

Detection of bluetongue virus RNA in cell cultures and in the central nervous system of experimentally infected mice using *in situ* hybridization

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ABSTRACT

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Two radiolabelled complementary DNA (cDNA) probes (1663 bp and 200 bp respectively) were prepared from the genome segment that encodes the non-structural protein 1 (NS1) of bluetongue virus serotype 4 (BTV4). The probes were used to optimize the *in situ* hybridization (ISH) method on baby hamster kidney-21 (BHK-21) cells and to investigate the use of the technique as a diagnostic procedure.

Cells were infected with BTV4 at a multiplicity of infection of 0,5 PFU/cell. An intense cytoplasmic hybridization signal could be detected from 3 hours post-infection onwards, reaching a peak at 17 hours. The ISH procedure has potential use as a diagnostic technique, but will probably find a wider application in pathogenesis studies.

An *in situ* hybridization method was also developed for the detection of BTV RNA in the central nervous system of newborn mice after intracranial inoculation with BTV10. Viral RNA-positive cells were detected from Day 3 onwards, predominantly in areas where the virus caused necrotic encephalitis.

INTRODUCTION

Bluetongue is an insect-transmitted disease of domestic and wild ruminants and has important economic implications, particularly in sheep. Bluetongue virus (BTV), the etiological agent, is the prototype of the Orbiviruses and 24 different serotypes are recognized worldwide (Jeggo & Wardley 1985; B. J. Erasmus, Onderstepoort Veterinary Institute, personal communication 1991). The Orbiviruses comprise a large genus within the Reoviridae family (Verwoerd, Huismans & Erasmus 1979; Gorman, Taylor & Walker 1983). The genome of

Orbiviruses consists of 10 double-stranded RNA (dsRNA) segments, each of which encode at least 1 viral protein (VP). The virion has a core consisting of 2 major proteins, VP3 and VP7, and 3 minor proteins VP1, VP4 and VP6. The core is surrounded by a diffuse protein layer consisting of 2 proteins, VP2 and VP5. The BTV genome also encodes 3 non-structural (NS) proteins, NS1, NS2 and NS3 (Huismans 1979; Van Dijk & Huismans 1988).

Currently, the routine diagnosis of BTV infection relies primarily on a variety of serological tests and isolation of the virus in tissue culture and embryonated eggs (Osburn, McGowan, Heron, Loomis, Bushnell, Stott & Utterback 1981; Squire, Stott, Dangler & Osburn 1987). Viral proteins of BTV can be detected by using immunoperoxidase staining techniques (Anderson, Phillips, Waldvogel & Osburn 1989; MacLachlan, Jagels, Rossitto, Moore

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& Heidner 1990). Nucleic acid hybridization is becoming an increasingly useful technique for the detection of viral infection (Haase, Brahic, Stowring & Blum 1984; Warford 1988) and has many applications in veterinary infectious diseases (Paul 1990). The use of hybridization methods to detect BTV in dot-spot samples from infected tissue cultures (Huismans & Cloete 1987; Squire, Chuang, Chuang, Doi & Osburn 1985; Squire *et al.* 1987; Venter, Viljoen, Nel, Huismans & Van Dijk 1991) and *in situ* hybridization (ISH) (Dangler, Dunn, Squire, Stott & Osburn 1988) has been reported. Recently, the ISH technique for the detection of BTV in cell cultures was found to be less sensitive when compared with viral isolation (Schoepp, Blair, Roy & Beaty 1991). However, the potential diagnostic usefulness of ISH has been widely accepted (Grody, Cheng & Lewis 1987; Unger & Brigati 1989).

Huismans & Cloete (1987) and Venter *et al.* (1991) described the use of the BTV NS1-gene as a group-specific probe to detect the virus. Furthermore, the frequency of transcription of the NS1 mRNA during replication of the virus is 4–5 × higher than the transcription of the other genome segments (Huismans & Verwoerd 1973; Huismans, Bremer & Barber 1979). The NS1 gene probe can thus be used to detect viral RNA at an early stage of replication.

