Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Bovine Coronavirus Antigen in the Host Cell Plasmalemma

H. R. PAYNE, 1,2 J. STORZ, 1 AND W. G. HENK 3

Departments of 1Veterinary Microbiology and Parasitology and 2Veterinary Anatomy and Fine Structure, Louisiana State University, Baton Rouge, Louisiana 70803

Received October 4, 1989, and in revised form July 19, 1990

Expression of bovine coronavirus (BCV) antigen in the plasmalemma of epithelioid human rectal tumor (HRT-18) and fibroblastic bovine fetal spleen (BFS) cell lines was traced by immunofluorescence and immunoelectron microscopy facilitated by colloidal gold. Cytoplasmic fluorescence was first observed at 12 hr postinfection (h.p.i) in infected HRT-18 cultures. This fluorescence coincided with the appearance of cell surface antigen reacting with colloidal gold-labeled antibodies to BCV antigens. At 24 h.p.i the amount of viral antigens at the surface of HRT-18 had increased, although cytoplasmic fluorescence remained constant. Infected BFS cells but not HRT-18 cells formed polykaryons when incubated in the presence of trypsin. One viral antigen in the plasma membrane of BFS cells was thus identified as the S glycoprotein with a fusion domain. In contrast to HRT-18 cells, the overall amount of BCV antigens at the surface of BFS cells remained constant after the onset of fusion. Analysis of the labeling characteristics established that the gold-marked-sites represented de novo expression of BCV antigen in the plasma membrane of infected cells. © 1990 Academic Press, Inc.

INTRODUCTION

Bovine coronavirus (BCV) infections are associated with enteric disease of viral etiology in newborn calves (Mebus et al., 1973; Doughri et al., 1976). The envelope of BCV virions contains two types of morphologically and functionally distinct spikes containing glycoproteins (Storz et al., 1981; King et al., 1985). The major envelope-associated glycoprotein S has a molecular mass of 185 kDa which is cleaved into S1 and S2 (100–110 kDa) while HE has 62 kDa in the reduced and 140 kDa in the nonreduced form. S carries the structural sites responsible for virus attachment and fusion of host cells (Collins et al., 1982; St. Cyr-Coats et al., 1988; Deregt et al., 1987). Hemagglutination of rodent red blood cells and acetyl esterase activity is associated with HE of BCV (King et al., 1985; Vlasak et al., 1988; Cavanagh et al., 1990). The integral membrane glycoprotein M has a molecular mass of 24–26 kDa. The nucleocapsid protein N is phosphorylated (King and Brian, 1982; Lapps et al., 1987).

The initial adaptation of wild-type strains of BCV to different cultured cells is difficult but most successful in the human rectal tumor (HRT-18) cell line which was derived from an adenocarcinoma (Tomkins et al., 1974; Laporte et al., 1979; St. Cyr-Coats and Storz, 1988). Monolayers of these polarized epithelioid cells resemble enterocytes, the type of cells targeted by BCV in natural infections (Doughri and Storz, 1977). Cultures of different bovine fetal cells support noncytotoxic multiplication of the cell-adapted strain of BCV (Mebus et al., 1978). Bovine fetal spleen (BFS) cells are highly susceptible to BCV-induced cell fusion if trypsin is added to the medium of infected cultures. Cell fusion depends on
PLASMALEMM Antigen of BCV-infected cells

Trypsin cleavage of the 185-kDa precursor to the 100- to 110-kDa S1 and S2 (St. Cyr-Coats et al., 1988; Storz et al., 1981; Cavanagh et al., 1990). This phenomenon implies that virally encoded macromolecules are expressed in the plasma membrane (White et al., 1983). Viral proteins in the host plasma membrane are not necessary for BCV maturation because coronaviral particles are enveloped by budding into intracellular compartments (Sturman and Holmes, 1982). Viral components at the cell surface contribute to intracellular spread of the infection by fusion with uninfected cells, and they serve as targets for immune surveillance. Immunoelectron and immunofluorescence microscopy were used in this investigation to analyze the expression of virus-specific antigen in the plasmalemma of cells infected with BCV.

