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Cell Culture Propagation of a Coronavirus Isolated from Cows with Winter Dysentery†

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Fecal filtrates from cows with winter dysentery were inoculated into gnotobiotic and conventional calves, and a coronavirus was isolated from calf feces. Cytopathic effects were observed on human rectal tumor cells but not bovine cell cultures. The winter dysentery isolates morphologically and antigenically resembled the Mebus strain of bovine coronavirus.

Winter dysentery (WD) is an acute diarrheal disease of adult beef and dairy cattle in the northern United States (5, 20, 24, 35) and other cattle-producing countries (1, 2, 6, 13, 15, 18, 19, 22, 23, 25, 32). Although the etiology of this disease remains undetermined, coronavirus particles have been observed by electron microscopy in feces of cattle with WD (12, 14, 18, 28, 32). Recently, coronavirus particles were identified by immune electron microscopy (IEM) in feces of cows with WD but not in feces of clinically normal cows in a closed dairy herd (30). A significant increase in antibody titers to bovine coronavirus was also detected in convalescent-phase sera from cows with WD. When fecal filtrates prepared from specimens of cows with WD were orally inoculated into gnotobiotic calves, these animals developed diarrhea and shed coronavirus in feces (L. J. Saif, D. R. Redman, K. V. Brock, R. A. Heckert, and E. M. Kohler, Abstr. 69th Annu. Meet. Conf. Res. Workers Anim. Dis., November 1988, Chicago, Ill., abstr. no. 74). Although the electron microscopy and serologic data implicate a coronavirus in the etiology of WD, the virus has not been isolated from cattle in the United States. A coronaviruslike agent has been isolated from feces of cows with WD in Japan and Belgium (2, 4), but it is not known if this agent is identical to the WD coronavirus prevalent in the United States.

In an effort to isolate the WD coronavirus, we prepared feces from a colostrum-deprived calf (C2421) and two gnotobiotic calves (C270 and C935) which had been orally inoculated with fecal filtrates from cows with WD for virus isolation as previously described (29). Filtrates of the calf feces were inoculated onto 4-day-old monolayers of primary bovine turbinate and bovine lung cells (M. L. Vickers, South Dakota State University, Brookings), Madin-Darby bovine kidney (MDBK) cells, and human rectal tumor (HRT-18) cells seeded in either six-well dishes (Costar, Cambridge, Mass.) or roller tubes (13 by 100 mm). Each cell type was washed twice prior to inoculation with a 1:25 dilution of each fecal sample. Mock-infected bovine turbinate, bovine lung, MDBK, and HRT-18 cells served as controls. Cells were maintained on serum-free media containing 2.5 μg of pancreatin (4× NF [National Formulary] [1× = 2.5 g/liter]; GIBCO Laboratories, Grand Island, N.Y.) per ml. Inoculated and control cells were observed daily for cytopathic effects (CPE), and if CPE were not evident after 7 days, a scraping of each culture was removed for immunofluorescence as previously described (3). Fluorescein-conjugated bovine anti-bovine coronavirus (Mebus strain) serum and porcine anti-transmissible gastroenteritis virus serum were used as conjugates (29, 30, 33). The prototype Mebus strain of bovine coronavirus (MBCV) was propagated in HRT-18 cells as previously described (17), except that 2.5 μg of pancreatin per ml was added to the maintenance medium.

CPE or virus antigens were not detected after three blind passages of each isolate in stationary or roller cultures of bovine cells. However, an initial passage of C270 and C2421, virus antigens were detected in the cytoplasm of HRT-18 cells 7 days postinoculation. Similar results were obtained with the C935 isolate on passage 2 in HRT-18 cells (Table 1). CPE were not observed in mock-infected HRT-18 cells (Fig. 1A), whereas CPE were usually evident in inoculated HRT-18 cells at 2 to 3 days postinoculation and consisted of granular, swollen, or enlarged cells (Fig. 1B). The membranes of the enlarged cells appeared to be fused and resembled syncytia (Fig. 1B), which are often observed in HRT-18 cells inoculated with MBCV (11, 31, 34). Usually the rounded, swollen cells detached, and as CPE progressed, focal to diffuse cytoplasmic vacuolation was prominent at 4 to 7 days postinoculation. Virus replication was confined to the cytoplasm, and virus antigens were demonstrated with the fluorescein-conjugated anti-bovine coronavirus serum but not the anti-transmissible gastroenteritis virus serum. The cytoplasmic fluorescence consisted of granular or globular areas of virus antigen in the early stages of infection (Fig. 2) and progressed to a diffuse distribution of antigen throughout the cytoplasm late in infection. The CPE and immunofluorescence staining patterns of the three WD isolates were identical to those obtained with MBCV.