Conventional ISH on histological tissue sections allows the sensitive and specific localization of viral nucleic acids with preservation of the morphology of cells and tissues. The technique has been widely used in the study of the pathogenesis of viral disease and can give more insight into the spread of the virus, mechanisms of tissue damage and virus-host interactions in disease (Haase 1986). It has been suggested that the differential pathogenesis of BTV infection in sheep and cattle results in part from differences in target cell specificity (Ellis, Luedke, Davis, Wechsler, Mecham, Pratt & Elliott 1990). The ISH technique has potential application in the study of the pathogenesis of BTV infection by identifying the tissues and cell type involved in viral replication in the vertebrate host.

The aim of this study was to optimize the ISH method for the detection of BTV in cell cultures using group-specific probes and to investigate its use as a diagnostic procedure. An ISH technique was subsequently developed for pathogenesis studies to localize BTV RNA in the central nervous system of experimentally infected mice.

MATERIALS AND METHODS

Viruses

A South African attenuated strain of BTV4 (Huismans & Cloete 1987) was used to infect the cell

cultures. The virus was propagated in monolayer baby hamster kidney-21 (BHK-21) cells according to the method described by Verwoerd (1969). A recently isolated BTV10 field strain, demonstrated to be pathogenic for mice, was used to infect newborn mice. The virus was passaged once in embryonated chicken eggs and twice in BHK-21 cells.

Tissue culture cells

BHK-21 and Vero cells, originally obtained from the American type culture collection, (ATCC, 12301 Parklawn Drive, Rockville, Maryland, USA 20825) were used for the propagation and titration of the BTV strains respectively. The cells were grown in Roux flasks or roller bottles as monolayers in modified Eagle's medium supplemented with 5 % irradiated bovine serum (Verwoerd, Oellerman, Broekman & Weiss 1967).

Pretreatment of slides

Microscope slides were cleaned to limit non-specific binding of the probes. The slides were sequentially immersed in 0,5 % (v/v) liquid detergent (Decon 75, Atomic Export Import, RSA) for 30 min, washed in running tap water (30 min), rinsed in ultra-pure water (2 × 3 min), rinsed in 99 % ethanol (2 × 5 min) and allowed to air-dry. The slides were then coated in the adhesive 2 % (v/v) 3-aminopropyltriethoxysilane (APES) in dry acetone for 5 sec, and then rinsed in acetone (2 × 1 min) and ultra-pure water (2 × 1 min) and allowed to dry overnight at 42 °C.

Preparation of infected tissue culture cells

Tissue culture cells were grown on sterile APES-coated slides in petri dishes. After 24 h incubation the cells were infected with BTV4 at 0,5 pfu/cell. The virus was allowed to adsorb to the cells for 45 min at 37 °C and the cells were then washed once with Eagle's medium. Fresh medium was added and the petri dishes were incubated at 37 °C. Slides were removed from the petri dishes after various periods post infection (p.i.) and fixed in a freshly prepared 4 % paraformaldehyde, 2 × SSC (1 × SSC is: 0,15 M NaCl, 0,015 M sodium citrate, pH 7,0) solution containing 5 mM MgCl₂. The slides were subsequently dehydrated in a series of increasing ethanol concentrations and stored dust free and dry at room temperature.

Animals and animal inoculations

Two-day-old MF-1 inbred mice of either sex were used. Their parents were obtained from the National Institute of Virology (Johannesburg, RSA). Mice were inoculated by the intracerebral route (n=60) with BTV10 at 6 × 10² plaque forming units/0,03 ml

of Eagle's medium. Uninfected control mice ($n=12$) were inoculated with 0,03 ml Eagle's medium.

Preparation of tissue sections

For ISH, 4 infected mice were randomly selected and sacrificed on a daily basis for 6 d, while 2 uninfected control mice were sacrificed on Days 2, 4 and 6. Mice were euthanized with ether and the entire brain removed. The brains were fixed by immersion in 10 % buffered neutral formalin for 18 h and then cut into coronal or transverse slices. Tissues were routinely embedded in paraffin wax and 5–6 μm thick sections were prepared. Sections for histological examination were stained with haematoxylin and eosin (HE).