MATERIALS AND METHODS

Monolayers of HRT-18 cells (Tompkins et al., 1974) were maintained in Dulbecco's modified Eagle's medium (DMEM) buffered with 44 mM NaHCO₃ and supplemented with 5% fetal calf serum. The D2 strain of the BFS cell line was maintained in minimal essential medium (MEM) buffered with 25 mM N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid and supplemented with 10% fetal calf serum. Working stocks of the Mebus strain L9 of BCV (Mebus et al., 1978) with 10⁶ to 10⁷ p.f.u. per milliliter were prepared in HRT-18 cells.

Monolayers were grown in 20-cm² solvent-resistant culture dishes for immunogold labeling experiments and in multiwell chamber slides for immunofluorescence studies. The cells were washed with serum-free medium, infected with BCV (m.o.i. = 2 to 20 p.f.u. per cell) for 60 min at 37°C, covered with fresh medium, and incubated for various periods. Infected BFS cultures were incubated with and without the addition of 0.2 µg of trypsin (Type XIII, Sigma Chemical Co.), per milliliter.

We used a polyclonal rabbit antibody against purified BCV. This antiserum had a neutralization index of 4.5 and reacted in Western blots with all BCV proteins. The IgG fraction was obtained by protein-A-Sepharose column chromatography of antiserum and normal serum from rabbits.

Cultures for electron microscopy were fixed for 30 min at 4°C in 1% glutaraldehyde in 0.01 M NaPO₄, 0.14 M NaCl at pH 7.3 (PBS), washed overnight in PBS, and then treated for 10 min with 1 mM glycyglycine to quench reactive aldehyde groups. The fixed monolayers were incubated at 4°C in rabbit anti-BCV IgG or normal rabbit IgG, washed in PBS, and then incubated with goat anti-rabbit IgG conjugated to 5-nm colloidal gold (Janssen Life Sciences Products, Beerse, Belgium). These monolayers were fixed again in 1% glutaraldehyde in PBS for 1 hr at 25°C, postfixed for 1 hr in a solution of 1% osmium tetroxide and 1% potassium ferrocyanide in PBS, washed in 0.2 M sodium acetate buffer, pH 3.5, and stained en bloc for 1 hr with 0.2% uranyl acetate in acetate buffer. After being washed in distilled water, the cells were dehydrated in an ascending alcohol gradient, embedded in situ in Spurr's epoxy resin (Spurr, 1969), and polymerized at 60°C. The embedded monolayers were peeled from the plastic substrate and reembedded. Thin sections were cut in a plane perpendicular to the monolayer, stained with lead citrate, and viewed with a Zeiss EM-10 transmission electron microscope at 80 kV. The density of labeling on the cell surface was quantitated by counting the number of gold particles per length of plasmalemma in thin-sectioned cells. An
image analyzing system (Sigma Scan, Jandel Scientific) was employed to measure membrane length directly from electron microscope negatives.

Preparations for immunofluorescence microscopy were fixed for 10 min in 4% formaldehyde in PBS with 0.005% CaCl₂ 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.

RESULTS

Accumulation of BCV antigen in HRT-18 cells. Antigen expression was analyzed in HRT-18 cultures which share distinctive morphological features with absorptive cells of the intestinal mucosa. Virus-specific fluorescence following infection at high MOI was observed in approximately 10% of the cell population at 12 to 36 hr postinfection.

Thin sections of immunolabeled HRT-18 monolayers revealed BCV antigen at the cell surface. The label did not attach to cells prior to 9 h postinfection but gold particles were observed at widely scattered locations on the apical surfaces of cells fixed at 12 hr (Fig. 1a). Infected HRT-18 cells (Fig. 1b) were labeled abun-

---

**Fig. 1.** Electron microscopy of infected HRT-18 cells incubated with immunogold reagents. (a) Cells fixed at 12 hr postinfection. Gold particles (arrowheads) marked widely scattered locations on the plasma membrane. (b) Cells that were fixed at 24 hr postinfection were labeled abundantly by the gold probe. Viral antigens were detected on both microvilli (inset) and on planar portions of the plasmalemma. Bar marker represents 200 nm for (a) and (b), 100 nm for inset.
dantly at 24 hr with $9.5 \pm 2.7$ gold particles bound per micrometer length of plasma membrane. BCV antigen was located on both microvilli and planar portions of the apical plasma membrane. The gold particles were spatially separated in general, but observations of clustered BCV antigen in the plasma membrane were made as well. Neighboring cells without evidence of infection were practically devoid of label with fewer than 0.5 gold particles per micrometer of plasmalemma.

