Ultrastructural Distribution of the Major Capsid Proteins within Bluetongue Virus and Infected Cells

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SUMMARY

Core proteins VP7 and VP3 have been localized in bluetongue virus (BTV) and BTV-infected cells by immunoelectron microscopy. Gold-labelled monoclonal antibodies to VP7 gave intense labelling with purified BTV core particles and weaker labelling with both directly visualized viral particles, which require no purification, and purified virus particles. It is believed that VP7 is, in a small number of viruses, accessible from the outer surface. The intensity of labelling by anti-VP7 antibodies was markedly increased by treatment of the virus with methanol. Intracellularly, VP7 antibodies also reacted with virus-like particles which appeared to be leaving virus inclusion bodies (VIB), the presumed site of virus synthesis and assembly. These antibodies also reacted with virus-like particles which were bound to the cytoskeleton and did not appear to be virus cores because they also reacted with gold-labelled antibody to the outer coat protein VP2. VP3 was not detected immunologically in either virus or core particles nor in cytoskeleton-associated virus-like particles suggesting an inner core location. VP7 and to a lesser extent VP3 were localized within the matrix of VIB. Virus tubules, a major structure found in infected cells and known to contain the non-structural protein NS1, were found to react with antibodies to both VP3 and VP7.

INTRODUCTION

The genus orbivirus is one of six in the family Reoviridae and is divided into 13 serogroups on the basis of group-reactive tests. The bluetongue serogroup comprises 24 virus serotypes which have been identified using virus neutralization tests. Bluetongue virus (BTV) contains 10 dsRNA segments (Verwoerd et al., 1979; Gorman et al., 1983) each of which codes for a single protein (Grubman et al., 1983; Mertens et al., 1984). Morphologically, the virus consists of a core, composed of 32 capsomeres arranged in icosahedral symmetry, surrounded by an outer fibrillar coat (Bowne & Richie, 1970; Verwoerd et al., 1972). The outer coat layer is composed of two major structural polypeptides (VP2 and VP5) of which VP2 is the dominant BTV serotype-specific antigen (Huismans & Erasmus, 1981; Appleton & Letchworth, 1983). The core contains two major (VP7 and VP3) and three minor (VP1, VP4 and VP6) structural polypeptides. Recent evidence indicates that VP7, the major bluetongue serogroup-reactive antigen (Huismans & Erasmus, 1981; Gumm & Newman, 1982), is the major constituent of the capsomeres on the surface of BTV core particles (Huismans et al., 1987). VP3 has also been shown to contain group-reactive sites (Huismans & Erasmus, 1981).

Cells infected with BTV contain two major non-structural proteins (NS1 and NS2) in addition to the seven structural proteins outlined above (Huismans & Els, 1979; Huismans & Basson, 1983). The cytoplasm of BTV-infected cells is characterized by the presence of fibrillar virus inclusion bodies (VIB) and virus-specific tubules (VT) (Lecatsas, 1968). VIB are believed to be the site of virus morphogenesis. Tubules contain predominantly NS1 (Huismans & Els, 1979) and their function in replication is obscure. We have recently used monoclonal antibodies to NS2, VP2 and NS1 in immunofluorescence and immunogold procedures to localize virus...
proteins in BTV-infected cells and cytoskeletons (Eaton et al., 1987, 1988). In the experiments reported here we have used monoclonal antibodies to locate the major core proteins VP7 and VP3 in infected cells and cytoskeletons and in virus and core particles of BTV. The results confirm the recent finding by Huismans et al. (1987) that VP7 is the major surface component of core particles. In addition, the data indicate that VP7 is present within VIB, is found in association with VTs and is weakly expressed at the surface of some virus particles.

**METHODS**

*Virus, cells and monoclonal antibodies.* An Australian isolate of BTV-1 (CSIRO 156) (St. George et al., 1980) was plaque-purified and used to infect SVP cell monolayers at a multiplicity of approximately 5 p.f.u. per cell as previously described (Eaton et al., 1987). Virus and core particles were purified as described by Mertens et al. (1987). Virus in the cytoplasm of infected cells was harvested at approximately 36 h post-infection (p.i.) by treating infected cells with 1% NP40 in STM (10 mM-Tris-HCl, 0.15 M-NaCl, 5 mM-MgCl₂, pH 7.4). Nuclei were removed by centrifugation and the cytoplasmic supernatant was pelleted through 40% sucrose at 39000 r.p.m, in a Beckman SW41 rotor, for 90 min at 4°C. Virus was resuspended in STM and the preparation referred to as cytoplasmic (crude) virus.

