Human Enteric Coronaviruses: Further Characterization and Immunoblotting of Viral Proteins

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for giardiasis. Further studies on larger numbers of patients will, however, be necessary to determine the course of time for developing this antibody and its persistence in the convalescent period.

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Human Enteric Coronaviruses: Further Characterization and Immunoblotting of Viral Proteins

Strains of coronavirus (CV) can be responsible for acute diarrhea in different animal species [1]. The enteropathogenic role of CVs in humans, however, has not been definitely established. Evidence for association of CV infection and gastroenteritis in man has been obtained mainly by using electron microscopy to detect CV-like particles in the feces of patients with diarrhea [2-4]. Because of the extreme difficulty with isolation and propagation in tissue culture, the candidate human enteric CVs (HECVs) have not been adequately characterized. Recently, we reported the possible implication of HECV strains in acute infantile gastroenteritis [5]. By immune electron microscopy (IEM) these strains appeared to be antigenically related to human coronavirus (HCV) OC43, a known respiratory pathogenic virus, but further characterization of the HECV strains was hampered by failure to isolate and propagate them in tissue culture. Beards et al. [6] described other fringed pleomorphic viral particles detected by electron microscopy in stools from children and adults with diarrhea. These viral particles were morphologically more similar to toroviruses of the Berne-Breda group (to which they were shown by IEM to be antigenically related) than to typical CVs. In this report, we attempted further characterization of two HECV strains purified from stools

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of two infants with acute gastroenteritis. Although the amounts of virus were low, we could determine the pattern of migration of their polypeptides in SDS-PAGE and examine the antibody response to the viral proteins electrophoretically transferred to nitrocellulose after SDS-PAGE. In addition, we investigated by IEM the antigenic relation between the HECV strains and viruses of the Berne-Breda group.

Materials and Methods

Viruses and antisera. Two strains of HECV, HECV-24 and HECV-35 [5], were purified from stools of two infants with acute gastroenteritis by ultracentrifugation in sucrose density gradients by using the procedure described for bovine CV by Sharpee et al. [7]. The preparation of antisera to HECV, propagation and assay of HCV OC43, and the preparation of antisera to HCV OC43 have been reported previously [5, 8]. Berne virus, strain P138/72, and rabbit antiserum were kindly provided by Dr. Marianne Weiss, Institute of Bacteriology, Veterinary Faculty, University of Berne, Berne, Switzerland. Berne virus was propagated in the E. Derm (NBL-6) cell line (American Type Culture Collection, Rockville, Md) essentially as reported by Weiss et al. [9] for Berne propagation in embryonic mule skin cells. Stools and convalescent sera from gnotobiotic calves infected with Breda type 1 and Breda type 2 viruses [10] were obtained from Dr. G. N. Woode, College of Veterinary Medicine, Iowa State University, Ames, Iowa.

SDS-PAGE and immunoblotting. SDS-PAGE in reducing conditions was performed as described by Laemmli [11] by using 10% polyacrylamide gel slabs 1.5 mm thick. Samples were electrophoresed in a water-cooled Protean II vertical cell (BioRad Laboratories, Richmond, Calif) for ~1 hr at a constant current of 25 mA/gel and then at 35 mA/gel for ~2 hr (until the bromphenol blue tracking dye reached 2–3 mm from the bottom of gel). High- and low-molecular-weight markers (BioRad Laboratories) were included in each gel run. Proteins were then either stained with ammonia silver or transversely electrophoresed onto nitrocellulose paper (0.2-μm pore size, BA83; Schleicher & Schuell, Dassel, West Germany) in a Tris-glycine-methanol buffer containing 0.1% (wt/vol) SDS. Electrophoretic transfer was done at a constant voltage of 3 V/cm for 20 hr in a water-cooled Transblot® cell (BioRad Laboratories). Under these conditions, proteins <200 kilodaltons (kDa) were transferred at a very high efficiency, as determined by silver staining of portions of the same gels before and after transfer and by staining of nitrocellulose blots with india ink or with colloidal gold (Aurodye, Janssen Pharmaceutica, Beerse, Belgium). The immunological detection of electrophoretically transferred proteins was performed by indirect immunoperoxidase, essentially as reported for dot-immunobinding by Hawkes et al. [12]. Test sera were examined at 1:50 and 1:100 dilutions. The working dilutions of conjugates (Cappel Laboratories, Cooper Biomedical, Malvern, Pa) were determined by checkerboard titration against serial dilutions of IgG of the appropriate animal species in a dot-immunobinding procedure [12]. The relative molecular weights of immunochemically detected proteins were determined by comparison of migration with molecular weight markers visualized on blots by india ink and colloidal gold.