Infectivity and hemagglutination titers were determined as previously described (2, 8, 11, 31, 34), and the lysates were also examined by IEM with a bovine anti-bovine coronavirus serum (26). Results of the infectivity and hemagglutination assays are summarized in Table 1 for WD isolate C935. Infectivity titers ranged from 316,000 to 15,000,000 and were generally higher than titers obtained with MBCV. Similar infectivity titers were obtained with isolates C270 and C2421. However, these two WD isolates did not hemagglutinate either mouse or rat erythrocytes, while hemagglutination titers obtained for C935 and MBCV were similar (Table 1). Although the C270 and C2421 WD isolates did not

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TABLE 1. Adaptation of WD virus (C935) to HRT-18 cells and infectivity, hemagglutination, and viral neutralization titers[a]

Fecal sample	Passage on HRT-18 cells	% of monolayer with CPE	% Fluorescent cells[b]	Infectivity titer (log_{10} $TCID_{50}$)[c]	Hemagglutination titer to[d]:		Viral neutralization titer of B173[e]
					Mouse erythrocytes	Rat erythrocytes	
C935	1	0	0	ND[f]	ND	ND	ND
	2	25	60	5.5	<2	<2	640
	3	50	>90	6.5	64	64	ND
	4	80	>90	6.5	64	128	ND
	5	80	>90	6.5	32	64	ND
	6	80	>90	7.2	ND	ND	ND
MBCV	9	60	>90	5.8	ND	256	5,120
	10	60	>90	4.2	16	128	ND

[a] Results obtained with isolates C270 and C2421 were similar to those obtained with isolate C395, except for the absence of hemagglutination.
[b] Percentage of cells in a scraping containing virus antigens detected by immunofluorescence.
[c] $TCID_{50}$, 50% tissue culture infective dose, determined by the method of Spearman and Karber (8).
[d] Expressed as the reciprocal of the highest dilution of virus causing complete hemagglutination of a 1% suspension of mouse or rat erythrocytes.
[e] Expressed as the reciprocal of the highest dilution of serum causing complete neutralization of the virus. B173 is a hyperimmune serum prepared in gnotobiotic calves to the Mebus cell culture-adapted strain of bovine coronavirus. The viral neutralization titers of B173 for C270 and C2421 were 320 and 160, respectively.
[f] ND, Not done.

hemagglutinate, we demonstrated hemadsorption of rat erythrocytes by each of the three WD isolates and MBCV by a previously described method (34). Aggregates of 10 to 20 virus particles uniformly coated with antibody were observed on IEM with lysates from inoculated but not uninoculated HRT-18 cells (Fig. 3). The virus particles were pleomorphic, were 80 to 120 nm in diameter, and had surface projections typical of coronaviruses.

In an effort to determine the antigenic relatedness of the WD isolates to MBCV, we mixed twofold serial dilutions of a hyperimmune antiserum (B173; courtesy of K. Theil, Ohio Agricultural Research and Development Center, Wooster) beginning at 1:10 with an equal volume of 100 to 300 50% tissue culture infective doses of each WD isolate or MBCV per 100 μl (2, 8, 11, 34). After 5 days, the neutralization titer was expressed as the reciprocal of the highest dilution of serum which prevented CPE in all inoculated wells. An 8- to 32-fold difference in neutralization titers was obtained between the WD coronaviruses and MBCV (Table 1).

In the present study, a coronavirus was isolated from each of three calves inoculated with fecal filtrates from cows with WD. All isolates were coronaviruses, as determined by the similarity of CPE to those produced by MBCV, morphology on electron microscopy, agglutination of virus particles observed by IEM with antiserum to MBCV, detection of virus antigens in the cytoplasm of inoculated HRT-18 cells, and neutralization of the WD isolates by antiserum to MBCV. Our results extend and concur with those of previous reports from Japan describing the isolation of a coronavirus (Kakegawa strain) from a cow with a clinical disease resembling WD (2, 32). The successful isolation of coronaviruses from cases of WD in the present study may be related to three factors. First, either the passage of feces from clinically ill cows in calves resulted in amplification of