All of the monoclonal antibodies except 20H(2) were derived from subcloned hybridoma cell lines and obtained from J. R. White (unpublished data) as Protein A-purified mouse ascitic fluid. 20H(2) was a hybridoma cell supernatant. Monoclonal antibodies (MAB) used in this study reacted with VP7 (20E9/B7/G2) and (20A11/10), VP3 (7A4/A11), NS2 (20H(2)), NS1 (20E6/A4) and VP2 (9J3/G3). Monoclonal antibody (20E9/B7/G2) and MAB (7A4/A11) precipitated VP7 and VP3 respectively. MAB (20A11/10) reacted with VP7 in Western blots and MAB (9J3/G3) precipitated VP2 and neutralized the virus. The antibodies were conjugated directly to either 6 nm or 14 nm gold particles as described by Slot & Geuze (1985). Monoclonal antibodies are referred to hereafter as MAB aVP7, aVP3, aNS2, aVP2 and aNS1. Except where otherwise stated the aVP7 antibody utilized was 20E9/B7/G2. Antibodies conjugated to gold are designated as follows, Au-MAB aVP7 and Au-MAB zVP2.

*Immunofluorescence.* Cells for immunofluorescence studies were grown on glass coverslips and infected with BTV at an m.o.i. of 5 p.f.u. per cell. Mock-infected and BTV-infected cells (18 to 20 h p.i.) were washed in phosphate-buffered saline (PBS) and treated sequentially with methanol and acetone at −20°C and then air-dried. Viral antigen distribution was determined using appropriate antibodies which were localized with biotinylated anti-mouse antiserum and fluorescein–streptavidin (Amersham). Primary incubations in double labelling experiments were carried out with fixed cells using MAB aVP7 and fluorescein-conjugated anti-mouse IgG serum. Secondary incubations utilized biotinylated MAB aNS2 followed by Texas red–streptavidin (Amersham). Cytoskeletons were prepared by treatment of cells with PBS containing 1% NP40 and 0.1% glutaraldehyde. Following two washes with PBS and PBS containing 1% bovine serum albumin (BSA), (PBS-BSA), and without drying, cytoskeletons were probed with MAB aVP2 or MAB aVP7 as described above. To enhance the fluorescence achieved with MAB aVP7 and to demonstrate activity with MAB aVP3 and MAB aNS1, cytoskeletons were treated with methanol at −20°C prior to washing with PBS-BSA.

*Negative contrast immune electron microscopy (NCIEM).* Cells grown and infected on electron microscope grids (grid cell cultures, GCC) as described by Hyatt et al. (1987) were rinsed three times in PBS. Cells were either fixed for 2 min in PBS containing 0.1% glutaraldehyde or cytoskeletons were generated from them and fixed in PBS containing 0.1% glutaraldehyde and 1% NP40. Cells and cytoskeletons were washed three times (5 min per wash) with PBS. In experiments involving the use of MAB aVP7 and MAB aNS1, cytoskeletons and cells were treated with methanol at −20°C for 2 min. Cells and cytoskeletons were washed six times with PBS-BSA prior to incubation with the appropriate antibody for 1 h at 37°C. Incubation with MAB which was not conjugated directly to gold was followed by six washes with PBS-BSA and a further incubation with Protein A–gold (prepared as described by Slot & Geuze, 1985) at 37°C for 1 h. Gold-labelled viruses and cytoskeletons were rinsed six times in PBS-BSA, post-fixed and stained or critical point-dried as described by Hyatt et al. (1987). Double labelling experiments involved primary incubations with Au–MAB aVP2 (14 nm) as described above followed by a further incubation with Au–MAB aVP7 (6 nm) at 37°C for 1 h prior to washing and fixation.

NCIEM was also performed on purified virus particles and cores adsorbed onto parlodion-filmed copper grids for 2 min. The procedure used for labelling and staining was identical to that outlined above. Preparations were stained with 2% phosphotungstic acid and buffered to pH 6.5 with KOH (KPT).

*Thin section, post- and pre-embedding immunogold labelling.* To probe post-embedded cells, monolayers in plastic tissue culture flasks were infected with BTV at 5 p.f.u. per cell and at 20 h p.i. scraped from the flasks. Infected cells were fixed with 0.25% glutaraldehyde in 0.1% cacodylate buffer (300 mmol/kg, pH 7.2), rinsed in cacodylate buffer, dehydrated in ethanol and infiltrated and embedded in Lowicryl K4M (4°C to −25°C).

