Replication of epizootic haemorrhagic disease and bluetongue viruses in DH82 cells

E.W. Howerth[(1)], G.S. Parlavantzas[(2)] & D.E. Stallknecht[(3)]

(1) Department of Pathology, College of Veterinary Medicine, University of Georgia, Athens, GA 30605, United States of America
(2) 1226 Clairmont Rd, Decatur, Georgia 30030, United States of America
(3) Department of Medical Microbiology, College of Veterinary Medicine, University of Georgia, Athens, GA 30605, United States of America

Summary

Bluetongue viruses (BTV) and epizootic haemorrhagic disease viruses (EHDV) can be propagated in a variety of mammalian cell lines, but in the ruminant host these viruses infect and replicate in endothelium and monocytes/macrophages. *In vitro* studies of virus-monocyte interactions typically rely on primary ruminant macrophage cultures that cannot be propagated and a continuous cell line of monocytes would be useful in the study of these interactions. The objective of this study was to determine if DH82 cells, a continuous canine macrophage-monocyte cell line, are susceptible to BTV and EHDV infection. We found DH82 cells susceptible to both BTV and EHDV infection resulting in high titres and the development of massive numbers of viral-associated tubules. This system could be useful for viral-monocyte receptor and viral-associated cytokine expression studies, and the production of large quantities of viral-associated tubules for structural or functional studies of these tubules.

Keywords

Bluetongue virus– DH82 cells – Electron microscopy – Epizootic haemorrhagic disease – Viral-associated tubules – Virus isolation.

Introduction

Bluetongue viruses (BTV) and epizootic haemorrhagic disease viruses (EHDV) can be propagated in a variety of mammalian cell lines ranging from green monkey fibroblasts (Vero cells) to bovine nasal turbinate cells (13). However, in the ruminant host, these viruses infect and replicate in endothelium and monocytes/macrophages (5, 7). Primary ruminant endothelial cell cultures that can be used to study virus-endothelial interactions are available and can be propagated and passed numerous times (14). On the other hand, continuous ruminant macrophage-monocyte cultures are not available and *in vitro* studies of virus-monocyte interactions rely on primary macrophage-monocyte cultures that cannot be propagated. Dogs were shown to be susceptible to BTV infection when pregnant bitches died of bluetongue following inoculation of a BTV-contaminated vaccine (3). We hypothesised that DH82 cells, a commercially available continuous canine macrophage-monocyte cell line, would be susceptible to these viruses and could prove useful in the study of virus-monocyte interactions (15). The objective of this study was to determine if DH82 cells are susceptible to BTV and EHDV infection.

Materials and methods

Cells

DH82 cells (American type culture collection CRL-10389) were propagated in minimum essential medium (MEM) with non-essential amino acids, 2 mM L-glutamine, and 10% foetal calf serum (MEM growth media). Baby hamster kidney (BHK-21) cells were propagated as described previously (8).

Viral inoculums

Flasks monolayered with BHK-21 cells were inoculated with EHDV serotype 2 (EHDV-2) or

BTV serotype 10 (BTV-10). At 80% cytopathic effect, the flasks were scraped and infected cells and supernatant were collected and centrifuged. The resulting pellet was resuspended in Dulbecco's phosphate-buffered saline, sonicated, recentrifuged and aliquots frozen at –70°C. The viral titre was determined by endpoint titration in BHK-21 cells. The EHDV-2 was originally isolated from a white-tailed deer lymph node in BHK-21 cells, and the BTV-10 was from stock originally obtained from the National Veterinary Services Laboratories, USDA, in Ames, Iowa.

