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Scanning Electron Microscopic Characterization of Bovine Coronavirus Plaques in HRT Cells

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With 5 figures

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Summary

The ecology of cytopathic expression of bovine coronavirus (BCV) in HRT-18 cells was analyzed within virus-induced plaques by scanning electron microscopy. Virus replication was cytocidal for many HRT-18 cells, a function enhanced in the presence of trypsin. A monolayer of cells remained that imparted a characteristic turbidity to the plaque. These structurally normal, lysis-resistant cells did not stain with fluorescent antibodies specific for BCV antigens, failed to adsorb virus particles or mouse erythrocytes in contrast to the susceptible cells. The survival of cells in the plaque interior reflects a non-productively infected population with evidence of viral persistence.

Introduction

Cytopathic interaction of bovine coronavirus (BCV) with intestinal cells induces serious enteric disease in neonatal calves (DOUGHRI and STORZ, 1977; DOUGHRI et al., 1977; MEBUS et al., 1973). Some strains of BCV can be adapted and propagated in several cell lines permitting *in vitro* investigations of the cytopathic effects (SHARPEE et al., 1976; DEA et al., 1980; LAPORTE et al., 1979; STORZ et al., 1981; TEKTOFF et al., 1983; TOTH, 1982). Many field strains of BCV fail to replicate in cultured bovine cells, but can be cultivated in HRT-18 cells (ST. CYR-COATS and STORZ, 1988), an intestinal cell line derived from an adenocarcinoma in a human rectum (TOMPKINS et al., 1974). A large percentage of the HRT-18 cells survive in the infected culture even when viral cytopathic expression is enhanced by the inclusion of trypsin in the culture medium (ST. CYR-COATS et al., 1988). These surviving cells impart a characteristic turbidity to BCV-induced plaques. We analyzed the surviving HRT-18 cells that are resistant to the BCV cytocidal effects for viral adsorption, hemadsorption, and antigen production. Similarities between these cells and persistently infected HRT-18 cultures became apparent.

Material and Methods

Cells and BCV virus strain

Monolayers of the human rectal tumor cell line HRT-18 (TOMPKINS et al., 1974) were maintained in Dulbecco modified Eagle medium (DMEM) buffered with 44 mM $NaHCO_3$ and supplemented with 5 % fetal calf serum. Stock preparations of the Mebus strain L9 of BCV (SHARPEE

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et al., 1976) were propagated in HRT-18 cells. Virus stocks were generated from cells infected at a multiplicity of approximately 0.01 PFU per cell, incubated for 4 to 5 days at 37 °C in serum-free DMEM and harvested by freeze-thawing. Viral titers obtained in these preparations ranged from 10^6 to 10^7 PFU per ml.

Plaque formation

Plaques were evaluated after seeding the virus on monolayers of HRT-18 cells grown in 6-well tissue culture dishes. The monolayers were incubated for 1 h at 37 °C with 5 to 50 plaque-forming units of BCV in DMEM. The virus-adsorbed monolayers were washed with DMEM and covered with an overlay consisting of DMEM with 0.6 % agarose (Bethesda Research Laboratories) and 5 μg of trypsin (Difco Laboratories) per ml to enhance plaque formation (STORZ et al., 1981). The plates were incubated for 2 to 4 d at 37 °C in an atmosphere of 5 % CO_2. After plaque development, the agarose overlay was removed from the cultures with a spatula. The monolayers were washed with 0.01 M $NaPO_4$, 0.14 M NaCl at pH 7.3 (PBS) and fixed at 4 °C for 30 min in 2 % glutaraldehyde and 2 % formaldehyde buffered with 0.1 M sodium cacodylate, pH 7.4.

Hemadsorption by cells within plaques

Cells within the plaques bearing viral hemagglutinin were identified by their ability to adsorb erythrocytes to the cell surface. The agarose was removed at 60 h post infection from monolayers containing trypsin-enhanced plaques. The monolayers were washed three times with PBS to remove the residual agarose, covered with a suspension of 6×10^6 mouse erythrocytes per ml in PBS, and then incubated for 20 min at 25 °C to allow hemadsorption. The cell cultures were washed twice with PBS to remove unbound erythrocytes and fixed at 4 °C for 30 min in 2 % glutaraldehyde and 2 % formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4.

