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.
Short Communication

Analysis of bovine parainfluenza-3 virus replication in bovine embryonic lung cells by indirect fluorescent antibody and hemadsorption assays

Thomas E. Toth and Deborah Jankura
Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24061, U.S.A.
(Accepted 5 September 1989)

Summary

The temporal manifestation of bovine parainfluenza-3 virus (BPI3V) proteins in the cytoplasm and on the surface of bovine embryonic lung (BEL) cells was characterized by indirect fluorescent antibody (IFA) and hemadsorption (HAd) assays. Intracellular proteins appeared earliest at 0.5 h post inoculation (p.i.) and the infection spread to virtually all cells by 48 h p.i. Viral proteins on the surface of cells were seen first at 16 h p.i., and by 48 h p.i. the entire cell monolayer was IFA positive. Hundred-fold less virus in the inoculum delayed appearance of the intracellular as well as cell-surface viral proteins by 24 h and allowed the infection of only about 1/3 of the cells by 48 h p.i. Kinetics of the development in the proportion of HAd-positive cells correlated with those of the surface fluorescence-positive cells. Morphology of the manifestation of BPI3V proteins is characterized by microphotography.

Bovine parainfluenza-3 virus; Bovine embryonic lung; Indirect fluorescent antibody; Hemadsorption

Bovine parainfluenza-3 virus, a member of the family Paramyxoviridae, is an important agent of the bovine respiratory disease complex (Kahrs, 1981). The in vitro replication of this virus was analyzed in bovine embryonic spleen (Tsai and
Thomson, 1975a), calf testicle (Gratzek et al., 1967), Madin Darby Bovine Kidney (Crandell and Michuda, 1972; Tsai and Thomson, 1975a) and most frequently in bovine embryonic kidney cells (Hamdy, 1965; Frank, 1970; Tsai and Thomson, 1975a; Fernandez et al., 1981). However, little information is available about the behavior of BPI3V in in vitro culture of bovine lung cells. Bovine embryonic lung cells were used to titrate the infectivity of BPI3V samples in a study of the aerosol stability of the virus (Elazhary and Derbyshire, 1979). Hemadsorption and bacterial adsorption to BPI3V-inoculated bovine embryonic lung (BEL) cells was also studied (Toth and Gates, 1981). The purpose of the present study was to characterize the kinetics of the expression, the morphology and location of BPI3V proteins in the cytoplasm and on the surface of BEL cells by fluorescence microscopy, and to correlate hemadsorption of guinea pig erythrocytes (GPE) with cell-surface fluorescence.

Cell culture

Bovine embryonic lung (Toth, 1982) cells were propagated with Eagle’s minimum essential medium (EMEM) (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Sterile Systems, Logan, Utah), penicillin, streptomycin, and fungizone at concentrations of 100 units, 100 µg, and 2.5 µg/ml, respectively. For the experiments about 40,000 BEL cells were seeded into each 1 cm² chamber of 8-chamber slides (Lab-Tek Products, Miles Laboratories, Elkhart, IN) in EMEM containing 10% FBS.

Virus replication in chamber slides

The SF-4 strain of BPI3V, propagated in BEL cells, and having a titer of $10^7$ median tissue culture infective dose (TCID₅₀)/ml, was used as stock virus. Monolayers of BEL cells were inoculated with $10^5$ or $10^3$ TCID₅₀ of BPI3V/chamber in 0.1 ml volume. The slides were incubated for 60 min at 37°C, the inoculum was removed and the cells were washed twice with EMEM containing 1% FBS. Then 0.4 ml of the same medium was added to each chamber and the slides were incubated at 37°C in 5% CO₂-containing, humidified air. The completed virus adsorption was considered 0 time. At 0.5, 1, 2, 4, 8, 16, 24, 30, 36, 42 and 48 h p.i., slides were removed from the incubator for demonstration of viral proteins. Negative controls were chambers with BEL monolayers that were sham inoculated with virus-free EMEM, and processed as the virus inoculated chambers. For each virus dose/p.i. time combination duplicate chambers were analyzed in each of 2 experiments.