Negative control preparations included (i) uninfected HRT-18 cells that had been incubated with anti-BCV antibody and (ii) infected cells that had been incubated with the IgG fraction of normal rabbit serum. These cells were not labeled by the gold probe, confirming that the antibody bound only to virus-specific antigen. The plasma membrane was not labeled on uninfected HRT-18 cells adsorbed at 4°C with BCV and incubated with these immunoreagents. This observation implies that binding of whole virus or subviral components to cellular receptors did not contribute to our observation of gold association with the plasma membrane.

Accumulation of BCV antigen in BFS cells. Polykaryocytosis was evident by 18 hr in infected BFS cells incubated in medium with trypsin but cell fusion was not observed in the absence of trypsin. We used immunofluorescence and immunogold electron microscopy to correlate the onset of cell fusion with the synthesis of viral antigen.

Faint fluorescence was first recognized in BFS cultures at 9 hr postinfection (Fig. 2a). Fluorescent cells constituted 25 to 30% of the cell population at 15 and 24 hr postinfection in cultures incubated without trypsin. Cultures that were incubated with trypsin contained large polykaryons with cytoplasmic fluorescence at 24 hr (Fig. 2b) while cultures without trypsin contained only mono- and binucleated cells (Fig. 2c). Fewer than 10% of the remaining single cells in trypsin-treated cultures were nonfluorescent at 24 hr. This decrease in the number of

![Fig. 2. Immunofluorescence of virus-infected BFS cells. (a) Cytoplasmic fluorescence evidenced in cells fixed after 15 hr infection in the presence of trypsin. (b) Fluorescent polykaryons predominated in cultures incubated in the presence of trypsin for 24 hr. (c) Cultures incubated in the absence of trypsin contained only mono- and binucleated cells at 24 hr postinfection. Bar marker represents 25 μm.](image-url)
uninfected cells evidently resulted from recruitment of uninfected cells into the enlarging polykaryons.

Electron microscopic preparations revealed no evidence of membrane fusion in BFS monolayers fixed after 15 h of infection in the presence of trypsin (Fig. 3a). At the same time, multiple sites of BCV antigen in the plasma membrane were labeled (Fig. 3b), indicating that antigen appearance at the plasma membrane preceded the onset of cell fusion by several hours. Viral antigen did not increase in the plasma membrane after the onset of cell fusion in infected BFS cell cultures. Vesicles filled with virus particles and continuous with the plasma membrane were observed as early as 15 hr after infection in BFS cells (Fig. 3c). This event appeared to represent exocytosis of newly formed coronavirus particles.

The abundance of gold binding sites on polykaryons at 24 hr (Fig. 4a) was approximately equal to the number seen on BFS cells not treated with trypsin (Fig. 4b). Evidently, the addition of trypsin did not alter the amount of viral antigen at the cell surface but influenced its function.

DISCUSSION

Our results are in agreement with reports of viral proteins at the surface of cells infected with mouse coronaviruses. Collins et al. (1982) used immunoferritin electron microscopy to demonstrate that the 170-kDa protein of MHV-4 is present at the surface of infected L-241 cells at 6 hr postinfection. Other evidence for coronavirus-specific proteins at the cell surface relied on inhibition of virus-induced cell fusion by antibodies specific for envelope proteins (Holmes et al., 1982; Sturman et al., 1985) and on fusion foci experiments (Mizzen et al., 1983; Tooze and Tooze, 1985). Our immunogold procedure directly demonstrates the presence

![Fig. 3. Electron microscopy of immunogold-labeled BFS cells at 15 hr postinfection in the presence of trypsin. (a) Cell fusion had not yet begun, although, in (b), viral antigens were located on the plasmalemma. (c) Vesicle filled with virus particles was continuous (arrowhead) with the plasmalemma. Bar marker represents 1.0 μm for (a), 100 nm for (b), and (c).]
of coronaviral antigen in the plasma membrane and extends the earlier observations to include the bovine coronavirus.