Viral assays

For the viral assay, 4 infected mice were sacrificed on a daily basis for 6 d. Two uninfected control mice were also sacrificed on Days 2, 4 and 6. A plaque reduction-neutralization test was used for the assay of BTV (Howell, Verwoerd & Oellermann 1967). BTV inoculums were titrated in monolayer Vero cells. Infected mouse brains were titrated after each day of harvesting. Titers were calculated by the method of Karber (1931), expressed as PFU/ml and the values were averaged for each time point.

Preparation of cDNA probe

A cloned, truncated 1663 base pair (bp) BTV4 NS1-gene (lacking c. 100 bp from the 3'-terminal end) as well as a 200 bp 3'-end of this truncated gene were used as DNA probes. The 1663 bp NS1-gene was cloned as a Pst I fragment in pBR322 and designated p42 (Huismans & Cloete 1987). The 200 bp 3'-terminal fragment of the truncated NS1-gene was subcloned as a Hind III fragment from p42. Both probes were prepared by cutting the respective NS1-fragments from a gel using a modified freeze squeeze method (Heery, Gannon & Powell 1990). They were labelled with ^{32}P -dCTP (1 000 $\mu\text{Ci}/\text{ml}$) using a random priming method (Amersham). The specific activity of the probes was generally in the region of $5\text{--}8 \times 10^8$ cpm/ μg DNA. The infected tissue culture cells and the tissue sections were probed with 30 ng of probe. An African horsesickness virus serotype 3 (AHSV 3) probe was derived from the cloned NS1-gene (Bremer, Huismans & Van Dijk 1990).

In situ hybridization

Slides with cultured cells were incubated in 100 mM glycine, $2 \times \text{SSC}$ for 15 min at 37 °C. They were then acetylated (2,5 % tri-ethanolamine in acetic anhydride; 2×5 min), rinsed in 50 % formamide, $2 \times \text{SSPE}$ for 5 min and prehybridized (50 % formamide, 10 % dextran sulphate, $2 \times \text{SSPE}$, 100 mM

glycine, 0,1 % SDS, 2 % 50 \times Denhardt's, 10 mM Tris pH 7,4 and 200 $\mu\text{g}/\text{ml}$ salmon sperm DNA) for 30 min at 52 °C prior to the application of the probe solution. Between 5–10 μl of heat-denatured probe solution was added to each tissue culture well and incubated for 4–5 h at 52 °C. Following hybridization the slides were washed twice for 1 h in a $2 \times \text{SSPE}/50$ % formamide solution and once in 50 % formamide, 0,1 % SDS, $2 \times \text{SSC}$ at 37 °C for 1 h. Slides were dehydrated in a series of ethanol concentrations of 50 %, 70 % and 90 % (each containing 0,3 M NH_4 acetate), and 100 %.

Tissue sections of mouse brain were dewaxed and rehydrated through xylene (3×5 min), ethanol and water, immersed in 0,2 N HCl at room temperature for 20 min, transferred into $2 \times \text{SSC}$ buffer at 70 °C for 10 min, rinsed in ultra-pure water and transferred to 0,05 M Tris-HCl pH 7,6 in ultra-pure water. Sections were then digested in $2 \times \text{SSC}$, 0,1 % SDS containing proteinase K (Boehringer Mannheim) at a concentration of 0,005 $\mu\text{g}/\text{ml}$ for 30 min at 37 °C. Sections were then post-fixed in 4 % paraformaldehyde, $2 \times \text{SSC}$ solution containing 5 mM MgCl_2 (5 min). They were then acetylated and rinsed in 50 % formamide, $2 \times \text{SSPE}$ solution and directly prehybridized as described for the tissue culture cells. Hybridization was performed at 52 °C for at least 16 h.

Detection of hybridized probes

Hybrids, formed with the radio-active probes, were detected after 4–7 d by autoradiography using a photographic emulsion (LM-1, Amersham) and following the instructions of the manufacturer. Slides were dipped in the emulsion, dried and exposed at 4 °C in the dark. After the appropriate period of exposure, the slides were developed for 5 min at 15 °C in Phenisol developer (Ilford), rinsed briefly in water and fixed for 5 min in Hypam fixative (Ilford). Cells were counterstained with HE, dehydrated and mounted. Hybridization signals were detected by light field microscopy.