Polymerization occurred at −25°C overnight under u.v. irradiation. Sections were cut onto nickel grids, washed with PBS–BSA (six times, 3 min each), incubated with normal rabbit serum (1 h, 37°C) followed by MAbs.
Results

Analysis of BTV particles by NCIEM

Cells grown on electron microscope grids were infected with BTV and 24 h later viruses released from the cells and adsorbed to the grid were probed with Au-MAb αVP7. These viruses (termed native viruses) reacted weakly but specifically with the antibody (Fig. 1a). Not all viruses were labelled and those which were possessed only one or two gold probes. Viruses isolated from the crude cytoplasmic fraction of infected cells and partially purified by sedimentation through a sucrose column gave more intense labelling when probed with Au-MAb αVP7 but differed morphologically from native viruses in that capsomeres could be observed underlying the outer fibrillar coat (Fig. 1d).

A significant proportion of VP7 in infected cells exists in a soluble form (Eaton et al., 1987). To eliminate the possibility that viruses may be contaminated with adventitiously adsorbed VP7, BTV was purified as described by Mertens et al. (1987) and probed with Au-MAb αVP7. Morphologically, these viruses possessed the outer fibrillar coat and reacted weakly with antibody. The results (not shown) were similar to those shown in Fig. 1(a). The intensity of labelling by Au-MAb αVP7 was increased by treatment of native, cytoplasmic or purified virus particles with methanol (Fig. 1b). In order to confirm that Au-MAb αVP7 was binding to complete viruses, GCC were incubated with Au–MAb αVP2 and both Au–MAb αVP2 (14 nm) and Au–MAb αVP7 (6 nm). The results (Fig. 2) show that such extracellular particles are labelled not only with Au–MAb αVP2 but with Au–MAb αVP7. It should be noted that although methanol treatment of the virus increases αVP7 activity we have found it to decrease αVP2 activity; hence the decreased number of Au–MAb αVP2 probes depicted in Fig. 2 (inset).

Unlike complete viruses, purified BTV core particles labelled intensely with Au–MAb αVP7 in the absence of any unmasking agent (Fig. 3). The identity of the particles as cores was confirmed by the presence of conspicuous capsomeres. When purified virus and core particles were incubated with MAb αVP3 and Protein A–gold, no labelling occurred suggesting that either the VP3 epitope underlies VP7 in BTV cores or the VP3 epitope is not presented to the antibody in an appropriate conformation for reaction.

Fluorescence

Immunofluorescence studies using fixed BTV-infected cells showed VP7 to be distributed throughout the cytoplasm. Both VP7 MAbs used in this study generated similar fluorescent patterns in fixed cells. The pattern for MAB 20E9/B7/G2 is shown in Fig. 4(a). Superimposed on a faint fluorescence distributed throughout the cytoplasm are small discrete foci and large circular bodies of fluorescence. Double labelling experiments (Fig. 4a, b) with MAB αVP7 and MAB αNS2 [NS2 is a component of virus inclusion bodies (Eaton et al., 1987)] show a coincidence of labelling, indicating that the circular, fluorescent structures are VIB and that they have a VP7 component. Immunofluorescent probing of detergent-generated cytoskeletons with MAB αVP7 (20E9/B7/G2) revealed an additional labelling pattern. As seen in Fig. 4(c) the cytoplasm contained many short fluorescent rods. MAB αVP7 (20A10/A11) did not reveal these structures. Immunogold procedures were utilized to determine the nature of the VP7-containing structures associated with the cytoskeleton of BTV-infected cells.
Fig. 1. Electron micrographs of BTV and core particles labelled with Au–MAb αVP7 (14 nm). (a) Native viruses prepared from GCC. Preparation was negatively stained with KPT. (b) Viruses prepared by GCC technique and treated, prior to labelling, with methanol. The preparation was critical
Fig. 2. Electron micrograph of a negatively stained, VP2-labelled GCC infected with BTV. Inset, double-labelled (Au-MAb αVP2, 14 nm; Au-MAb αVP7, 6 nm) virus. Cell (c) viruses (v). Bar markers represent 100 nm.

Intracellular distribution of VP7 and VP3 by immunogold-labelled MAb

Due to the low antigenic mass of some viral proteins, particularly on the surface of embedded sections, both pre- and post-embedding immunolabelling procedures were used in an attempt to optimize the localization of intracellular capsid proteins.