Processing for scanning electron microscopy

Monolayers fixed for scanning electron microscopy were washed in 0.1 M sodium cacodylate buffer pH 7.4 and gently detached from the plastic culture dishes with a cell scraper. Areas of interest were cut from the monolayers with razor blades into segments approximately 5 mm^2. The segments were incubated at 25 °C in sodium cacodylate buffered solutions (pH 7) of 1 % osmium tetroxide for 30 min, 1 % tannic acid for 15 min, and again in 1 % osmium tetroxide for 15 min to deposit a conductive coating on the cell surface. After a final rinse in distilled water, the samples were dehydrated in an alcohol series, dried from CO_2 in a critical point drying apparatus (Poloron Equipment), mounted with tape on aluminum stubs, and coated with an alloy of gold and palladium. The specimens were examined with a Cambridge S-150 scanning electron microscope.

Indirect immunofluorescence

Viral antigens in cells were traced with fluorescent antibody. The infected monolayers were fixed for 10 min in 4 % formaldehyde in PBS with 0.005 % $CaCl_2$ and 0.11 M sucrose, permeabilized for 5 min with acetone at −20 °C, incubated with rabbit anti-BCV antibody, and reacted with goat anti-rabbit antibody conjugated to fluorescein isothiocyanate. The preparations were viewed with a Leitz fluorescent microscope using epifluorescence.

Establishment of persistently infected cultures

Monolayers of HRT-18 cells were grown in 25 cm^2 flasks and inoculated with BCV at a multiplicity of approximately 0.01 PFU per cell to establish persistent infections. The cultures were incubated at 37 °C for 6 to 12 days in serum-free medium. Trypsin (5 μg per ml) was added to the incubation medium of certain cultures to enhance the cytopathic expression of the virus during the establishment of persistence. Supernatant fluid containing released virus was harvested at weekly intervals for hemagglutination and infectivity assays. The cultures were passaged and maintained with DMEM supplemented with 5 % fetal calf serum.

Results

Characteristics of viral plaques in HRT-18 cells

A scanning electron micrograph of the apical surface of an HRT-18 cell monolayer is illustrated in Figure 1. The epithelioid appearance of the confluent monolayer derived from the close associations between the polyclonal cells. The apical surface of each cell was

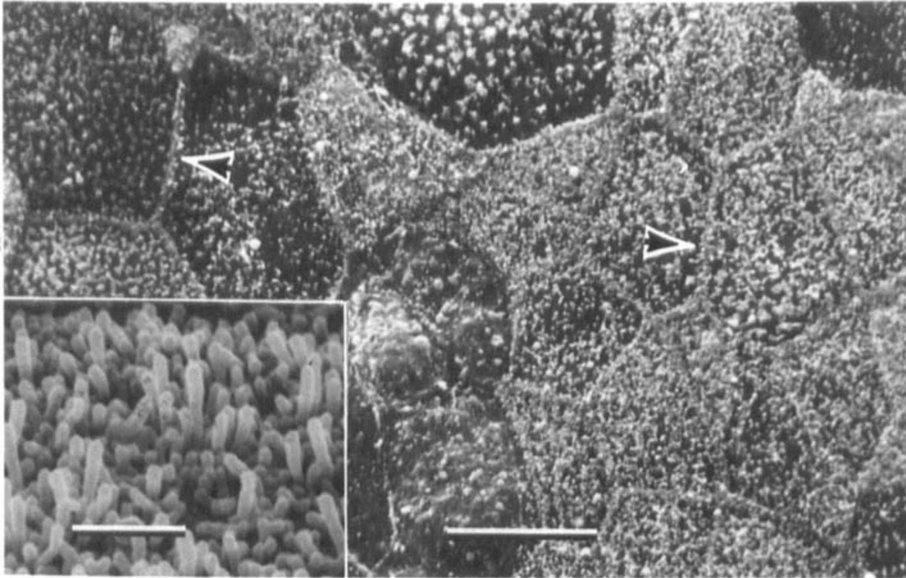

Fig. 1. Scanning electron micrographs of a control monolayer of uninfected HRT-18 cells. Cell borders are denoted by arrowheads. Bar = 10 µm. Inset, microvilli at apical cell surface are filiform. Bar = 1 µm

modified by a variable number of small filiform microvilli which were most numerous at the cell margins.