RESULTS

Detection of BTV nucleic acids in tissue culture cells

The BHK-21 cells were grown as monolayers and infected with BTV4 at a multiplicity of infection (MOI) of 0,5 PFU/cell. Slides were removed from the petri dishes at 1, 2, 3, 4, 7, 9, 13, 17 and 25 h p.i. and fixed. Controls included uninfected slides probed to the BTV4 NS1-gene probe as well as BTV infected slides probed to the AHSV NS1-gene probe at 13 h p.i.

No hybridization signals to BTV nucleic acids were detected at 1 h p.i. and 2 h p.i. (Fig. 1), while at 3 h p.i. visible signals could be detected (Fig. 2). The

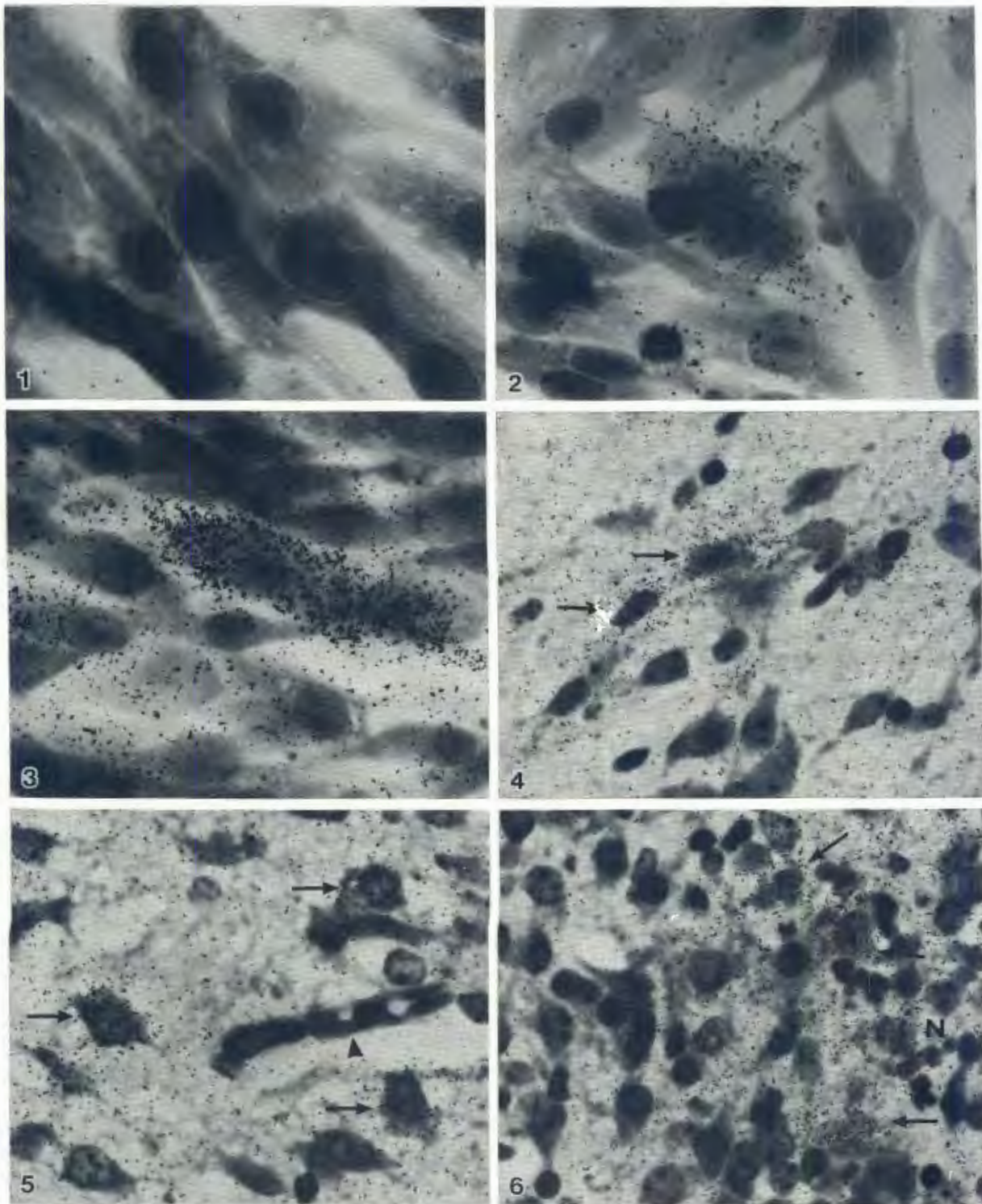


FIG. 1–3 *In situ* detection of viral RNA in BTV-infected BHK cell cultures fixed at 2 h p.i. (Fig. 1), 3 h p.i. (Fig. 2) and 13 h p.i. (Fig. 3). There is an increase in the number of infected cells as well as the hybridization signal at 13 h p.i. compared to 3 h p.i. No specific signal is detected at 2 h p.i.

FIG. 4–6 Localization of viral RNA in sections of BTV-infected mouse brain

FIG. 4 Grains overlying 2 neural cells (arrows) in the thalamus of a mouse at 4 d p.i.

FIG. 5 Several positive cells (arrows) are present in the thalamus at Day 5. Note that viral RNA is not detected in endothelial cells or in the vascular lumen (arrowhead)

FIG. 6 Cells containing viral RNA (arrows) on the edge of an area of necrotic encephalitis (N) at Day 6

number of positive cells and the hybridization signals increased thereafter (Fig. 3) and reached a peak at 17 h (not shown). At 25 h p.i. less staining was observed (not shown). Uninfected cells were negative, as were the BTV infected tissue culture cells probed with the AHSV 3 NS1-gene probe (not shown).

Clinical signs in the mice

Neurological signs developed 4–5 d after inoculation. Affected animals became somnolent, were found separated from their mothers and littermates and usually died within a day. Clinical signs were not present in the control mice during the course of the experiment.

