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## Bovine Coronavirus Antigen in the Host Cell Plasmalemma

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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 acetylesterase 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 (Tompkins *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

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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 NaHCO<sub>3</sub> 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 10<sup>6</sup> to 10<sup>7</sup> p.f.u. per milliliter were prepared in HRT-18 cells.

Monolayers were grown in 20-cm<sup>2</sup> 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-Sephadex 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 NaPO<sub>4</sub>, 0.14 M NaCl at pH 7.3 (PBS), washed overnight in PBS, and then treated for 10 min with 1 mM glycylglycine 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 h 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 h 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%  $\text{CaCl}_2$  and 0.11 M sucrose, permeabilized for 5 min with acetone at  $-20^\circ\text{C}$ , 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-

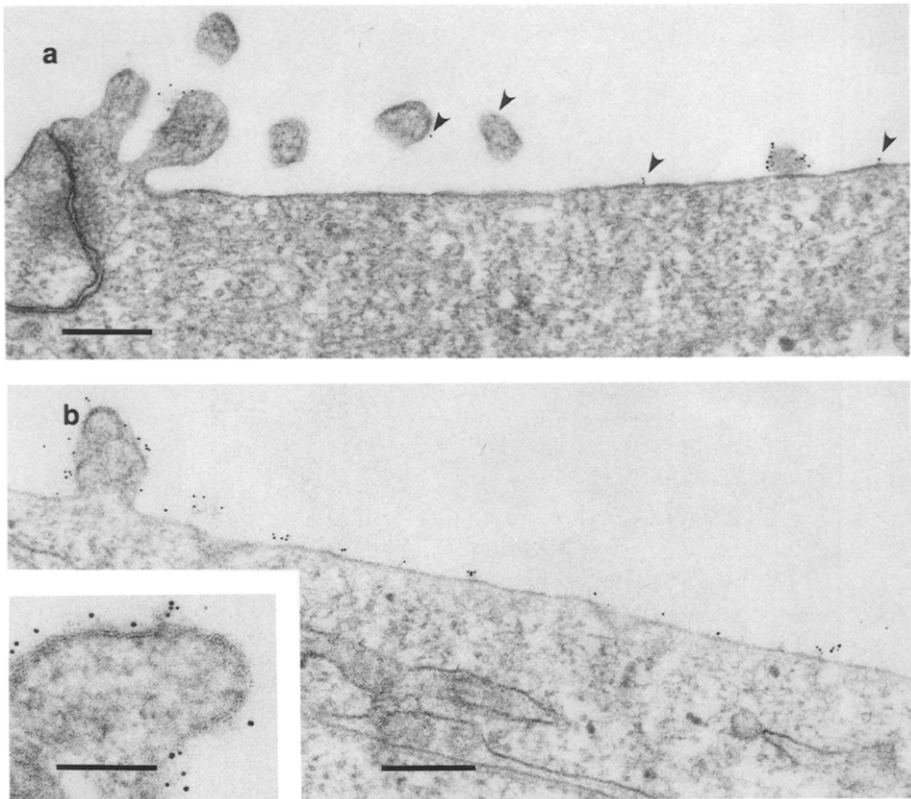


FIG. 1. Electron microscopy of infected HRT-18 cells incubated with immunogold reagents. (a) Cells fixed at 12 hr postinfection. Gold particles (arrowheads) marked widely scattered locations on the plasma membrane. (b) Cells that were fixed at 24 hr postinfection were labeled abundantly by the gold probe. 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