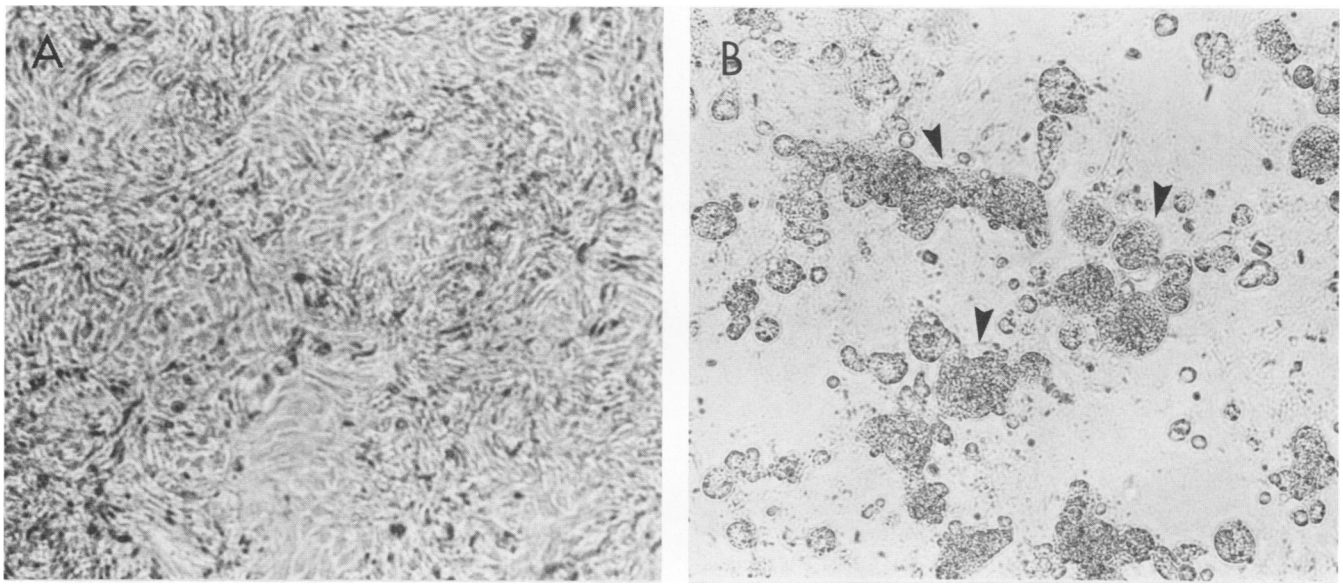

FIG. 1. (A) Uninoculated monolayer of HRT-18 cells 4 days after seeding. (B) CPE at 2 days postinoculation induced by the C935 isolate of WD virus on passage 3 in HRT-18 cells. The CPE were characterized by enlarged, rounded, and densely granular cells that occurred singly or in clusters (arrowheads). Magnification, ×240.

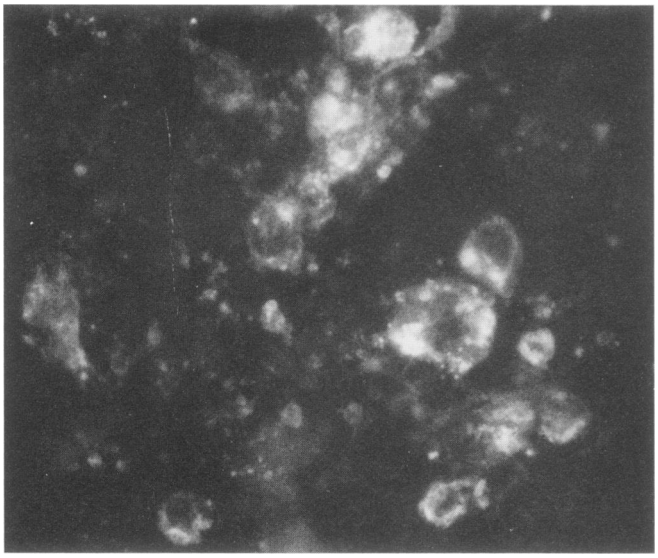

FIG. 2. Immunofluorescence of HRT-18 cells 4 days after inoculation with the C935 isolate of WD virus. The fluorescence was restricted to the cytoplasm. Cells were stained with fluorescein-conjugated bovine anti-bovine coronavirus (Mebus strain) serum. Magnification, ×600.

the virus, providing a pool of high-titered material for isolation, or coronavirus-antibody complexes reported to exist in the feces of cows (9, 10) were dissociated upon passage through the intestinal tracts of inoculated calves.