Morphological changes in the central nervous system

Macroscopical lesions in which the cerebrum appeared abnormally soft were limited to animals that died on Days 5 and 6. On histological examination scattered neural cells in the subventricular zone were necrotic on Day 2, and the number of necrotic cells increased on subsequent days. Towards Days 3 and 4 the necrosis also involved neural cells in the olfactory lobe. There was an associated inflammatory infiltrate composed of small numbers of neutrophils. By Days 5 and 6 randomly distributed foci of necrosis were present in the subventricular area, caudate/putamen, subcortical gray matter and olfactory lobe. Necrotic foci often became confluent and contained accumulations of neutrophils, while the adjacent brain substance was oedematous. Capillaries in the affected gray and white matter appeared more prominent than normal due to endothelial cell hypertrophy and an attendant perivascular cuffing of round cells was evident. Small numbers of neutrophils infiltrated the choroid plexus.

Detection of BTV nucleic acids in sections of mouse brain

The presence of viral RNA was indicated by silver grains overlaying cells. In control mice the background was low and no definite signals were evident. No virus RNA-positive cells were detected on Days 1 and 2 in infected mice. On Days 3 and 4 individual or small groups of positive cells were present predominantly in areas of necrosis and inflammation in the subventricular zone and olfactory lobe (Fig. 4). On Days 5 and 6 positive cells were most abundant immediately adjacent to areas of necrosis, as well as in regions of the cerebrum not morphologically affected particularly in the hippocampus (Fig. 5 & 6). Viral RNA was not observed in endothelial cells and vascular lumina, ependymal cells, or in the choroid plexus (Fig. 5).

