polypeptides were separated by SDS-PAGE as described above, but no urea was used in the electrophoretic buffer. The polypeptides were electrotransferred onto a nitrocellulose membrane in Tris-glycine buffer containing 20% methanol for 2.5 h at 65 V (23). The resulting blots were incubated for 2 h at room temperature with antisera in washing buffer (0.01 M phosphate-buffered saline containing 0.3% Tween 20 and 0.05% nonfat milk) and then washed four times with washing buffer. The blots were blocked by incubation with 5% nonfat milk in phosphate-buffered saline and washed. Bound antibodies were detected by using a rabbit antibody prepared against human immunoglobulin G that was conjugated with horseradish peroxidase (Dako). The reactivity of animal antisera prepared against a variety of different animal rotaviruses was examined, and antibodies were detected by using species-specific peroxidase conjugates (goat immunoglobulin to guinea pig [Dako], rabbit immunoglobulin to swine [Accurate], goat immunoglobulin to rat [Accurate], goat immunoglobulin to bovine [Accurate], swine immunoglobulin to rabbit [Dako], and goat immunoglobulin to mouse [Dako]). After being washed, the blot was developed for 10 min with 0.05% diaminobenzidine in phosphate-buffered saline containing 0.1% of 3% H₂O₂ and washed with H₂O. Biotinylated molecular weight markers were provided by R. Combs of the Centers for Disease Control.

**EM.** During each step of purification, virus was monitored by EM. Samples were absorbed for 15 s on carbon-coated grids, stained with 2% phosphotungstic acid (pH 4.6) for 15 s, and examined in a Phillips 300 electron microscope (18).
RESULTS

Characterization of purified virions. ADRV has the same distinctive morphology as group A rotavirus, with a double-shelled capsid and spikes arranged in wheel-like fashion (Fig. 1). The complete particle measures ~70 nm in diameter, single-shelled viral particles purified in CsCl gradient after EDTA treatment are ~60 nm in diameter, and viral cores prepared by CaCl₂ treatment of single-shelled particles have a hexagonal outline with a diameter of ~47 nm.

By isopycnic centrifugation in CsCl, complete viral particles banded at a density of 1.373 g/ml and single-shelled particles banded at 1.435 g/ml (Fig. 2). Freshly prepared double-shelled virus had a maximum UV absorption at 259 nm and a minimum UV absorption at 247 nm, and single-shelled particles had a maximum UV absorption at 259 nm and a minimum UV absorption at 244 nm (Fig. 3). These values are identical to the values observed with the group A rotavirus. The ratio of UV absorbance at 260 nm to that at 280 nm (A₂₆₀/A₂₈₀), an indicator of RNA content, was ~1.43 for double-shelled particles and ~1.49 for single-shelled particles.

ADRV structural polypeptides. The structural polypeptides of ADRV were examined by SDS-PAGE, and their molecular weights were estimated by direct comparison with reference markers and with group A rotavirus controls electrophoresed in companion lanes (Fig. 4). Seven distinct polypeptide bands were visualized; molecular masses were approximately 136, 113, 84, 64, 61, 47, and 41 kilodaltons (kDa). Comparing the sizes and staining intensities of these proteins with those of the group A rotaviruses suggests that the polypeptides of 136, 113, 64, 47, and 41 kDa may be comparable with VP1, VP2, VP5, VP6, and VP7, respectively. Several polypeptides smaller than VP7 were visualized in the double-shelled group A rotavirus, of which only one appeared in the single-shelled particle. The 28-kDa polypeptide reacted strongly with convalescent sera and may represent VP8 (Fig. 5). Others may represent degradation products since they show a marginal response to convalescent sera.

Removal of the outer capsid of ADRV by EDTA treatment
was associated with the loss of three polypeptides with molecular masses of 64, 61, and 41 kDa (Fig. 4). Similarly, EDTA treatment of group A rotavirus resulted in the loss of three proteins, VP5, VP5a, and VP7. Removal of the inner capsid of ADRV by CaCl₂ treatment was associated with the loss of one polypeptide with a molecular mass of 47 kDa. Two polypeptides of 136 and 113 kDa remained in the viral cores. Some protein bands were located within the VP3 and VP4 range, but their identities were unclear. Endoglycosidase treatment of group B rotavirus resulted in the loss of one polypeptide with a molecular mass of 47 kDa. Positive control (lanes P) was a human convalescent-phase serum with 10- to 20-fold-higher titer than paired convalescent-phase sera (lane C). Human sera to group A rotavirus (group A lanes) was obtained from the recipient of rhesus rotavirus vaccine. Guinea pig sera were obtained pre- and postimmunization with SA11. Human sera to ADRV (group B lanes) were obtained from two patients recovering from recent infection. Rabbit sera were obtained from The Baylor College of Medicine, Houston, Tex. (see Materials and Methods.)
demonstrated that viruses and determined the ratio of convalescent-phase sera. Immuno-blotting was performed as described in the legend to Fig. 5. The dilutions of antisera are shown in each lane.