Immune electron microscopy. IEM was performed, following the procedures reported previously for antigenic characterization of HECV by IEM [5], on the following: (*1*) HECV-24 and HECV-35 purified from stools; (*2*) crude harvests of cell culture–adapted HCV OC43 and Berne virus; and (*3*) fecal extracts from gnotobiotic calves infected with Breda 1 and Breda 2 viruses.

Results

Polypeptides of HECV strains. Silver staining of purified HECV-24 and HECV-35 viruses disrupted and run in SDS-PAGE slabs showed at least four major proteins, with apparent molecular weights of ~62 kDa, 60 kDa, 34 kDa, and 32 kDa. In addition to the four major proteins, minor protein bands of 110 kDa, 97 kDa, 80 kDa, 77 kDa, and 56 kDa were detected. The pattern of polypeptide migration of HECV-35 by SDS-PAGE was indistinguishable from that of HECV-24 (data not shown).

Antibody response to HECV by immunoblotting. Mouse antiserum to HCV OC43 was unreactive with HECV polypeptides separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose. Guinea pig antisera to HECV strains immunochemically stained mainly two proteins of apparent molecular masses of 62 kDa and 56 kDa. Minor bands of 110 kDa, 97 kDa, 80 kDa, and 77 kDa were also stained, although weakly. Preimmunization sera were unreactive. Acute-phase serum from a patient with acute gastroenteritis, whose stools were shown to contain large amounts of HECV particles, reacted weakly with 62-kDa band; convalescent serum from the same patient showed a stronger reaction with the same 62-kDa band and a very weak reaction with the 56-kDa band. The immunological reactivities of polypeptides from the two HECV strains were indistinguishable from each other. Rabbit Berne antiserum and convalescent sera from gnotobiotic calves infected with Breda 1 and Breda 2 viruses did not react at all with polypeptides of either HECV strain. Rabbit Berne antiserum was also examined by immunoblotting with homologous virus, and it detected Berne proteins of ~39 kDa, 20.5 kDa, and 18.5 kDa. We did not detect antibody reactivity to Berne polypeptides in mouse antiserum to HCV OC43 or in guinea pig antisera to HECV-24 and HECV-35.

IEM reactivity of coronavirus and torovirus strains.

Results obtained by IEM by using animal antisera and human convalescent-phase sera are summarized in table 1. Mouse antiserum to HCV OC43 reacted very strongly with HCV OC43 and moderately with HECV-24 and HECV-35, but it did not react with Berne, Breda 1, or Breda 2 virus. Antisera to HECV-24 and HECV-35 reacted strongly with both HECV strains and weakly to moderately with HCV OC43, but they did not react with Berne, Breda 1, or Breda 2. The IEM reactivity of HCV OC43 and HECV with human convalescent-phase sera was similar to that with the corresponding animal antisera. Rabbit Berne antiserum reacted strongly with Berne and weakly with Breda 1 and Breda 2, but it did not react with any of the CV strains tested. Conversely, Breda calf immune sera reacted strongly with both Breda serotypes and weakly to moderately with Berne, but they were unreactive with HCV OC43, HECV-24, and HECV-35.

Discussion

In this paper we report partial characterization of polypeptides of two strains of HECV purified from stools of two patients with gastroenteritis. As previously reported, these agents possess a typical coronavirus morphology and cannot be successfully isolated and propagated in cell cultures [5]. The HRT 18 cell line [13] was partially susceptible to HECV, but only at low population-doubling levels, and we could not serially propagate strains of HECV. Although the amount of virus purified from stools was low, we could separate HECV proteins by SDS-PAGE, detect them by the silver staining technique, and electrophoretically transfer them onto nitrocellulose supports for determination of antibody. The electrophoretic patterns of HECV-24 and HECV-35 (four major proteins of 62 kDa, 60 kDa, 34 kDa, and 32 kDa) closely resemble those of the HECV strains recently reported by Resta et al. [14], who isolated them from stools of two infants with necrotizing enterocolitis and propagated them in organ cultures of human fetal intestine. These strains appeared to be antigenically distinct from known human and animal CVs as well as from Breda 1 and 2 viruses, as determined by single radial hemolysis and by enzyme-linked immunosorbent assay [14].