BCV-induced plaques at 3 days after plating in the presence of trypsin were round, 1 to 2 mm in diameter, and had sharp edges. Interior regions of the plaques were visibly turbid. The plaque margins were defined by a peripheral area of extensive cell destruction (Fig. 2). Many of the cells had detached from this marginal region leaving holes in the monolayer. The remaining cells presented evidence of viral cytopathic expression consisting of separation from adjoining cells, rounding, plasma membrane damage, and cytolysis. Cell damage was less within interior areas of the plaques where unaffected cells predominated.

Hemadsorption by plaques

Virus particles, 100 to 130 nm in size, were observed on the surfaces of HRT-18 cells within plaques (Fig. 3). The abundance of virus particles varied widely from cell to cell. Unaffected cells in the interior regions of plaques generally possessed few or no surface particles of BCV size and shape. We incubated plaques with erythrocytes to test for the hemadsorbing ability of these cells. Mouse erythrocytes were adsorbed to the microvilli of cells bearing surface virions (Fig. 4). The virions contributed to erythrocyte-cell binding by bridging the membrane surfaces. Hemadsorbing cells were most prominent near the plaque margin (Fig. 5 A). This region of infected cells extended at least 100 µm beyond the margin of the plaque as defined by structural cell damage. Damaged cells were also present at interior sites of the plaques where more than 90% of the cells were free of erythrocytes (Fig. 5 B). The hemadsorbing cells had signs of viral cytopathic effects. The cells not adsorbing erythrocytes were structurally normal except for some microvillous clustering which appeared to be a response to the agarose overlay. The monolayer at the plaque interior was less densely populated with cells than the normal monolayer.

HRT-18 cultures persistently infected with BCV

We inoculated monolayers of HRT-18 cells with BCV and maintained the refractory population through 8 serial passages to monitor viral persistence. Low grade cytopathic expression of the virus consisted of cell rounding, some cell detachment and, in cultures incubated with trypsin, the appearance of some polykaryons. These effects were apparent at 4 to 5 d post infection, but the infected monolayers remained nearly confluent. Monolayers incubated with trypsin eventually detached. Numerous cells in cultures without trypsin survived the initial infection. They rapidly established confluent mono-