Experimental design

DH82 cells scraped from a 25 cm^2 flask were resuspended in 14 ml of MEM growth media and fourteen wells of a 24-well plate were each seeded with 1 ml of resuspended cells. Five wells were inoculated directly with $10^{2.5}TCID_{50}$ of EHDV-2 and five wells were inoculated directly with $10^{2.5}TCID_{50}$ of BTV-10. The other four wells containing cells served as uninoculated negative control wells to compare cytopathic effects and for immuno-cytochemistry. The remaining ten wells contained MEM growth media, but no cells, and served as virus controls: five were inoculated with $10^{2.5}TCID_{50}$ of EHDV-2 and five with $10^{2.5}TCID_{50}$ of BTV-10. On days 1 to 5 post inoculation (pi), one DH82 and one cell-free virus inoculated well for each virus was harvested. The DH82 wells were scraped and the cells resuspended in the media and 500 µl was removed for virus endpoint titration. The remaining contents of the virus-inoculated DH82 wells were cytocentrifuged onto glass slides, fixed by immersion in cold acetone for 10 min, and stained for EHDV or BTV by immunocytochemistry. On days 2 to 5 pi, one uninoculated DH82 well was scraped and the contents prepared for immunocytochemistry by cytocentrifugation.

Virus endpoint titrations

Viral titres were quantified by endpoint titration in 96-well tissue culture plates using BHK-21 cells. Six ten-fold dilutions were made and eight replicate wells were used for each dilution; titres were determined as described by Reed and Muench (9).

Immunocytochemistry

Fixed slides were hydrated in phosphate-buffered saline for 15 min and then treated with 1.0 mM EDTA solution, pH 6.8 for 30 min at 37°C. Slides were rinsed, blocked with 0.05% casein solution for 30 sec, rinsed, and then either rabbit anti-BTV-10 (1:1 000) or rabbit anti-EHDV-2 (1:2 000) was applied for 2 h at room temperature. After rinsing, antibody was detected using a commercially available avidin-biotin alkaline phosphatase technique (BioGenex) with fast red as the chromagen (BioGenex). In duplicate slides, rabbit anti-*Helicobacter* antibody replaced the primary antibody as a negative control.

Transmission electron microscopy

A 25 cm^2 flask of DH82 cells was inoculated with$10^{4.43}TCID_{50}$ of EHDV-2. On day 1 pi, 50% of the flask was scraped and the cells suspended in the media of the flask. The media was then harvested and centrifuged and the resulting pellet was fixed in 2% paraformaldehyde, 2% glutaraldehyde, 0.2% picric acid in 0.1 M Cacocylate-HCl buffer. Media was replaced in the flask and the process repeated on days 2 and 3 pi. In addition, an aliquot of media was removed for virus titration on days 2 and 3 pi. After fixation, cells were pelleted, enrobed in agar, post-fixed in 1% osmium tetroxide and embedded in Epon-Araldite. Thin sections were post-stained with uranyl acetate and lead citrate.

Results

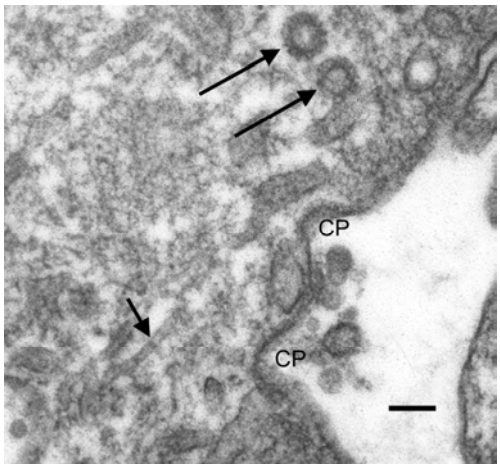
Viral titres in DH82 cells inoculated with both EHDV and BTV were greatly increased over controls (virus and media without cells) by day 2 pi (Table I). Titres peaked slightly on day 3 pi, but remained high until termination on day 5 pi. Titres were similar for both viruses, varying by less than 0.5 logs. Virus was detected in the control (no-cell) wells until day 5 pi, but never exceeded the titre of the original inoculum. Discrete plaques were not appreciated microscopically. By immuno-cytochemistry, rare DH82 cells in cytocentrifuge preparations were positive for either BTV or EHDV on day 1 pi, but by day 2 pi, and up to day 5 pi, approximately 10% of the cells were immunopositive for BTV or EHDV.

