Animal Science Journal (2006) **77,** 253–257 doi: 10.1111/j.1740-0929.2006.00346.x

RAPID COMMUNICATION

Propagation of bovine coronavirus in clones of the Caco-2 cell line showing different levels of alkaline phosphatase activity

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ABSTRACT

The aim of the present study was to determine whether bovine coronavirus (BCV) has the ability to initiate infection in a human colon carcinoma cell line, Caco-2, that has been established to spontaneously differentiate after confluence. When Caco-2 cells were infected with BCV, a titer of 5.5×10^6 plaque-forming units (p.f.u.)/mL was found in the culture supernatant at 5 days postinfection. Two clones, Caco-2/CA1 and Caco-2/CA2, were then isolated by monitoring alkaline phosphatase (ALP) and cell proliferation activities. The ALP activity level of CA1 cells was significantly higher than that of CA2 cells, while the level of cell proliferation activity of CA1 was significantly lower than that of CA2. When CA1 and CA2 cells were infected with BCV at confluence, virus hemagglutination (HA) was detected in the culture supernatant at 5 days postinfection for CA1 cells and at 8 days postinfection for CA2 cells. Thus, BCV propagation was substantially delayed in CA2 cells, suggesting that a cellular factor(s) that appears at the differentiation stage may control BCV propagation. BCV-susceptible CA1 and CA2 cells showing different levels of ALP activity would be useful for further experiments to elucidate the mechanism of BCV propagation.

Key words: *alkaline phosphatase, bovine coronavirus, Caco-2, differentiation.*

INTRODUCTION

Bovine coronavirus (BCV), a member of the family *Cronaviridae*, is an enveloped virus containing a helical nucleocapsid structure composed of a single-stranded, positive-polarity RNA of approximately 32 kb plus poly(A) tail in length. BCV causes respiratory and enteric diseases in cattle (Cho *et al*. 2000; Niskanen *et al*. 2002). The enteric BCV attacks intestinal epithelial cells, resulting in diarrhea and in economic losses due to reduction in production of milk and lost weight (Tråvén *et al*. 2001). A high rate of BCV isolation from nasal specimens of feedlot cattle with signs of respiratory tract disease has also been reported (Lin *et al*. 2000).

The intestinal epithelium, which is functionally divided into a zone of proliferation confined to crypts and a zone of differentiation situated in the villi, undergoes continuous renewal throughout the lifespan of an animal. The terminally differentiated cells of the epithelium are removed by a process of apoptosis occurring throughout the villus. Thus, the renewal process of the intestinal epithelium occurs along the crypt–villus axis (Hall *et al*. 1994). It has been reported that propagation of some viruses that cause enteropathy depends on the stage of differentiation of intestinal epithelial cells (Jarvis *et al*. 1999; Ciarlet *et al*. 2001). However, it is still unclear whether BCV propagation depends on the stage of differentiation of the intestinal epithelium.

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Received 1 September 2005; accepted for publication 28 November 2005.

Caco-2 cells, derived from human colon carcinoma, differentiate spontaneously in an *in vitro* culture system (Fogh *et al*. 1977; Pinto *et al*. 1983), as they do *in vivo* in the normal intestine, and exhibit many of the morphological and functional properties of enterocytes (Hidalgo *et al*. 1989). They express disaccharidases, peptidases and alkaline phosphatase (ALP) found in cells of the small intestinal villi *in vivo*, and they form domes on impermeable substrates, transport ions and water, develop tight junctions that prevent the passage of macromolecules through underlying permeable membranes, and develop transepithelial electrical resistance typical of the polarized epithelium in *in vitro* cultivations (Eaton & Simons 1995). Thus, Caco-2 cells may be suitable for study of BCV propagation in the intestinal epithelia.

The purpose of the present study was to examine whether BCV has the ability to initiate infection in differentiated Caco-2 cells. We also selected clones showing different activities of ALP and examined the infectivity of BCV to the clones.