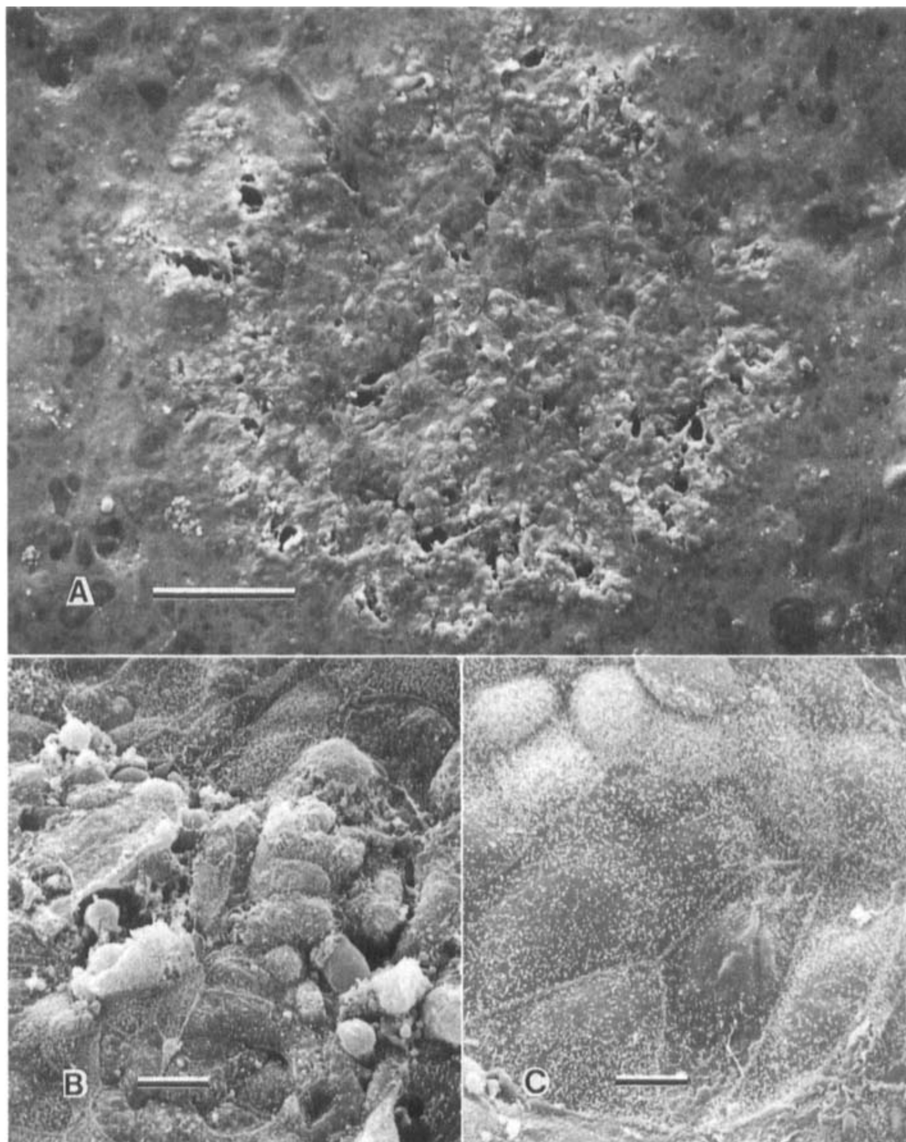

Fig. 2. Plaque induced by BCV replication for 60 h in HRT-18 monolayer. (A) Monolayer disruption is greatest at margin of entire plaque. (B) Depiction of cell damage at the plaque margin. (C) A community of undamaged cells in plaque center. Bars = 0.2 μm in (A) and 10 μm in (B, C)

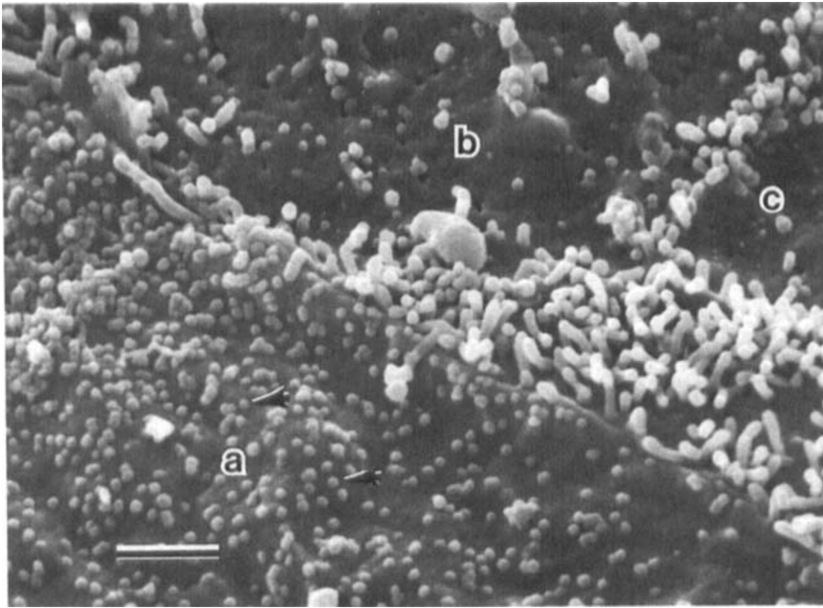

Fig. 3. Adsorption of virus particles to the cell surface. Virus particles (arrowheads) are abundant on cell (a) but less numerous on adjacent cells (b, c). Bar = 1 μm

layers when the infected cultures were passaged and incubated with DMEM supplemented with 5 % serum free of BCV antibodies. The cell number increased at least 10-fold at each passage. Viral cytopathogenic functions were not readily evident. These cultures carried the virus infection for 8 passages at which time the experiment was terminated. Immunofluorescence analysis revealed that only 10 to 15 % of the cells in these carrier cultures contained viral antigens. The hemagglutinating titer varied from 4 to 64, and the infectivity titers ranged from 7.0×10^5 to 7.2×10^6 PFU per ml of culture supernatants.