In preliminary experiments, cytoskeletons were prepared by NP40 treatment of BTV-infected cells on grids and were then probed with Au-MAb αVP7. Labelling of virus-like particles was intense after exposure of the cytoskeleton to methanol (Fig. 1 c) and weak without methanol treatment (data not shown). Sections of Au-MAb αVP7 pre-labelled cytoskeletons revealed gold label, not only with virus particles throughout the cytoplasmic regions and at the cell periphery but also with virus particles which were intimately associated with the VIBs and/or fibrillar material morphologically similar to that constituting the VIB (Fig. 5 a). Lowicryl sections labelled with MAb αVP7 gave similar results but the degree of labelling was substantially reduced (Fig. 5 f). Sections of Au-MAb αVP2 pre-labelled cytoskeletons revealed gold label associated with both cytoskeleton-associated particles (Fig. 5 b) and those apparently leaving the VIB (Fig. 5 c). This suggests that these intracellular particles, although labelled with Au-MAb αVP7 are unlikely to be cores because they possess at least some of the outer coat protein VP2.
Fig. 3. Purified BTV core particles labelled with Au-MAb zVP7. The preparation was negatively stained with KPT. Bar marker represents 50 nm.

Fig. 4. Immunofluorescent labelling of BTV-infected cells and cytoskeletons. (a) Cells fixed with glutaraldehyde and labelled with MAAb zVP7 and fluorescein. (b) Same cell but labelled also with antibody zNS2 and streptavidin–Texas red. (c) Cytoskeleton labelled with antibody zVP7 and streptavidin–fluorescein. VIB (arrows), short fluorescent rods (r).

Fig. 5. Electron micrographs of pre-embedded and post-embedded gold-labelled sections of BTV-infected cells. (a) Distribution of VP7 in an infected cytoskeleton. The cells were treated with methanol prior to labelling, leading to a slight alteration in virus and tubule morphology. VIB (arrowhead), viruses (v). (b) Association of VP2 with cytoskeleton-associated viruses (v), virus-specified tubules (vt). (c) Association of VP2 with viruses (v) emerging from a VIB (arrowhead). (d) Labelled Lowicryl section showing the association of NS2 with VIB (arrowheads). (e, f) Labelled Lowicryl section showing distribution of VP7. Viruses (v), VIB (arrowhead). Bar markers represent 100 nm.
Distribution of bluetongue virus proteins
Virus inclusion bodies within Lowicryl sections were identified by their ability to bind MAb αNS2 (Fig. 5d). Labelling of the internal matrix of VIBs was also achieved with MAb αVP7 (Fig. 5a) but not with MAb αVP2 (data not shown). The gold probes associated with MAb αVP7 did not appear to be associated with the dense, virus-like structures within VIBs. The data suggest that VP7 is part of the VIB matrix.

**Capsid proteins and virus tubules**

Previous work has shown that antibodies to VP3 react with tubules in infected cells (Eaton et al., 1987). Immunogold labelling of thin sections and cytoskeletons confirms the association of VP3 with virus-specified tubules (Fig. 6a). No labelling with MAb αVP3 was observed for virus particles and only very weak labelling (one or two particles per VIB) was associated with the VIBs. Fluorescence studies (Fig. 4c) suggested that VP7 may also be associated with tubules. To
help clarify this relationship between VP7 and tubules, cytoskeletons were labelled with MAb αVP7 (20E9/B7/G2) (Protein A-gold system). Preparations were subsequently processed for thin sections and critical point-dried GCCs. Both procedures gave similar results in that VTs were specifically labelled. Fig. 6(b) unlike Fig. 6(a) illustrates the results (MAb αVP7) obtained by thin section electron microscopy.

**DISCUSSION**

Core proteins VP7 and VP3 have been localized in BTV and BTV-infected cells by immunoelectron microscopy. The results indicate that VP7 is associated predominantly with the surface of the virus core or nucleocapsid as reported by Huismans et al. (1987). The protein is also found associated with VIBs, VTs and may be accessible at the surface of both native and purified viruses from infected cells.