Indirect fluorescence antibody staining of intracellular viral proteins

The primary antibody was BPI3V hyperimmune serum (Dr. Glynn Frank, National Animal Disease Center, Ames, IA, U.S.A.) diluted 1:600 in 0.01 M phos-
phate-buffered saline (PBS). The secondary antibody was fluorescent isothiocyanate (FITC)-conjugated anti-bovine IgG (Miles Laboratories, Elkhart, IN) diluted 1:80 in 0.01 M PBS. Slides were washed twice with 0.01 M PBS, air dried, fixed for 5 min in ice-cold acetone, air dried and stored at -80°C until stained. Before staining the slides were rehydrated for 5 min in deionized water. Antibodies were incubated on the slides at room temperature for 30 min, with 20-min rinses between antibodies, in 0.01 M PBS. The slides were observed on an Olympus incident light fluorescence microscope and photographed with Ektachrome 1600 film. Proportions of cells positive for viral fluorescence were determined by scanning the whole surface of the chambers and recording the IFA positive cells according to the following scale: very few individual cells, less than 1%, or 5% of the monolayer, and in 5% increments if more than 5% of the monolayers were IFA positive. The averages of the observations in duplicate chambers of 2 experiments representing each p.i. time are given.

**Indirect FA staining of cell surface viral proteins**

Viral proteins expressed on the surface of infected cells were demonstrated by IFA staining of unfixed, viable cells. At the above p.i. times the culture fluid was aspirated from the chambers, the slides were rinsed with 0.1 M PBS, then IFA staining was performed on fresh slides as described for the fixed cells, substituting 0.1 M PBS for 0.01 M PBS in the rinsing steps. After the final rinse the slides were dipped once in deionized water to desalt the preparation, air dried, fixed in absolute ethanol for 5 minutes, and then handled as described for the intracellular IFA assay.

**Hemadsorption assay**

These assays were performed independently from the IFA assays. Eight-chamber culture slides with monolayers of BEL cells were inoculated as described. At the p.i. times given above the fluid was aspirated from the chambers and 0.2 ml of 0.5% suspension of GPE in 0.1 M PBS containing 0.1% FBS was added to each chamber. The slides were incubated for 30 minutes at room temperature. The GPE suspension was aspirated, the chambers and gaskets were removed. The slides were rinsed, air dried, stained by Giemsa, rinsed, air dried and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ). The proportions of cells adsorbing GPE were estimated and expressed in percentage of the HAd-positive monolayers by the scale used for the IFA assay.

**Results**

**Intracellular BP13V proteins**

The proportions of BEL cells positive in intracellular IFA tests are in Table 1. Fluorescence was detected earliest at 0.5 h p.i. in a single, very small focus in the
TABLE 1

Kinetics of the manifestation of intracellular and surface BPI3V proteins in cultured BEL cells

| Hours p.i. | % Cells with intracellular proteins IFA b | % Cells with surface proteins |
|------------|-----------------------------------------|-----------------------------|
|            | 10^3d                                   | 10^3                        | 10^3 | 10^3 |
| 0.5        | Ind.cells 0                             | 0                           | 0    | ND   |
| 1          | <1                                      | 0                           | 0    | ND   |
| 2          | 5                                       | 0                           | 0    | ND   |
| 4          | 10                                      | 0                           | 0    | ND   |
| 8          | 10                                      | 0                           | 0    | ND   |
| 16         | 15                                      | 0                           | 15   | Ind.cells 5 |
| 24         | 55                                      | <1                          | 35   | <1   |
| 30         | 85                                      | 5                           | 70   | 70   |
| 36         | 90                                      | 15                          | 90   | 95   |
| 42         | >95                                     | 15                          | >95  | 95   |
| 48         | >95                                     | 35                          | <95  | >95  |

bPercentage of cells positive for BPI3V proteins by IFA and HA d tests was determined by scanning the entire surface of 2 chambers of 1 cm² surface area. The values (average of 2 chambers in each of 2 experiments) were scored by a system of negative (0), very few individual cells (Ind. cell), <1%, 5% and in 5% increments above 5%.