Table I
Replication of bluetongue virus serotype 10 (BTV-10) and epizootic haemorrhagic disease virus serotype 2 (EHDV-2) in DH82 cells

Treatment	Virus titre ($log_{10}TCID_{50}$/ml) Day post inoculation				
	1	2	3	4	5
BTV-10 and DH82[a]	2.97	5.26	5.60	5.60	5.39
EHDV-2 and DH82[b]	2.17	4.80	5.26	5.14	5.14
BTV-10 + media[c]	2.26	2.20	2.20	2.20	2.20
EHDV-2 + media[d]	2.20	2.20	2.20	2.20	2.20

a) DH82 cells inoculated with $10^{2.5}TCID_{50}$ BTV-10
b) DH82 cells inoculated with $10^{2.5}TCID_{50}$ EHDV-2
c) Media (no cells) inoculated with $10^{2.5}TCID_{50}$ BTV-10
d) Media (no cells) inoculated with $10^{2.5}TCID_{50}$ EHDV-2

Ultrastructurally, day 1 pi, EHDV-infected cells had multiple coated pits on their surface, some of which contained extracellular viral particles, and a few viral particles and viral-associated tubules were present in the cytoplasm of some cells (Fig. 1). By day 2 pi, some cells were either swollen and lysed (necrotic) or shrunken and dark with condensed nuclei (apoptotic). Intact cells contained viral matrices, sometimes containing viral particles and viral tubules (Fig. 2). Groups of viral particles, matrix, and tubules were associated with the cytoplasmic remnants of lysed cells (Fig. 3). The shrunken dark cells did not appear to be infected. By day 3 pi, no lysed or shrunken dark cells were observed; however, many cells were infected and contained large viral matrices and significant numbers of viral-associated tubules (Fig. 4). Viral particles were scarce and, when observed, were near the plasma membrane, often in a cytoplasmic protrusion. Virus titres were $10^{7.03}$TCID$_{50}$/ml and $10^{5.80}$TCID$_{50}$/ml on days 2 and 3 pi, respectively.



CP: coated pits

Figure 1
Transmission electron micrograph of epizootic haemorrhagic disease virus serotype 2-infected DH82 cell on day 1 post inoculation
Long arrows: viral particles; short arrow: viral-associated tubules
Bar=100 nm

Discussion

DH82 cells were susceptible to both EHDV and BTV infection resulting in relatively high viral titres of similar magnitude. DH82 cells were derived from a canine malignant histiocytoma and were originally characterised as histiocytic by positive staining reactions for alpha naphthyl acetate esterase and acid phosphates, presence of Fc receptors, phagocytosis of latex beads and plastic adherence in culture (15). As EHDV and BTV can infect a variety of cell types, and have been shown to replicate in cow (16) and

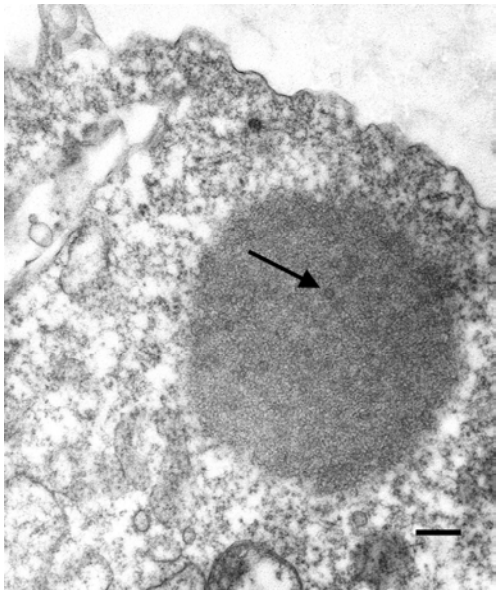


Figure 2
Transmission electron micrograph of epizootic haemorrhagic disease virus serotype 2-infected DH82 cells on day 2 post inoculation
Portion of viable cell containing viral matrix with developing viral particles (arrow demonstrating one)
Bar=200 nm