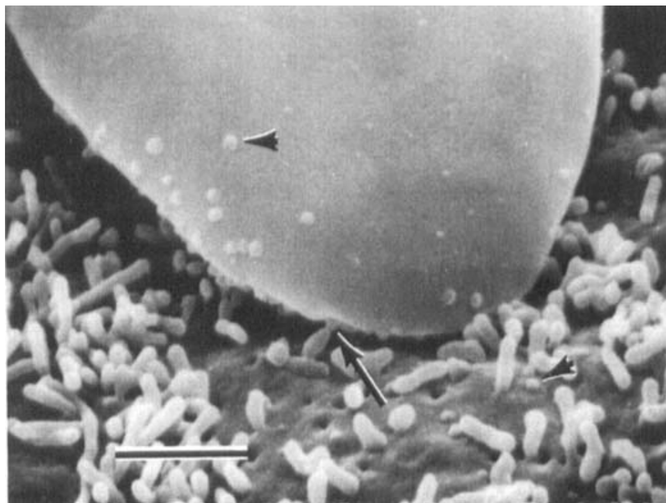

Fig. 4. Erythrocyte bound to surface of HRT-18 cell. Notice virus particles (arrowheads) on erythrocyte, on cell surface, and on microvillus linked to erythrocyte (arrow). Bar = 1 μm

Discussion

The high degree of heterogeneity of the HRT-18 cell line was recently studied with cloned sublines which differ in their susceptibility to viral cytopathic expression (ST. CYR-COATS and STORZ, 1988). The insight gained by analysis of surface properties of these cells