MATERIALS AND METHODS

Cell line and virus

Caco-2 and HRT-18G cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Caco-2 cells were propagated in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 20% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS), 1% nonessential amino acids (Gibco BRL), 100 IU/mL penicillin and 100 μg/mL streptomycin. HRT-18G cells, which show high susceptibility to BCV infection (Lin *et al*. 1997), were cultivated in DMEM supplemented with 5% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin. The culture medium was exchanged every other day.

Confluent monolayers of Caco-2 and HRT-18G cells in 6-well tissue culture plates were infected with the Kakegawa strain of BCV at a multiplicity of infection of 1 for 60 min at 37°C and then cultured in DMEM supplemented with 5% FBS for Caco-2 cells or with 2% FBS for HRT-18G cells. The culture supernatant was then collected at the indicated time and titrated by a plaque assay using HRT-18G cells, and plaque-forming units (p.f.u.) were determined as described previously (Matsumoto *et al*. 2005). A hemagglutination (HA) assay was performed using mouse erythrocytes as described previously (Matsumoto *et al*. 2005).

Determination of ALP and cell proliferation activities

Caco-2 clones were selected by single-cell cloning from Caco-2 cells via limiting dilution as described by Chantret *et al*. (1994). For measurement of ALP activity, a brush-border membrane-rich fraction was prepared by the method of Pinto *et al*. (1983). Briefly, the cell pellet was homogenized at 4°C in Tris-mannitol buffer (2 mmol/L Tris, 50 mmol/L mannitol, pH 7.1) and disrupted, and then solid $CaCl_2$ was added to a final concentration of 10 mmol/L. After incubation for 10 min at 4°C, the calcium-treated homogenate was centrifuged (10 min, 950 *g*, 4°C) and then the supernatant was further centrifuged (30 min, 33 500 *g*, 4°C) to yield a small pellet containing brush-border membranes. The pellet was resuspended in distilled water, and ALP activity was measured according to the method described by Garen and Levinthal (1960) with *p*-nitrophenyl phosphate as a substrate. A protein assay was conducted with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). A cell proliferation assay was performed by using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the method described by Chen *et al*. (2005). Briefly, cells were seeded in 96-well plates and cultured in the growth medium for 48 h. Then WST-8, which is a water-soluble tetrazolium salt, was added to each well and incubated for an additional 4 h at 37°C. The relative amounts of viable cells in triplicate wells were measured as the absorbance (450 nm) of reduced WST-8.

RESULTS AND DISCUSSION

In this study, we found for the first time that Caco-2 cells are susceptible to BCV infection. As shown in Figure 1, a titer of 5.5×10^6 p.f.u./mL was found in the culture supernatant of Caco-2 cells at 5 days postinfection, although Caco-2 cells yielded a slightly smaller amount of BCV than did HRT-18G cells. Furthermore, significant cell fusion as a sign of cytopathic changes was not observed in Caco-2 cells infected with BCV during cultivation (data not shown).

As it has been reported that post-confluent Caco-2 cells displayed a remarkable heterogeneity in their differentiated characteristics (Beaulieu & Quaroni 1991), we selected clones by monitoring ALP activity as a differentiation marker and examined the infectivity of BCV to the clones. ALP activities of brush-border membranes obtained from 18 clones of 10 days post-

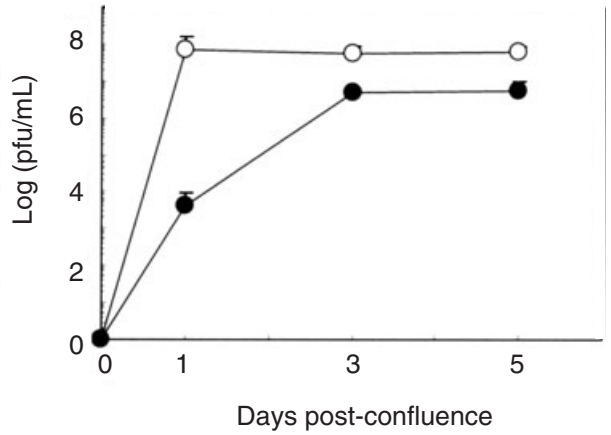

Figure 1 Bovine coronavirus propagation in (○) HRT-18G cells and (●) Caco-2 cells. Culture supernatant was collected at the indicated time and titrated by a plaque assay using HRT-18G cells, and then plaque-forming units (pfu) were determined.