We found that BCV antigen was expressed at the surface of infected cells despite the intracellular mode of maturation for this virus. The gold-labeled sites were observed in areas free of attached virions. Control preparations indicated that these sites represent de novo expression of virus-specific proteins in the plasma membrane.

BCV-infected BFS cells are highly susceptible to fusion in contrast to BCV-infected HRT-18 cells, which are relatively resistant to fusion (Payne and Storz, 1988). This difference in fusion capacity in the two cell types probably depends on differences in membrane structure such as the respective complements of membrane proteins, glycolipids, or the composition of the lipid bilayer (Sturman and Holmes, 1985; White et al., 1983). Infected BFS cells failed to accumulate surface viral antigen after the onset of cell fusion, but BCV-infected HRT-18 cells accumulated increasing amounts of viral antigen on the cell surface yet these cells failed to fuse. The differences among infected BFS and HRT-18 cells in the surface expression of viral antigen and fusion might result from the kinetics and mechanisms of protein expression and the differences in the composition of the two plasma membranes.

The expression of BCV antigen in the plasma membrane has important implications for host–virus interactions. The appearance of virus-specific surface components during eclipse, for example, renders the infected cell susceptible to specific antibody-dependent or cell-mediated host defense mechanisms. Cell fusion induced by BCV may be an important mechanism for cell-to-cell spread of the infection (Mebus et al., 1978). Here, we have shown that the appearance of viral antigen on the surface of BCV-infected cells precedes the onset of fusion. This fusion activity involves the S glycoprotein, which promotes fusion after trypsin cleavage of the 185-kDa precursor to the 100- to 110-kDa S1 and S2 (St. Cyr-Coats et al., 1988; Payne and Storz, 1988; Cavanagh et al., 1990). This BCV glycoprotein was thus identified by its function as a plasmalemma antigen of infected cells. The BCV hyperimmune serum used in our studies did not permit differentiation of the BCV glycoproteins. Monoclonal antibodies were not available when we initiated these investigations, which were designed to clarify whether BCV antigens are detectable at all in the plasmalemma of infected cells.
ACKNOWLEDGMENTS

This work was supported by Grants 80-CRSR-2-0650, 86-CRSR-2-2871 and 89-34116-4675 from the United States Department of Agriculture. This paper contains parts of a dissertation submitted by H. R. Payne to Louisiana State University in partial fulfillment of the requirements for the Ph.D. degree.

REFERENCES

CAVANAGH, D., BRIAN, D. A., ENUANES, L., HOLMES, K. V., LAI, M. M., LANDE, H., SIDDEL, S. G., SPAAN, W., TAGUSHI, F., and TALBOT, P. J. (1990). Recommendation of the coronavirus study group for the nomenclature of the structural proteins, mRNAs, and genes of coronaviruses. Virology 176, 306-307.

COLLINS, A. R., KNOBLER, R. L., POWELL, H., and BUCHMEIER, M. J. (1982). Monoclonal antibodies to murine hepatitis virus-4 (Strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. Virology 119, 358-371.

DEREGT, D., SABARA, M., and BABIUK, L. A. (1987). Structural proteins of bovine coronavirus and their intracellular processing. J. Gen. Virol. 68, 1863-2877.

DOUGHERTY, A. M., and STORZ, J. (1977). Light and ultrastructural pathological changes in intestinal coronavirus infection of newborn calves. Zentralbl. Veterinaermed. Reihe B 24, 367-385.

DOUGHERTY, A. M., STORZ, J., HAUSER, I., and FERNANDO, H. S. (1976). Morphology and morphogenesis of a coronavirus infecting intestinal epithelial cells of newborn calves. Exp. Mol. Pathol. 25, 355-370.

HOLMES, K. V., and BEHNKE, J. N. (1982). Evolution of a coronavirus during persistent infection in vitro. Adv. Exp. Med. Biol. 142, 287-299.

