Immunofluorescence studies of biotype-specific expression of bovine viral diarrhoea virus epitopes in infected cells

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The expression of biotype-specific epitopes in cells infected with cytopathic (cp) and non-cytopathic (ncp) bovine viral diarrhoea virus (BVDV) was analysed by immunofluorescence. Four monoclonal antibodies (MAbs) directed against different epitopes on the viral glycoprotein gp48 were used. With cells infected with cpBVDV strain NADL, the four MAbs yielded a strong and granular cytoplasmic fluorescence. The same pattern was observed when cells were infected with ncpBVDV 7443 with two of the MAbs (BVD/C12, BVD/C42). In contrast, reactivity with the other two MAbs (BVD/C38, BVD/C46) was restricted to a narrow perinuclear zone. These biotype-specific differences were not observed either with a gp53-specific MAb, or with an MAb specific for the non-structural protein p125/p80. Double immunofluorescence staining of living cells with a polyclonal BVDV-specific serum and with the MAbs revealed that expression of viral proteins on the surface of cells infected with cp- or ncpBVDV, respectively, was not detectable.

Bovine viral diarrhoea virus (BVDV), a positive-strand RNA animal virus, together with border disease virus of sheep and hog cholera virus (HCV), once held generic status in the family Togaviridae (Westaway et al., 1985), but because of their similarities in genome organization and replication strategy to flaviviruses (Collett et al., 1988, 1991) reclassification to the family Flaviviridae became necessary (Horzinek, 1991). Two biotypes of BVDV, cytopathic (cp) and non-cytopathic (ncp), can be distinguished on the basis of their effect on cultured bovine cells. Only cpBVDV induces cytoplasmic vacuolation and progressing changes resulting in cell death 2 to 3 days after inoculation of susceptible cell cultures (Lee & Gillespie, 1957; Gillespie et al., 1960). The cp biotype plays a key role in the pathogenesis of BVDV-induced fatal mucosal disease (Brownlie et al., 1984; Bolin et al., 1985). Although only ncpBVDV strains are isolated from persistently infected cattle, a mixture of both biotypes is generally found in animals with mucosal disease (Liess et al., 1983; McClurkin et al., 1985).

In the present study, immunofluorescence techniques using monoclonal antibodies (MAbs) were applied to obtain information on the distribution of BVDV antigens in cells infected with each biotype. For this, MAbs to epitopes on the putative structural glycoproteins, gp48 and gp53, and the non-structural (ns) protein, p125/p80, of BVDV were used. In addition, expression of viral proteins on the surface of live infected cells was analysed.

The isolation and initial characterization of MAbs BVD/C12, BVD/C16 and BVD/C38 (Peters et al., 1986; Greiser-Wilke et al., 1991) and BVD/CA3 (Bolin et al., 1988) have been described. MAbs BVD/C42 and BVD/C46 were generated using the same method (Peters et al., 1986). The homologous antigen for all hybridomas was the cpBVDV strain NADL. Hybridomas were propagated in Dulbecco's modification of Eagle's MEM (DME) supplemented with 10% donor horse serum (Biochrom). MAbs were purified from cell culture supernatants by immunoaffinity chromatography using a column containing purified goat anti-mouse IgG covalently coupled to CNBr-activated Sepharose 4B beads (Pharmacia). The protein content (Lowry et al., 1951) of the eluted antibody fractions ranged from 0.8 to 1.2 mg/ml.

The protein specificity of the MAbs was determined by radioimmunoprecipitation (not shown) as described previously (Greiser-Wilke et al., 1990). MAbs BVD/C12, BVD/C38, BVD/C42 and BVD/C46 reacted with the minor viral glycoprotein gp48, and BVD/CA3 was specific for the major viral glycoprotein gp53. The pestivirus group-specific MAb BVD/C16 (Cay et al., 1989) was directed against an epitope on the ns protein p125/p80.

Reciprocal antibody blocking assays were performed to establish the topological relationship of the epitopes defined by the four gp48-specific MAbs. The assays were repeated twice in an infected monolayer enzyme im-
munoassay (IM-EIA) (Holm-Jensen, 1981), as described
previously (Greiser-Wilke et al., 1990), with foetal calf
kidney cells (FCKC) infected with the cpBVDV strain
NADL. Unlabelled MAbs were diluted in twofold steps
with the antigen, starting with 2.5 μg per test. At this
concentration, all MAbs were in excess over the antigen
(not shown). Immediately afterwards, a fixed dilution of
peroxidase (PO)-labelled MAbs, yielding \( A_{405} \) values
between 0.5 and 1.0, was added. After 2 h of incubation
at room temperature, excess MAbs were removed by
washing. As a substrate for PO, ABTS [2,2'-azino-bis(3-
ethylbenzthiazoline-6-sulphonic acid)] was used (0.5
mg/ml in 0.05 m-citric acid, 0.1 mM-Na₂HPO₄ pH 4.4,
0.05% H₂O₂). Microtitre plates were read in an ELISA
reader (SLT-Labinstruments) at a wavelength of 405 nm.
As a control, the blocking assays were performed with an
unrelated MAb (no blocking) and with each of the
homologous labelled MAbs.