Antibody reactivity to HECV polypeptides separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose could be demonstrated in HECV antisera and in convalescent serum from a patient with acute gastroenteritis excreting HECV particles in feces during the acute phase of illness. Sera specifically reacted with proteins of 62 kDa and 56 kDa and gave immunoblotting patterns very similar to those reported by Resta et al. [14] for their HECV strains. No antigenic relatedness to HCV OC43, Berne, or Breda serotype 1 or 2 could be detected by immunoblotting. Although rabbit Berne antiserum immunochemically stained in homologous immunoblots bands of 39 kDa, 20.5 kDa, and 18.5 kDa, probably corresponding to the Berne envelope, nucleocapsid, and a nucleocapside cleavage product, respectively [15], Berne proteins were not detectable by HCV OC43 antiserum or HECV antisera.

By using IEM, we again confirmed the antigenic relatedness of HECV to OC43 [5]. The two HECV strains were antigenically distinct from viruses of the Berne-Breda group and indistinguishable from each other. Thus, these HECV strains are probably different from the Breda-like enveloped viral particles found by Beards et al. [6] in stools from children and adults with diarrhea. The immunoblotting results are in contrast with the antigenic relation between HECV and OC43 detected by IEM. It seems reasonable, however, that denaturation of HECV proteins in the presence of dissociating and reducing agents could have destroyed the antigenic determinant(s) responsible for their IEM cross-reactivity with HCV OC43. Unfortunately, the amounts of purified HECV were too scarce to allow further immunoblotting studies with unreduced viral proteins and extensive serological examinations of patients with gastroenteritis.

Table 1. IEM reactivity of strains of coronavirus and torovirus.

	IEM reactivity of					
Serum, source	HCV OC43	HECV-24	HECV-35	Berne	Breda 1	Breda 2
HCV OC43, mouse	+ + + +	+ +	+ +	−	−	−
HECV-24, guinea pig	+	+ + +	+ + +	−	−	−
HECV-35, guinea pig	+ / + +	+ + +	+ + +	−	−	−
HCV OC43, human convalescent-phase	+ + +	+ +	+ +	−	−	−
HECV, human convalescent-phase	+ +	+ + +	+ + +	−	−	−
Berne, rabbit	−	−	−	+ + +	+	+
Breda 1, calf	−	−	−	+ / + +	+ + +	+ + +
Breda 2, calf	−	−	−	+ / + +	+ + / + + +	+ + +

NOTE. The IEM reactivity (per 400 mesh grid square) was scored as follows: −, no immune aggregates; ±, <1 immune aggregate; +, 1 aggregate; + +, 2–5 aggregates; + + +, 6–10 aggregates; and + + + +, >10 aggregates.

In conclusion, HECVs appear to be antigenically distinct from viruses of the Berne-Breda group and antigenically related to HCV OC43. The preliminary characterization of HECV polypeptides appears promising and suggests that more-extensive studies on HECV should be done to obtain futher data on its biology and to further define its epidemiology and its pathological role.

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Fulminant Necrotizing Fasciitis and Nonsteroidal Anti-Inflammatory Drugs

Necrotizing fasciitis is a distinct clinical entity usually caused by *Steptococcus pyogenes*. Although accurately defined by Wilson [1] in 1952, the disease is often unrecognized. It is potentially fatal, especially when it becomes fulminant. Even though the host factors and the infectious agent responsible for initiating and spreading necrotizing fasciitis have been identified, the pathogenesis of the disease is unclear. After examining all cases of necrotizing fasciitis occurring in the intensive medical care unit of this hospital between 1983 and 1985, we found that five of seven had a fulminant evolution. This fulminant evolution apparently resulted from nonsteroidal anti-inflammatory therapy.

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Patients

We included only those patients who had necrotizing fasciitis that fulfilled strict criteria: a soft-tissue infection involving the superficial fascia and resulting in extensive undermining of surrounding tissues. The infection was considered fulminant when the delay between the first functional and local signs and surgery did not exceed six days (table 1).

All patients had previously been healthy, and none had a predisposing condition—previous surgery, chronic ve-