HOLMES, K. V., DOLLER, E. W., and BEHNKE, J. N. (1982). Analysis of the functions of coronavirus glycoproteins by differential inhibition of synthesis with tunicamycin. Adv. Exp. Med. Biol. 142, 133-142.

KING, B., and BRIAN, D. A. (1982). Bovine coronavirus structural proteins. J. Virol. 42, 700-707.

KING, B., POTTs, B. J., and BRIAN, D. A. (1985). Bovine coronavirus hemagglutinin protein. Virus Res. 2, 53-59.

LAFORTE, J., L'HARIDON, R., and DOBULESCU, P. (1979). In vitro culture of bovine enteric coronavirus (BEC). Inst. Nat. Sante Rech. Med. Colloq. 90, 99-102.

LAPPS, W., HOGUE, B. G., and BRIAN, D. A. (1987). Sequence analysis of the bovine coronavirus nucleocapsid and matrix protein genes. Virology 157, 47-57.

MEBUS, C. A., STAIR, E. L., RHODES, M. B., and TWIEHAUS, M. J. (1973). Neonatal calf diarrhea: Propagation, attenuation, and characteristics of a corona-like agent. Amer. J. Vet. Res. 34, 145-150.

MEBUS, C. A., RHODES, M. B., and UNDERDAHL, N. R. (1978). Neonatal calf diarrhea caused by a virus that induces villous epithelial cell syncytia. Amer. J. Vet. Res. 39, 1223-1228.

MIZZEN, L., CHELEY, S., RAO, M., WOLF, R., and ANDERSON, R. (1983). Fusion resistance and decreased infectability as major host determinants of coronavirus persistence. Virology 128, 407-417.

PAYNE, H. R., and STORZ, J. (1989). Analysis of cell fusion induced by bovine coronavirus infection. Arch. Virol. 103, 27-34.

SPURR, A. R. (1969). A low viscosity embedding medium for electron microscopy. J. Ultrastruct. Res. 26, 31-43.

ST. CYR-COATS, K., and STORZ, J. (1988). Bovine coronavirus-induced cytopathic expression and plaque formation: Host cell and virus strain determine trypsin dependence. J. Vet. Med. Biol. 35, 48-56.

ST. CYR-COATS, K., PAYNE, H. R., and STORZ, J. (1988). The influence of the host cell and trypsin treatment on bovine coronavirus infectivity. J. Vet. Med. Biol. 35, 752-759.

ST. CYR-COATS, K., STORZ, J., HUSKAIN, K. A., and SCHNORR, K. L. (1988). Structural proteins of bovine coronavirus strain L9: Effects of the host cell and trypsin treatment. Arch. Virol. 103, 35-43.

STORZ, J., ROTT, R., and KALUZA, G. (1981). Enhancement of plaque formation and cell fusion of an enteropathogenic coronavirus by trypsin treatment. Infect. Immum. 31, 1214-1222.

STURMAN, L. S., and HOLMES, K. V. (1982). The molecular biology of coronaviruses. Adv. Virus Res. 28, 35-112.

STURMAN, L. S., RICARD, C. S., and HOLMES, K. V. (1985). Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: Activation of cell fusing activity of virions by trypsin and separation of two different 90K cleavage fragments. J. Virol. 56, 904-911.
TOMPKINS, W. A. F., WATRACH, A. M., SCHMALE, J. D., SCHULTZ, R. M., and HARRIS, J. A. (1974). Cultural and antigenic properties of newly established cell strains from adenocarcinomas of human colon and rectum. *J. Natl. Cancer Inst.*, 52, 101-106.

TOOZE, J., and TOOZE, S. A. (1985). Infection of AtT20 murine pituitary tumour cells by mouse hepatitis virus strain A59: Virus budding is restricted to the Golgi region. *Eur. Mol. Biol. Org. J.* 37, 203-212.

VLASAK, R., LYNTJES, W., LEIDER, J., SPAAN, W., and PALESE, P. (1988). The E3 protein of bovine coronavirus is a receptor-destroying enzyme with acetylersterase activity. *J. Virology* 62, 4686-4690.

WHITE, J., KIELIAN, M., and HELENIUS, A. (1983). Membrane fusion proteins of enveloped animal viruses. *Q. Rev. Biophys.* 16, 151-195.