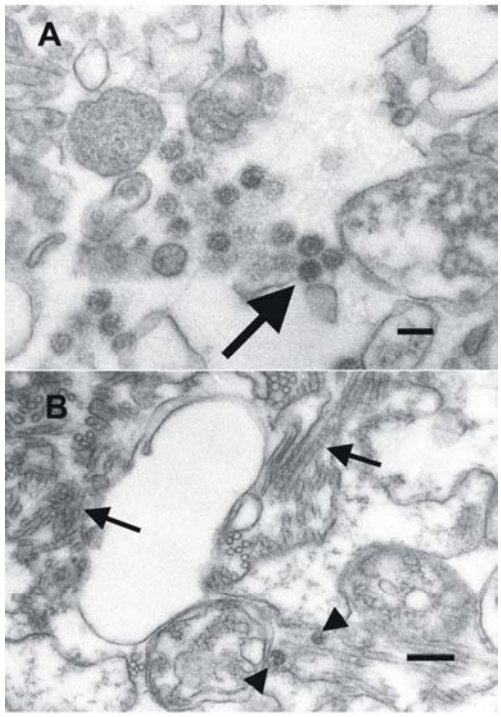


Figure 3
Transmission electron micrographs of epizootic haemorrhagic disease virus serotype 2-infected DH82 cells on day 2 post inoculation
A: Viral particles (arrow) associated with remnants of lysed cell
Bar=100 nm
B: Viral particle (arrow heads) adjacent to tubules and massive numbers of tubules (arrows) associated with remnants of lysed cell
Bar=100 nm

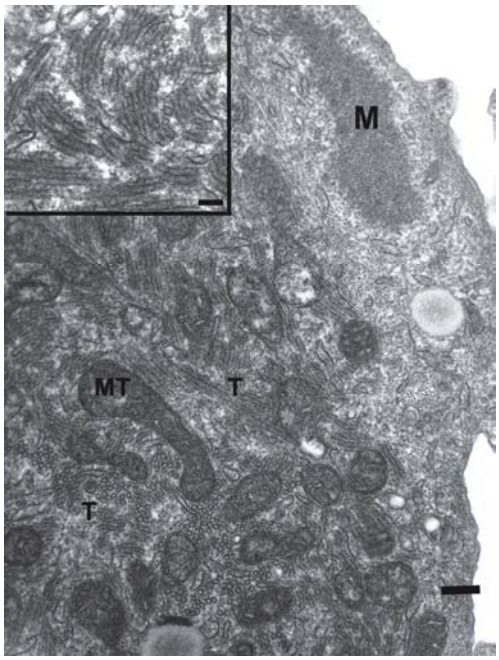


Figure 4
Transmission electron micrographs of epizootic haemorrhagic disease virus serotype-2 infected DH82 cells on day 3 post inoculation
Cell cytoplasm contains viral matrix (M) and numerous virus-associated tubules (T) among mitochondria (MT)
Bar=200 nm
Inset: Higher magnification of tubules
Bar=100 nm

deer (11) peripheral blood monocytes *in vitro*, it is not surprising that DH82 cells were susceptible to infection. Unlike peripheral blood monocytes, DH82 macrophage-monocytic cells can be continually propagated in culture facilitating *in vitro* studies. Infection of large numbers of monocytic cells with these viruses, as seen in this system, would assist viral-monocyte receptor studies. DH82 cell surface antigens have been partially characterised and the cells are known to express CD14, and partially express CD5 and CD45 (1).

Cytopathic effects were difficult to appreciate in infected DH82 cell cultures despite the relatively high viral titres. This may be partially explained by the immunocytochemistry results, where, despite high titres, only about 10% of the cells appeared to be infected.

Curiously, in the ultrastructural study, evidence of viral induced DH82 cell death was present on day 2 pi, but had disappeared by day 3 pi. This is unlike infection in other cell systems, such as BHK-21 and cow pulmonary artery endothelial cells, where cell death is progressive (E.W. Howerth, personal observation), and the possibility of persistent infection of DH82 cells needs to be addressed. Also intriguing in the ultrastructural study was the presence of cell death by necrosis (lysis) in obviously infected cells and death by apoptosis in apparently uninfected cells. It is possible that the apoptosis was induced by cytokines released from infected cells rather than by direct viral induced damage. This is similar to what has been observed in BTV-infected endothelial cultures where cell death by apoptosis appears to be, at least partially, mediated by interleukin-1 (IL-1) rather than direct viral replication (2). DH82 cells are known to produce tumour necrosis factor alpha (TNF-α), IL-1, IL-6, IL-5, IL-8, and IL-10, so these cells could be used to study cytokine expression by BTV or EHDV infected monocytes (1).