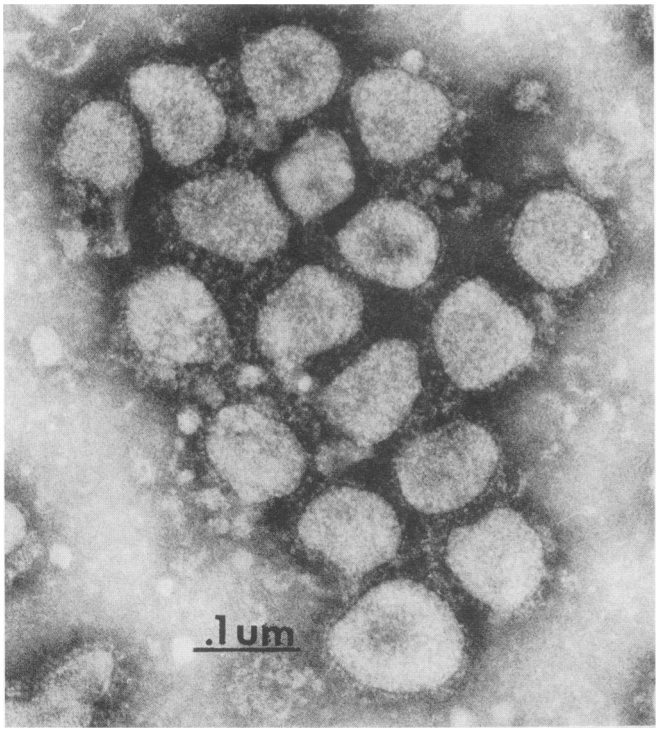

FIG. 3. Electron micrograph of IEM of the C2421 isolate (passage 3 on HRT-18 cells) of WD virus and a hyperimmune bovine anti-bovine coronavirus (Mebus strain) serum. Aggregates typically contained 10 to 20 virions. Phosphotungstic acid negative staining. Magnification, ×130,500. um, Micrometer.

Second, HRT-18 cells, which are permissive for several coronaviruses (17), were the only cells permissive for the WD coronaviruses. Although we could not demonstrate the presence of virus or virus antigens in bovine turbinate, bovine lung, or MDBK cells, others have cultivated cell culture-adapted MBCV in bovine lung (34) and MDBK (11, 27) cells. However, bovine cells have not always proved convenient and satisfactory for the primary isolation of bovine coronaviruses from feces or intestinal contents (11), and HRT-18 cells may prove to be more sensitive for the initial isolation of these viruses from field cases. Finally, proteolytic enzymes (trypsin, chymotrypsin, and pancreatin) enhance the replication of several coronaviruses, including bovine coronavirus (11, 16, 21, 27, 31, 34). The formation of polykaryons, a common CPE of coronaviruses (16, 31, 34), is dependent on the presence of proteolytic enzymes in the medium. We did not determine if the presence of proteolytic enzymes in the medium was an absolute requirement for the propagation of the three WD isolates described in this study, but the absence of pancreatin in the medium resulted in a reduction in the visible CPE (data not shown).

While the virus neutralization data suggest possible antigenic differences between the WD isolates and bovine coronavirus, further studies with two-way cross-neutralization assays and plaque-purified viruses will have to be done to confirm these differences. Our neutralization results are in contrast with those of previous reports (2, 4) which indicated that the Kakegawa and Belgium strains of WD are antigenically identical to MBCV. Also, only one of the three WD isolates consistently hemagglutinated mouse and rat erythrocytes, a characteristic common to the Mebus and Kakegawa strains of bovine coronaviruses. Perhaps not all isolates of bovine coronavirus hemagglutinate or perhaps hemagglutination is a transient event with the WD isolates as compared with bovine coronavirus.

Our results represent the first report of the successful isolation of a coronavirus from cows with WD. While normal cattle shed coronavirus or coronavirus antigens in feces (7, 9, 10), our samples originated from a herd in which cows with clinical signs of WD shed coronaviruses, while clinically normal cows did not (28). A coronavirus etiology of WD would explain the explosive nature of this disease among confined cattle in the winter months, since coronaviruses are readily disseminated in winter, when lower temperatures and UV light intensity result in increased stability of the viruses in the environment.

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