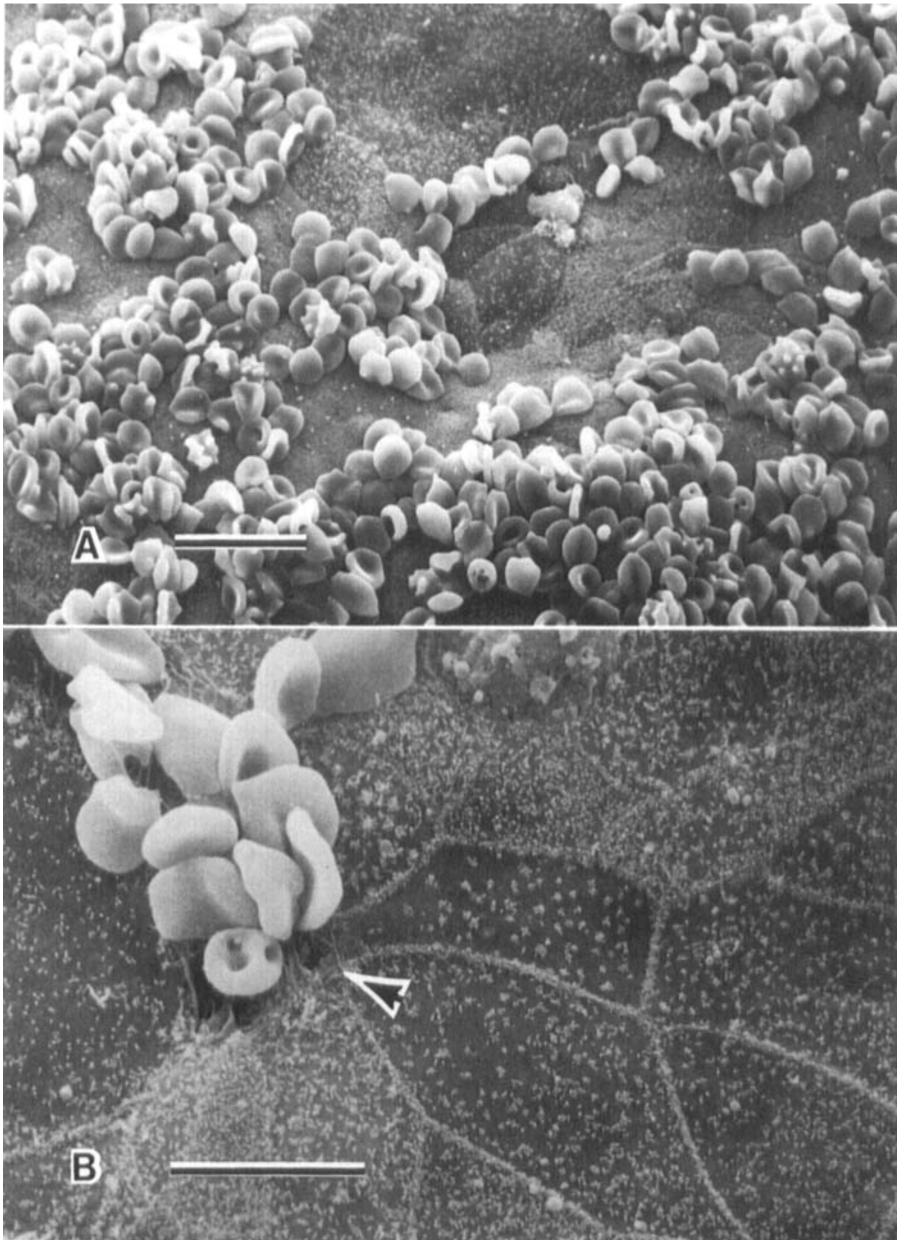

Fig. 5. Plaque incubated with erythrocytes. (A) Erythrocytes bind to many cells at plaque margin. (B) Central regions of the plaque contain few cells that have adsorbed erythrocytes (arrowhead). The structurally damaged cell is surrounded by normal cells that lack viral hemagglutinin. Bars = 20 µm in (A) and 10 µm in (B)

through scanning electron microscopy confirmed this observation. Cell borders were tightly apposed in confluent monolayers of the HRT-18 line. This association and the presence of filiform microvilli on the apical surface reflect the epithelial origin of the cell line. Scanning electron microscopy revealed a heterogenous mixture of cells in the monolayer. Cellular diversity was apparent through the number of microvilli at the apical surface and through adsorption of coronavirions to the plasmalemma in infected cultures. The abundance of surface-adsorbed coronavirions relates to the concentration of cellular receptors for the virus. Both of these features could be a function of the cell cycle or they may be characteristic of stable subpopulations in the culture.

The coronavirus multiplied in HRT-18 cells and, in the presence of trypsin, induced pronounced cytopathic changes in the monolayer. Plaques formed as the result of viral destruction of susceptible cells yet a residual turbidity remained. The outer margin of the plaque was defined by a peripheral zone of monolayer disruption. The central area was covered with a nearly confluent monolayer of structurally normal cells that lacked viral hemagglutinin. This type of plaque morphology evidently derived from a mixed population of susceptible and resistant cells and developed during the centrifugal progression of infections from the initial infectious center to neighboring cells. Viral cytopathogenic functions induced lysis of susceptible cells while the resistant cells, unaffected by the virus, remained and spread in the plaque interior. Susceptible cells are more abundant at the recently infected periphery of the plaque.

Cell proliferation in the absence of serum was probably not a major factor in maintaining the cell population within the plaques. Reestablishment of the monolayer within the plaque by the hemagglutinin-negative cells could involve a reorganization of their intercellular junctions in a process that may well be facilitated by partial proteolysis of the junctions. This effect would increase the mobility of HRT-18 cells and augment additional mechanisms that are responsible for trypsin enhancement of plaque formation such as activation of a viral fusion factor (St. Cyr-Coats et al., 1988 b; Storz et al., 1981). Polykaryons were not found within the plaques.

The hemagglutinin-negative cells coexisted with the infected cells at the plaque center in an association reminiscent of a persistent infection. We found that the BCV persisted in HRT-18 cultures. Viral antigens were produced by a minority of the cells in carrier HRT cultures and progeny virus was released for at least 8 passages. Cell growth was affected little by the infection. Cytopathic expression of the virus was minimal when the culture medium was supplemented with serum. These persistently BCV-infected cultures were readily established. Possible mechanisms involved in the establishment of persistent coronaviral infections include conditional interference by emerging temperature-sensitive or poorly growing mutants (Bang, 1982), the development of defective interfering virus particles (Makino et al., 1984), and the production of interferon (Bang, 1982). Cell cycle-dependent variations in the expression of cellular viral receptors may place a temporal limit on host susceptibility to BCV. This could allow continued HRT-18 cell growth and development of daughter cells in the culture. Cellular determinants are probably important factors in BCV persistence in these cultures because of the heterogeneity of HRT-18 cells and the presence of subpopulations with intrinsic resistance to the virus. Such cells have been found to be involved in the establishment of a persistent murine coronavirus infection on a subline of L cells (Mizzen et al., 1983).