Each of the four PO-labelled MAbs (BVD/C12-PO,
BVD/C38-PO, BVD/C42-PO and BVD/C46-PO) was
allowed to compete for its epitope on NADL-infected
cells. In addition, MAbs BVD/CA3 and BVD/C16 were
included. As shown in Fig. 1 (top), the blocking of the
homologous MAb pairs was always symmetrical, indicating
that the MAbs did not lose activity during the PO-
labelling process. Symmetrical blocking was also
observed between MAb pairs BVD/C12:BVD/C38,
BVD/C42:BVD/C46 and BVD/C38:BVD/C46. However,
with some MAb pairs differences in the extent of reciprocal
blocking were observed, e.g. whereas binding
of BVD/C12-PO was completely inhibited by BVD/C42,
BVD/C12 was blocked to only about 50% by BVD/C42-
PO. With BVD/C38 and BVD/C46, reciprocal blocking
was complete (> 70%). In contrast, symmetrical block-
ing between BVD/C12 and BVD/C46 was only partial
(about 40%). One-way reactions were observed between
BVD/C42 and BVD/C38-PO (100% blocking), or
BVD/C46-PO (50% blocking) (Fig. 1). BVD/C16 did not
interfere with the binding of any of the four gp48-specific
MAbs, but the gp53-specific MAb BVD/CA3 showed
symmetrical blocking with BVD/C42 and hindered the
binding of BVD/C38-PO (Fig. 1, bottom).

Immunofluorescence analysis using the gp48-specific
MAbs showed differing reactivity patterns in cells
infected with ncp- or cpBVDV strains. For these studies,
secondary FCKC (1 ml containing 4 x 10⁴ cells) were
seeded into vials (Liess & Prager, 1976) containing round
glass coverslips (1.5 cm diameter). After 24 h, the cells
were infected at an m.o.i. of 2 with the ncpBVDV strain
7443 or the cpBVDV strain NADL. Mock-infected cells
were used as controls. After 48 h incubation the
monolayers were air-dried and fixed with acetone for 10
min. Thereafter, the coverslips were incubated (1 h at
room temperature) with MAbs diluted 10-fold in 50 mm-
Na-HEPES pH 7.4, containing 100 mM-NaCl and 1
mg/ml BSA (HEPES buffer). After washing three times
with HEPES buffer, rhodamine-labelled goat anti-
mouse IgG (GAM-Rh, Dianova) and fluorescein iso-
thiocyanate-labelled polyclonal swine anti-BVDV im-
munoglobulins (BVD-FITC; Hyera et al., 1987) were
added. The latter conjugate was used as a positive control
for monitoring the number of BVDV-infected cells (Fig.
2a and b, left panels; Fig. 3a and c). Incubation and
washing were performed as before. The coverslips were
mounted in HEPES buffer on glass slides and examined
under a fluorescence microscope (Zeiss Axioplan).
Fig. 2. Double immunofluorescence analysis of monolayers infected with the cpBVDV strain NADL and the ncpBVDV strain 7443. (a) Acetone-fixed FCKC infected with NADL. (b) Acetone-fixed FCKC infected with 7443. (c) Staining of live FCKC infected with NADL. Left panels, reactivity of monolayers incubated with an FITC-conjugated swine anti-BVDV/HCV serum. Right panels, reactivity of monolayers incubated with MAbs and rhodamine-labelled goat anti-mouse IgG. (a and b) MAb BVD/CI6; (c) MAb BVD/CA27.

Excitation of green fluorescence was at 450 to 490 nm and for detection a 515 to 565 nm filter was used. For red fluorescence, excitation was at 530 to 585 nm and a > 615 nm filter was used for detection. Photographs were taken on Kodak Tmax 3200 films using a Zeiss 40 x PLAN NEOFLUAR objective and a 10 x ocular. Films were developed in Ultrafin Plus (Tetenal) as indicated by the manufacturer.