Indirect fluorescent antibody assay using anti-BPI3V hyperimmune serum and FITC-conjugated anti-bovine IgG antiserum of rabbit origin.

Hemadsorption test using 0.5% guinea pig erythrocytes.

Viral dose (TCID₅₀/ml) used to inoculate 1 cm² of BEL cell monolayer.

10^5 TCID₅₀-inoculated monolayer. By 1 h p.i., the fluorescence appeared as very fine, distinct cytoplasmic granules in an extremely few individual cells. At 4 and 8 h p.i. the cells contained distinct granules (Fig. 1A). At 16 h p.i. viral proteins appeared as fine-granular or diffuse fluorescence occupying a large portion of the cytoplasm (Fig. 1B). At 24 h p.i. the cytoplasm of the cells was filled with large aggregates of viral antigen which accumulated in some cells in the perinuclear region (Fig. 1C). The morphology of fluorescence was essentially the same till 48 h p.i. as seen at 24 h p.i.

Fig. 1. Intracellular fluorescence in BEL cells that were inoculated with BPI3V, and stained by indirect fluorescent antibody method as described in the text. Post-inoculation times: 4 h (A); 16 h (B); 24 h (C).
Viral proteins on the cell surface

The proportions of BEL cells positive in viable cell IFA assays are in Table 1. Viral proteins were detected first at 16 h p.i. as a faint-to-sharp outline of the cell membrane on cells inoculated with $10^5$ TCID$_{50}$ virus (Fig. 2A). By 24 h p.i. the surface of the cells acquired a diffuse 'glowing' in addition to the intensive fluorescence outlining the cells (Fig. 2B). At 48 h p.i. the cells had diffuse surface fluorescence, the cell membranes were intensively outlined and larger aggregates of surface antigens were seen on a few cells (Fig. 2C). In cells inoculated with $10^3$ TCID$_{50}$/chamber the appearance of viral proteins was delayed, but the character of fluorescence was similar to those in cells inoculated with the higher dose of BPI3V.

Hemadsorption

The proportion of HAd positive cells are in Table 1. At 16 h p.i. isolated foci of HAd-positive cells were seen. Subsequently, the increasing proportions of HAd-positive cells correlated with the increasing percentage of the cells positive for surface fluorescence.

All sham-inoculated monolayers of BEL cells were consistently negative for cytoplasmic or surface fluorescence and for hemadsorption.

Discussion

The post inoculation time at which we have seen the first intracellular BPI3V proteins corresponds with the results of Portner and Russel (1973) with measles virus, but precedes by about 8 h the first demonstration of intracellular measles virus by Norrby (1972) and by about 16 h the first appearance of intracellular bovine respiratory syncytial virus proteins (Rossi and Kiesel, 1977). Our results agree with those of Tsai and Thomson (1975a) who used fluorescence microscopy to demonstrate intracytoplasmic inclusions, as small granules, surrounding the nuclei in more than 90% of BPI3V-inoculated bovine embryonic kidney and spleen cultures 2 days p.i. As they have not tested the kinetics of the appearance of BPI3V
prior to 2 days p.i. we do not know how our results would have related to theirs in earlier times of virus replication. In our study 100-fold lower BPI3V inoculum resulted in a 24 h delay in the appearance of detectable intracellular viral proteins. Proteins of the BPI3V appeared 16 h later on the surface than in the cytoplasm of the cells. This agrees with the results of Norrby (1972) with measles virus membrane associated proteins and demonstrates the period necessary for the BPI3V envelope proteins to be inserted into the cell membrane. While the proportion of cells positive for intracellular FA increased within 22 h from 5% at 2 h p.i. to 55% at 24 h p.i., the proportion of surface FA positive cells increased within 24 h from <1% only to 35% (Table 1). This suggests that the rate of the insertion of the BPI3V proteins into cell-membranes lagged somewhat behind the rate of the appearance of the intracellular BPI3V proteins. One of the viral proteins on the surface of the cells, the hemagglutinin, is involved in binding red blood cells to the surface of the infected cells (Kahrs, 1981; Toth and Gates, 1981). The kinetics of the adherence of the GPEs to the BEL cells inoculated with the 2 different doses of BPI3V correlated closely with the kinetics of the appearance of the IFA positive cell-surface BPI3V antigens (Table 1). This finding indicates that the BPI3V hyperimmune serum has recognized BPI3V hemagglutinin on the surface of inoculated cells.