confluence were determined, and the two clones showing the highest and lowest levels of ALP activity were selected and named Caco-2/CA1 and Caco-2/CA2, respectively. A comparison of ALP activities in parental Caco-2, CA1 and CA2 cells showed that the activity levels of parental Caco-2 and CA1 cells in the brush-border membrane-rich fraction were markedly higher than those of CA2 cells over a period of 15 days post-confluence (Fig. 2a). However, the level of cell proliferation activity of CA2 cells in the exponential growth phase was significantly higher than the levels of parental Caco-2 and CA1 cells (Fig. 2b). The results suggest that both parental Caco-2 and CA1 cells were in a more differentiated state than were CA2 cells.

The next experiment was carried out to determine whether the propagation of BCV is controlled by the differentiation stage of the host cells and whether the times at which BCV appears in culture media during successive cultivation are different among the cells. HA activity was detected just after infection at 0, 3 and 10 days post-confluence in parental Caco-2 cells (data not shown). When CA1 and CA2 cells were infected with BCV at confluence, the intervals were 4 days postinfection for CA1 cells and 7 days postinfection for CA2 cells (Fig. 3a,b). Also, when CA1 and CA2 cells were infected at 3 days post-confluence, the virus was detected at 1 and 5 days postinfection, respectively (Fig. 3c,d). Interestingly, virus in the supernatant of both CA1 and CA2 cells was detected immediately after infection when both cells were infected with BCV at 10 days post-confluence (Fig. 3e,f). The results suggest that certain cellular factors that are expressed around 3 days post-confluence in CA1 cells and around 7 days post-confluence in CA2 cells control BCV propagation.

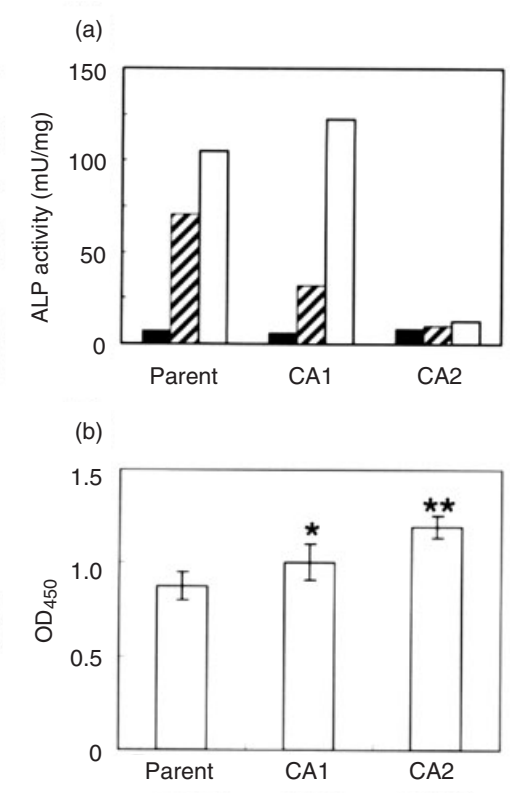

Figure 2 Characterization of parental Caco-2, CA1 and CA2 cells. (a) Alkaline phosphatase (ALP) activities in the brush-border membrane-rich faction were determined at (■) 0 days, (▨) 10 days and (□) 15 days post-confluence. (b) Cell proliferation activity (OD_{450}) of parental Caco-2, CA1 and CA2 cells in the exponential phase was assayed by a Cell Counting Kit-8. $*P < 0.05$ and $**P < 0.01$ versus parental Caco-2. Data are expressed as mean ± SD ($n = 4$).