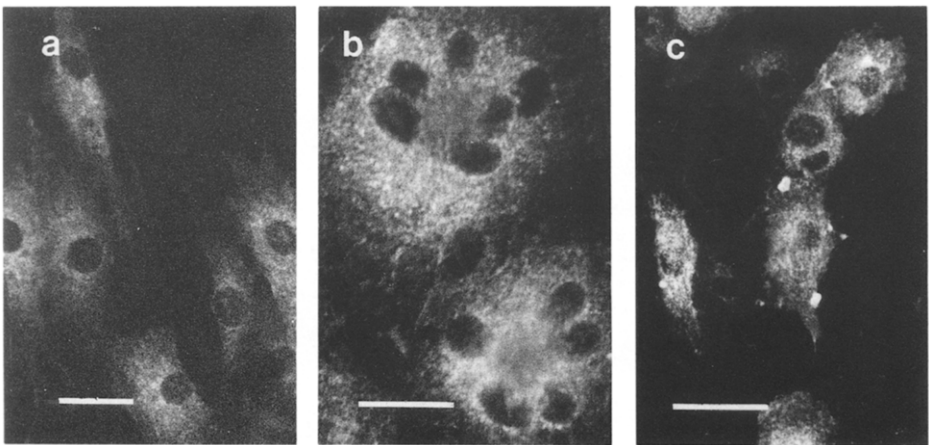


FIG. 2. Immunofluorescence of virus-infected BFS cells. (a) Cytoplasmic fluorescence evidenced in cells fixed after 15 hr infection in the presence of trypsin. (b) Fluorescent polykaryons predominated in cultures incubated in the presence of trypsin for 24 hr. (c) Cultures incubated in the absence of trypsin contained only mono- and binucleated cells at 24 hr postinfection. Bar marker represents 25  $\mu$ m.

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

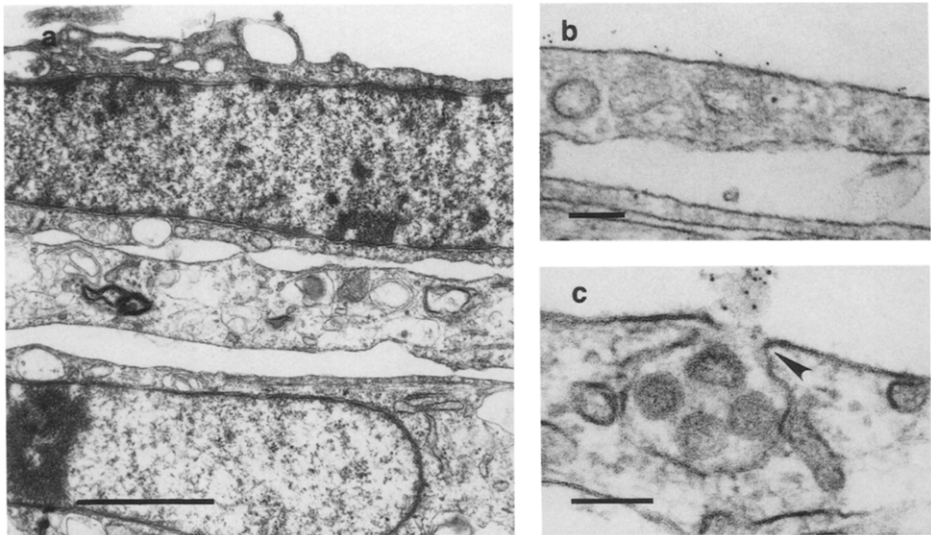


FIG. 3. Electron microscopy of immunogold-labeled BFS cells at 15 hr postinfection in the presence of trypsin. (a) Cell fusion had not yet begun, although, in (b), viral antigens were located on the plasmalemma. (c) Vesicle filled with virus particles was continuous (arrowhead) with the plasmalemma. Bar marker represents 1.0  $\mu\text{m}$  for (a), 100 nm for (b), and (c).

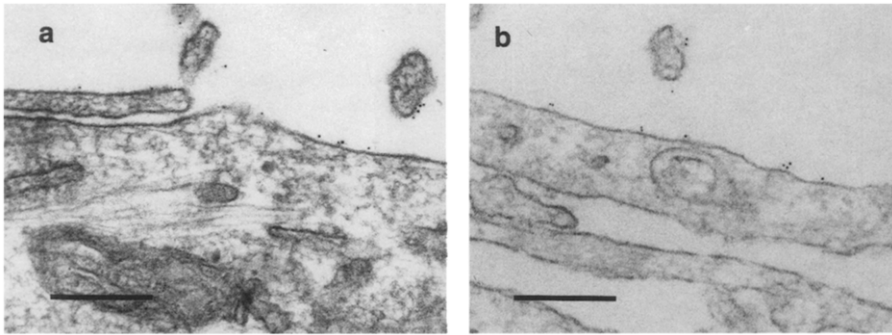


FIG. 4. Immunogold-labeled BFS cells at 24 hr postinfection. Incubated in the presence (a) and in the absence (b) of trypsin. Bar marker represents 200 nm.

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.

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