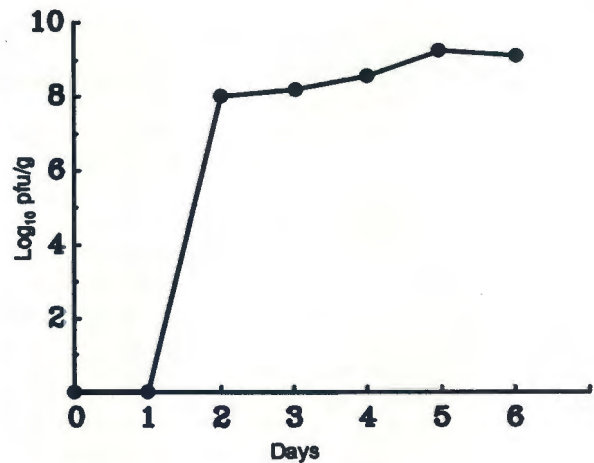


FIG. 7. Viral growth curve in the brains of mice after intracerebral inoculation with BTV

Viral assays

The amount of virus in the brains monitored by plaque titration assays increased rapidly on Day 2 and reached a peak on Day 5 (Fig. 7).

DISCUSSION

The initial aim of this study was to optimize the ISH technique on infected cell cultures and evaluate it as a diagnostic procedure. No visible differences in hybridization signal between the 2 probes were found indicating that the differences in length did not influence penetration of the target cells and hybridization. The probes detected BTV RNA in the cell cultures at 3 h p.i. These results, obtained with a MOI of 0.5 PFU/cell, corresponded to those obtained using a dot-spot ISH technique which detected viral ssRNA at 4 h p.i. in cultured cells infected at a MOI of 2 PFU/cell (Venter *et al.* 1991). BTV can therefore be detected within 3–4 d with both conventional and dot-spot ISH. Squire *et al.* (1987) reported positive identification of BTV RNA, which was extracted from infected embryonated chicken eggs, within 3 d. Although the potential diagnostic usefulness of ISH has been widely accepted (Unger & Brigati 1989), the technical burden of manual sample preparation and processing, as well as the lower sensitivity of ISH compared with viral isolation for the detection of BTV (Schoepp *et al.* 1991; Wechsler, Austin & Wilson 1990), may limit the use of conventional ISH in the clinical laboratory.

In order to apply ISH for pathogenesis studies, the technique was optimized using the NS1-gene probes for the detection of BTV RNA in the central nervous system of newborn mice after intracerebral inoculation. Viral RNA-positive cells were initially detected on Day 3. The number of positive cells increased towards Day 6 at which time the experi-

ment was terminated. Morphological changes and spread of the virus, as detected by virus RNA-positive cells in this study, were in agreement with those reported by Narayan & Johnson (1972) who studied the spread of BTV infection after intracranial inoculation in the CNS of newborn mice using immunofluorescent antibody staining. They found that the infection was limited to immature cells of the subventricular zone 2–4 d after inoculation and that subsequent spread of the infection over the next 4 d followed the migratory paths of these cells into the basal ganglia, cortex, olfactory bulbs and hippocampus. No attempt was made in this study to identify the specific cell type involved in viral replication in the mice. However, Waldvogel, Anderson, Higgins & Osburn (1987) concluded that both glial fibrillary acidic protein (GFAP)-positive astrocytes and GFAP-negative cells presumably immature glial cells were affected in BTV infection in newborn mice following subcutaneous inoculation. Viral antigen was not detected in endothelial cells in the present study which is in agreement with previous studies by Narayan & Johnson (1972) and Waldvogel *et al.* (1987).

Further application of this technique should be aimed at studying the pathogenesis of BTV infection in the vertebrate host. Previous studies on the pathogenesis of BT disease used a combination of techniques including titration of the virus in tissue samples collected from infected sheep, histology, and immunofluorescence and immunohistochemical staining of BTV antigens in tissues (Lawman, 1979; MacLachlan *et al.* 1990; Pini 1976; Stair 1968) as well as ISH for the detection of nucleic acids in blood mononuclear cells (Dangler, De La Concha-Bermejillo, Stott & Osburn 1990). A combined immunocytochemical and *in situ* hybridization procedure which allows concurrent localization of cell-specific antigen and viral nucleic acid in the same cell (Gendelman, Moench, Narayan, Griffin & Clements 1985) may have potential application in assessing target cell specificity in BTV infections.

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