Fluorescence was confined to the cytoplasm and the membrane of infected cells. Tsai and Thomson (1975a) have seen small eosinophilic inclusion bodies early in BPI3V-infected bovine embryonic kidney and spleen cultures which by fluorescence microscopy appeared as small fluorescing granules surrounding the nuclei. In the tracheal mucosa of calves infected with aerosolized BPI3V these aggregates were identified as viral nucleocapsids (Tsai and Thomson, 1975b). We have seen granules, presumably also viral nucleocapsids, relatively early, e.g. 4 and 8 h p.i.

In summary, our study demonstrated (a) intracellular viral BPI3V proteins very early in infected BEL cells, (b) followed by rapid replication resulting in full infection of the monolayers in 48 h, (c) a delay of 16 h in insertion of BPI3V proteins into the cell membranes, and (d) a general delay in the kinetics of the appearance of viral proteins when cells were infected with 100-fold less virus.

References

Crandell, R.A. and Michuda, L. (1972) Isolation and application of a bovine parainfluenza-3 virus variant to veterinary diagnostic medicine. Proc. 76th Ann. Meet. US Anim. Health Assoc. 745-757.
Elazhary, M.A.S.Y. and Derbyshire, J.B. (1979) Aerosol stability of bovine parainfluenza type 3 virus. Can. J. Comp. Med. 43, 295-304.
Frank, G.H. (1970) Serial passage of two plaque types of parainfluenza-3 virus: changes in hemagglutinating properties and cytopathic effect. Am. J. Vet. Res. 31, 1085-1091.
Fernandez, V.M., Correa, P.G. and Hernandez, P.J. (1981) Isolation and identification of parainfluenza-3 virus in bovines from Mexico. Proc. 24th Am. Assoc. Vet. Lab. Diagnosticians, 141-148.
Gratzek, J.B., Rosenbusch, R.F. and Buening, G.M. (1967) Plaque characteristics of four classes of bovine viruses. Am. J. Vet. Res. 28, 641-646.
Hamdy, A.H. (1965) Variants of myxovirus parainfluenza-3 virus isolated from calves clinically affected with shipping fever. Cornell Vet. 55, 623-630.
Kahrs, R.F. (1981) Viral diseases of cattle, 1st edit, pp. 171-181. Iowa State University Press, Ames, Iowa.
Norrby, E. (1972) Intracellular accumulation of measles virus nucleocapsid and envelope antigens. Microbios. 5, 31–40.
Portner, A. and Bussell, R.H. (1973) Measles virus ribonucleic acid and protein synthesis: effects of 6-azuridine and cycloheximide on viral replication. J. Virol. 11, 46–53.
Rossi, C.R. and Kiesel, G.K. (1977) Bovine respiratory syncytial virus infection of bovine embryonic lung cultures: a kinetic study by the fluorescent antibody technique. Am. J. Vet. Res. 38, 1901–1904.
Toth, T.E. and Gates, C. (1981) Lack of virus-specific bacterial adherence to bovine embryonic lung cells infected with bovine parainfluenza virus type 3. J. Clin. Microbiol. 17, 561–563.
Toth, T.E. (1982) Trypsin enhanced replication of bovine coronavirus. Am. J. Vet. Res. 43, 967–972.
Tsai, K. and Thomson, R.G. (1975a) Bovine parainfluenza type 3 virus infection: virus replication in bovine embryonic cell cultures and virion separation by rate-zonal centrifugation. Infect. Immun. 11, 770–782.
Tsai, K. and Thomson, R.G. (1975b) Bovine parainfluenza Type 3 infection: ultrastructural aspects of viral pathogenesis in the bovine respiratory tract. Infect. Immun. 11, 783–803.