Viruses analysed by the GCC technique as described by Hyatt et al. (1987), have not been subjected to any forces which could damage the outer coat. The ability of some of these viruses to bind Au–MAb αVP7 is therefore not an artefact of virus preparation but probably represents the *in vivo* state. The reaction of purified viruses with Au–MAb αVP7 strongly indicated that the occasional expression of VP7 on the surface of complete, native viruses was not due to adventitiously adsorbed protein. The possibility exists however that some damage, albeit slight, to the outer capsid layer may occur during sedimentation onto a sucrose cushion and so account for the low reactivity of Au–MAb αVP7. The number of labelled antibodies associated with the crude cytoplasmic preparation was greater than that found with purified virus particles. This observation may be correlated with a more conspicuous appearance of capsomeres which was suggested to be mainly, if not entirely, composed of VP7 (Huismans et al., 1987). The difference in morphology and degree of labelling of cytoplasmic and purified viruses may well be due to an alteration in, or damage to, the structure of the outer coat of the cytoplasmic virus induced by pelleting through sucrose and/or subsequent resuspension during partial purification. The low labelling of native viruses and the progressive increase of labelling of virus particles as they gain the morphological characteristics of cores, in the absence of methanol treatment, suggests that VP7 may project beyond the core surface into the outer coat where in a small proportion of viruses it may be able to interact with MAb αVP7. As stated above, it is unlikely that the outer capsid layer observed in the GCC of viruses have been damaged, therefore the Au–MAb αVP7-labelled native viruses may indicate a heterogeneity in the virus population. Such heterogeneity may be due to either the outer coat not covering the core in a consistent manner or the lability of the outer coat proteins following release from infected cells.

The use of methanol significantly increased the intensity of VP7 labelling in native or glutaraldehyde-treated viruses and virus-infected cells. Methanol, a known protein precipitant, presumably causes a distortion of the normal protein conformation by sequestering water molecules from the sphere of hydration of the virus proteins. Such distortion of the diffuse outer coat of BTV (i.e. VP2 and VP5) would easily result in an increased exposure of the underlying core protein VP7.

The absence of MAb αVP3 labelling with virus and core particles was not unexpected. Huismans et al. (1987) found that, following adsorption and penetration of infected cells, BTV particles lose not only VP2 and VP5, but also VP7-containing capsomeres of the virus core. The 'sub-core' particles thus generated contained VP3. The immunoelectron microscopical studies reported here support the conclusion that VP3 is present within core particles and underlies the VP7-containing capsomeres. Specifically the results imply that the epitope involved in binding to MAb αVP3 is buried within the virus core although the possibility that the remainder of the protein may be present on the core surface cannot be discounted. Recently, Huismans & Cloete (1987) found that the gene coding for VP3 (RNA 3) was highly conserved among BTV serotypes. In contrast, RNA 7, which codes for VP7, was not highly conserved. It is interesting to speculate that the variability in RNA 7 may be due, in part, to the selective immunological pressure placed on the potentially more exposed VP7 protein.

Intracellularly, VP7 was localized to virus particles and VIBs. Collectively, the labelling of infected cells with Au–MAb αVP7 and Au–MAb αVP2 shows that the core protein VP7 is
incorporated into virus particles within VIBs. Furthermore the particles emerge from the VIB with at least part of the outer coat VP2 component. Au–MAb αVP2 fails to react with the internal matrix of VIBs, thus the localized labelling of VP2 reported here may represent the sites of VP2 synthesis. VP3 may also be present within VIB. Although the labelling of VP3 within these structures is poor (approximately one or two gold particles per VIB) it should not be dismissed as negative. Within viruses, VP3 is present in the core at about half the amount of the other major core protein VP7 (Huismans et al., 1987). If this is representative of the VIB protein content, and considering the level of VP7 labelling of VIB, the low but specific level of VP3 labelling is not unexpected. It is also possible that the optimum conditions for VP3 labelling within VIBs have yet to be determined.

Virus-specified tubules, a structure common within BTV-infected cells, are known to contain predominantly NS1 (Huismans & Els, 1979). Results from this study show that VP3 and VP7 are also associated with the tubules. The labelling is specific and of the αVP7 MAbs only 20E9/B7/G2 labelled the tubules, suggesting that the specificity is real and not a consequence of protein contamination during cell extraction. The function of VTs still remains obscure but perhaps the finding that they are composed of a mixed protein population may aid the eventual elucidation of their role. The different labelling patterns observed with the two αVP MAbs, namely the ability of one to react with VTs suggests that they may react with different epitopes. Furthermore as both MAbs react with native ‘untreated’ and ‘treated’ viruses it is possible that at least two VP7 epitopes may on occasion be exposed on the surface of BTV particles.

Work on the distribution of other viral proteins such as NS2 and VP5 in viruses and cells is continuing.

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REFERENCES


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