For some coronaviruses, it has been observed that host cell restriction of propagation is due exclusively to the lack of an appropriate receptor (Sawicki *et al*. 1995; Maeda *et al*. 1997). Interestingly, Caco-2 cells have been shown to have high susceptibility to infection with severe acute respiratory syndrome coronavirus (SARS-CoV). In the case of SARS-CoV, angiotensin-converting enzyme 2 has been identified as a receptor (Li *et al*. 2003) and has been found to be the determinant of host cell restriction (Mossel *et al*. 2005). Although further studies are needed, it is possible that expression of cellular factors, including the BCV receptor, is responsible for BCV propagation in

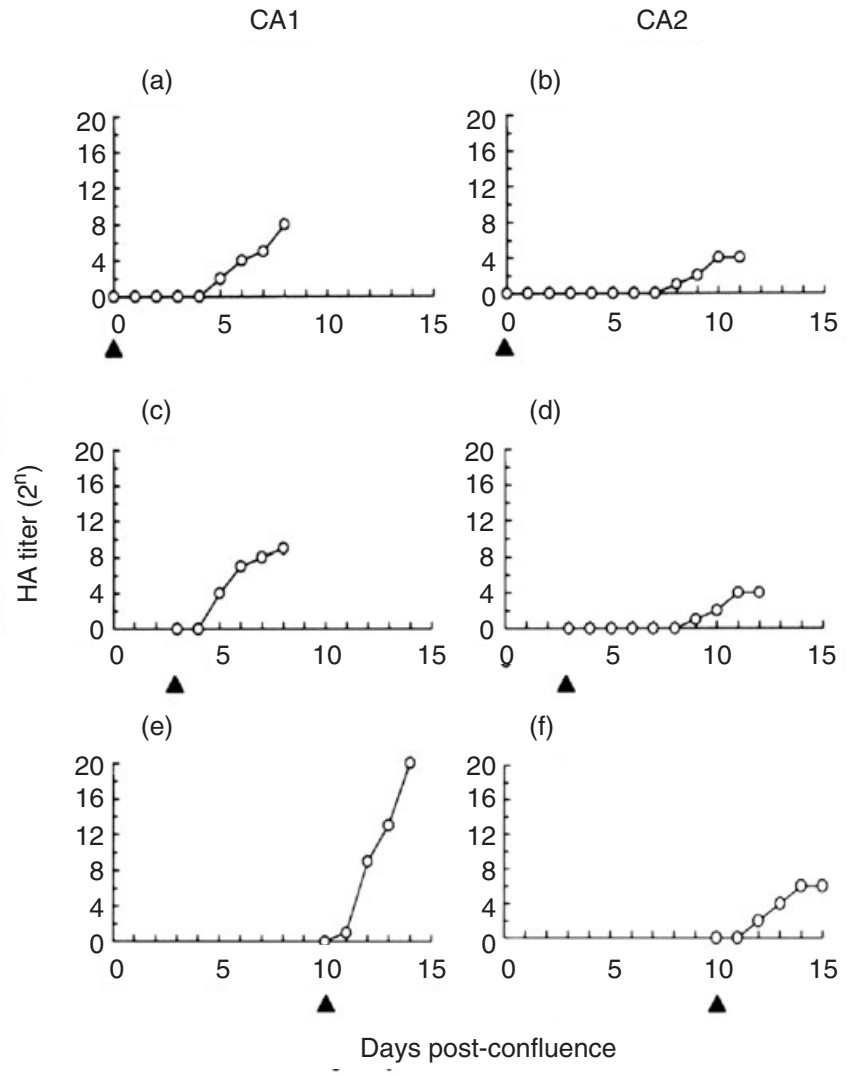

Figure 3 Profiles of bovine coronavirus (BCV) propagation in CA1 and CA2 cells. CA1 and CA2 cells were separately inoculated with BCV at: (a,b) 0 days, (c,d) 3 days, and (e,f) 10 days post-confluence. Hemagglutination of the supernatant was determined each day postinfection. Arrowheads represent times of infection with BCV after confluence. Data represent the means of four experiments.

CA1 and CA2 cells. Thus, CA1 and CA2 cells showing different levels of ALP activity should be good cells for studying the BCV propagation mechanisms.

ACKNOWLEDGMENTS

We thank Masato Watanabe for his technical assistance. This work was in part supported by the Japan Society for the Promotion of Science.

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