No cross-reactivity could be demonstrated with antisera against group A rotavirus tested by Western blot against group B proteins or antisera against ADRV tested against group A proteins (data not shown).

To determine the titer of convalescent-phase serum of one patient was determined to endpoint (Fig. 7). Antibody to the 47-kDa protein was present in highest titer (1:1,000), antibody to the 113-kDa protein was present in an intermediate titer (1:200), and antibodies to the 136-, 64- and 41-kDa proteins were present at the starting dilution (1:100).

Antisera raised against a variety of animal group B rotaviruses and tested in 1:200 dilution against human ADRV demonstrated strong reactivity to the 47-kDa polypeptide and some reactivity to the 113-kDa polypeptide (Fig. 8). Guinea pig antisera raised against ADRV, however, reacted strongly with all the viral polypeptides. None of the four MAbs prepared against ADRV were reactive to ADRV in Western blot analysis. However, the MAb prepared against IDIR demonstrated a strong reaction to the 47-kDa polypeptide.
DISCUSSION

The major polypeptides of ADRV migrate in a pattern generally comparable with that of group A rotavirus (15) and with that of the recently described group C rotavirus (2), although the apparent molecular weights of each polypeptide are distinct. Treatment of double-shelled particles with EDTA followed by CaCl₂ led to degradation of the complete virus into single-shelled particles and cores that were identified by EM. Comparison of the proteins of double, single, and core particles by SDS-PAGE analysis indicated that the outer capsid was composed of polypeptides of 64, 61, and 41 kDa and the inner capsid was composed of polypeptides of 47 kDa. On the basis of a comparison with the polypeptides of SA11, we have tentatively designated these polypeptides VP5, VP5a, and VP7, and VP6, respectively. The 41-kDa polypeptide is a glycoprotein which corresponds to VP7 of group A rotavirus. The 47-kDa polypeptide is recognized by antisera prepared against a variety of human and animal strains of group B rotavirus and appears to be common to all of them (20). It is also recognized strongly by the MAb prepared to IDIR, suggesting that this may be a particularly good diagnostic reagent. This 47-kDa polypeptide is most strongly antigenic in human convalescent-phase sera, perhaps because of its relative abundance in the virus. It is not removed by EDTA treatment of double-shelled virus but is removed by CaCl₂ treatment of single-shelled particles.

Other polypeptides of ADRV located in viral cores have been identified, assigned approximate molecular sizes, and given a tentative protein designation on the basis of the control preparation of SA11. These include the 136-kDa polypeptide, proposed to be VP1, a 113-kDa structural polypeptide, proposed to be VP2, and several proteins within the range of 100 to 72 kDa which may be VP3, VP4, or related products.

As determined by Western blot analysis, all the viral proteins are antigenic when tested with high-titer sera (1: 100). As the titer of convalescent-phase sera rose, antibodies to the 47-kDa polypeptide appeared at highest titer, with antibodies to the 113-, 64-, and 41-kDa polypeptides present at intermediate titers. The ratio of the antibody response to the 47- and 41-kDa polypeptides differed markedly between patients, suggesting a parallel with the dissociation of antibody responses to group A rotavirus between inner-capsid antigen tested by enzyme-linked immunosorbent assay and outer capsid antigen tested as a neutralization response.

The lack of cross-reactivity between antisera to group A and group B rotaviruses has been reported previously in studies using immunoelectron microscopy, immunofluorescence, and enzyme-linked immunosorbent assays (17, 20, 21). These studies suggest that these two viruses, though in the same family, are antigenically quite distinct. Our data extend these observations by examining individual proteins by Western blot. Initially, ADRV was hypothesized to have emerged by simple reassortment of human group A rotavirus with animal strains of group B rotavirus. The lack of cross-reactivity to the individual proteins suggests that major antigenic differences that we observed reflect a greater diversity of the polypeptides.

Efforts at understanding the biology and the origin of the non-group A rotaviruses, considerations of immunogenicity and control with vaccines, and improved diagnosis of this noncultivable agent will require identification of the genes encoding the inner and outer capsid proteins. This study has identified the structural proteins of these capsid structures and provides a basis for determining the gene-coding assignments for these proteins.

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LITERATURE CITED