Uninfected cells showed no fluorescence. Cells infected with either cp- or ncpBVDV reacted strongly with the polyclonal serum, yielding bright cytoplasmic fluorescence (Fig. 2a and b, left panels). A similar reaction extending from the perinuclear area and involving the entire cytoplasm was observed when MAbs BVD/CA3 (anti-gp53; not shown) and C16 (anti-p125/p80; Fig. 2a, and b, right panels) were used. Strong but granular cytoplasmic fluorescence was seen when NADL-infected cells were stained with each of the four gp58-specific MAbs. As an example, the patterns obtained with MAbs BVD/C12 and BVD/C46 are shown in Fig. 2(b) and (d), left panels, respectively. Completely different staining patterns were observed in cells infected with strain 7443. MAbs BVD/C12 (Fig. 3d, right panel) and BVD/C42 (not shown) generated a granular perinuclear fluorescence extending, with decreasing intensity, towards the cytoplasm in all infected cells. In contrast, reactivity of BVD/C38 (not shown) and BVD/C46 (Fig. 3b, right panel) was restricted to a narrow perinuclear zone only.

In order to elucidate whether biotype-specific expression of any of the three viral proteins gp48, gp53 and p125/80 could be distinguished on the outer cell surface, double immunofluorescence staining was performed on live cells. For this, coverslips with cells infected with cp- and ncpBVDV, respectively, were prepared as above, except that the cells were not fixed before incubation with MAbs. MAbs were diluted in DME
without sodium hydrogen carbonate, supplemented with 10 mg/ml BSA and 50 mM-HEPES pH 7.5. Three controls were included. (i) Infection of cells was verified on parallel slides fixed with acetone and stained with the polyclonal FITC conjugate (Fig. 2a and b, left panels). (ii) Binding of MAb to surface proteins of live cells was verified by using an MAb directed against a bovine cell surface antigen (BVD/CA27; Fig. 2c, right panel) related to a cellular receptor for BVDV (V. Moennig et al., unpublished results). Neither the BVDV-specific MAb (not shown) nor the polyclonal BVD-FITC (Fig. 2c, left panel) reacted with live infected cells. (iii) Identical results were obtained when the cells were fixed with 5% formaldehyde in PBS after incubation with the MAb in order to exclude capping on live cells.

Antibody blocking assays showed that the gp48-specific MAb bound to related epitopes on a single antigenic domain. Two classes of epitopes could be distinguished by their reactivity pattern in immunofluorescence. The first class, defined by MAb BVD/C12 and BVD/C42, was expressed equally in cells infected with cp- or ncpBVDV. In contrast, the epitopes defined by BVD/C38 and BVD/C46 showed a biotype-specific reactivity pattern. In addition, these two MAb interfered with the binding of a gp53-specific MAb in the blocking assays. This may be due to a close association of both proteins in the viral envelope.

The immunofluorescence studies with BVD/C38 and BVD/C46 extend the analysis performed by IM-EIA with BVD/C38. The MAb reacted with most strains tested, but clearly demonstrated biotypic differences. Although strong cytoplasmic staining was observed in between 80 and 100% of cells infected with different cpBVDV strains or isolates, most of the cells infected with ncpBVDV strains or isolates showed either no reaction, or only single cells or foci were stained (Greiser-Wilke et al., 1991). Our studies show that the epitopes for these MAb are accessible throughout the cytoplasm in cells infected with cp strains, whereas reactivity is more or less restricted to the perinuclear region in cells infected with ncp strains. It is not yet known whether these differences in epitope expression reflect biotype-specific differences in processing of gp48, thus allowing MAb BVD/C38 and BVD/C46 to bind with high affinity only to the gp48 of cpBVDV. On the molecular level, different protein profiles are obtained by radioimmunoprecipitation of lysates of cpBVDV- and ncpBVDV-infected cells. Two structurally related ns proteins (Purchio et al., 1984), p125 and p80, are precipitated from cpBVDV-infected cells, but the latter is missing in lysates from ncpBVDV-infected cells (Pocock et al., 1987; Donis & Dubovi, 1987; Magar et al., 1988). The expression of the epitopes for BVD/C38 or BVD/C46 could be an additional marker for cytopathogenicity.

Using immunofluorescence tests on viable infected cells neither the polyclonal serum nor the MAb reacted with viral antigens, indicating that none of the viral proteins is expressed on the surface of cells infected with either biotype. This is in accordance with the results obtained by electron microscopy. It was shown that virus replication takes place entirely within the cytoplasm in association with structures formed from modified endoplasmic reticulum. No budding through the cell membranes was observed (Gray & Nettleton, 1987).

By analogy, the maturation of flaviviruses also occurs on intracellular membranes and there is no evidence that the plasma membrane plays any role in replication or maturation (Brinton, 1986). In spite of this, it was found that both the non-structural NS1 and the envelope E proteins are present on the surface of cells infected with the flavivirus yellow fever virus (Schlesinger et al., 1990). Our findings further verify the theory that pestiviruses differ from flaviviruses with respect to morphogenesis and protein structure (Weiland et al., 1990).

The authors wish to thank M. Kaps and G. Müller for excellent technical assistance.

References


(Received 17 December 1990; Accepted 1 May 1991)