Acknowledgements

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References

Bang, F. B., 1982: The use of a genetically incompatible combination of host and virus (MHV) for the study of mechanisms of host resistance. Adv. Exp. Med. Biol. **142**, 359–374.

Baybutt, H. N., H. Wege, M. J. Carter, and V. ter Meulen, 1984: Adaptation of coronavirus JHM to persistent infection of murine sac(−) cells. J. Gen. Virol. **65**, 915—924.

Dea, S., R. S. Roy, and M. E. Begin, 1980: Bovine coronavirus isolation and cultivation in continuous cell lines. Am. J. Vet. Res. **41**, 30—38.

Doughri, A. M., and J. Storz, 1977: Light and ultrastructural pathological changes in intestinal coronavirus infection of newborn calves. Zbl. Vet. Med. B **24**, 367—385.

Doughri, A. M., J. Storz, I. Hajer, and H. S. Fernando, 1976: Morphology and morphogenesis of a coronavirus infecting intestinal epithelial cells of newborn calves. Exp. Mol. Pathol. **25**, 355—370.

Laporte, J., R. L'Haridon, and P. Bobulesco, 1979: *In vitro* culture of bovine enteritic coronavirus (BEC). INSERM Colloq. **90**, 99—102.

Makino, S., F. Taguchi, and D. Fujiware, 1984: Defective interfering particles of murine hepatitis virus. Virology **133**, 9—17.

Mebus, C. A., E. L. Stair, M. B. Rhodes, and M. J. Twiehaus, 1973: Neonatal calf diarrhea: propagation, attenuation, and characteristics of a coronavirus-like agent. Am. J. Vet. Res. **34**, 145—150.

Mizzen, L., S. Cheley, M. Rao, R. Wolf, and R. Anderson, 1983: Fusion resistance and decreased infectability as major host determinants of coronavirus persistence. Virology **128**, 407—417.

Sharpee, R. L., C. A. Mebus, and E. P. Bass, 1976: Characterization of a calf diarrheal coronavirus. Am. J. Vet. Res. **37**, 1031—1041.

St. Cyr-Coats, K., and J. Storz, 1988: Bovine coronavirus-induced cytopathic expression and plaque formation: Host cell and virus strain trypsin dependence. J. Vet. Med. **35**, 48—56.

St. Cyr-Coats, K., H. R. Payne, and J. Storz, 1988a: The influence of the host cell and trypsin treatment on bovine coronavirus infectivity. J. Vet. Med. **35**, 752—759.

St. Cyr-Coats, K., J. Storz, K. A. Hussain, and K. L. Schnorr, 1988b: Structural proteins of bovine coronavirus strain L9: effects of the host cell and trypsin treatment. Arch. Virol. **103**, 35—45.

Storz, J., R. Rott, and G. Kaluza, 1981: Enhancement of plaque formation and cell fusion of an enteropathogenic coronavirus by trypsin treatment. Infect. Immun. **31**, 1214—1222.

Tektoff, J., M. Dauvergene, M. Durafour, and J. P. Soulebot, 1983: Propagation of bovine coronavirus on VERO cell line: electron microscopic studies. Dev. Biol. Stand. **53**, 299—310.

Tompkins, W. A. F., A. M. Watrach, J. D. Schmale, R. M. Schultz, and J. A. Harris, 1974: Cultural and antigenic properties of newly established cell strains from adenocarcinomas of human colon and rectum. J. Natl. Cancer Inst. **52**, 101—106.

Toth, T. E., 1982: Trypsin-enhanced replication of neonatal calf diarrhea coronavirus in bovine embryonic lung cells. Am. J. Vet. Res. **43**, 967—972.