Although viral-associated tubules normally develop in BTV and EHDV infected cells, both *in vitro* and *in vivo* (4, 10, 12), massive quantities of these tubules developed in EHDV-infected DH82 cells in this study. These tubules are formed from the virus coded non-structural protein, NS1, but their function is poorly understood (10). The production of large quantities of these viral-associated tubules in DH82 cells might prove useful for structural or functional studies of these tubules.

DH82 cells are routinely used for the isolation of ehrlichial agents from deer, cattle and sheep (6, 17). Isolation of these agents could be confounded by the concurrent isolation of either EHDV or BTV from animals co-infected with both types of agents.

References

1. Barnes A., Bee A., Bell S., Gilmore W., Mee A., Morris R. & Carter S.D. (2000). – Immunological and inflammatory characterization of three canine cell lines: K1, K6 and DH82. *Vet. Immunol. Immunopathol.*, **75**, 9-25.
2. DeMaula C.D., Jutila M.A., Wilson D.W. & MacLachlan N.J. (2001). – Infection kinetics, prostacyclin release and cytokine-mediated modulation of the mechanism of cell death during bluetongue virus infection of cultured ovine and bovine pulmonary artery and lung microvascular endothelial cells. *J. Gen. Virol.*, **82**, 787-794.
3. Evermann J.F., McKeirnan A.J. & Wilbur L.A. (1994). – Canine fatalities associated with the use of a modified live vaccine administered during late stages of pregnancy. *J. Vet. Diag. Invest.*, **6**, 353-357.
4. Howerth E.W. & Tyler D.E. (1988). – Experimentally induced bluetongue virus infection in white-tailed deer: ultrastructural findings. *Am. J. Vet. Res.*, **49**, 1914-1922.
5. Howerth E.W., Stallknecht D.E. & Kirkland PD. (2001). – Bluetongue, epizootic haemorrhagic disease, and other orbivirus-related diseases. *In* Infectious diseases of wild mammals, 3rd Ed. (E.S. Williams & I.K. Barker eds) Iowa State Press, Ames, Iowa, 77-97.

6. Lockhart J.M., Davidson W.R., Stallknecht D.E., Dawson J.E. & Howerth E.W. (1997). – Isolation of *Ehrlichia chaffeensis* from wild white-tailed deer (*Odocoileus virginianus*) confirms their role as natural reservoir hosts. *J. Clin. Microbiol.*, **35**, 1681-1686.
7. MacLachlan N.J., Jagel G., Rossitto P.V., Moore P.F. & Heidner H.W. (1990). – The pathogenesis of experimental bluetongue virus infection of calves. *Vet. Pathol.*, **27**, 223-229.
8. Quist C.F., Howerth E.W., Stallknecht D.E., Brown J., Pisell T. & Nettles V.F. (1997). – Host defense responses associated with experimental haemorrhagic disease in white-tailed deer. *J. Wildl. Dis.*, **33**, 584-599.
9. Reed L.J & Muench H. (1938). – A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, **27**, 493-497.
10. Roy P. (2001). – Orbiviruses *In* Field's Virology, Vol. II (D.M. Knipe & P.M. Howley, eds). Lippincott, Williams & Wilkins, Philadelphia, 1835-1870.
11. Stallknecht D.E., Howerth E.W., Kellogg M.L., Quist C.F. & Pisell T. (1997). – *In vitro* replication of epizootic haemorrhagic disease and bluetongue viruses in white-tailed deer peripheral blood mononuclear cells and virus-cell association during *in vivo* infections. *J. Wildl. Dis.*, **33**, 574-583.
12. Tsai K. & Karstad L. (1973). – The pathogenesis of epizootic haemorrhagic disease of deer. *Am. J. Pathol.*, **70**, 379-400.
13. Wechsler S.J. & McHolland L.E. (1988). – Susceptibilities of 14 cell lines to bluetongue virus infection. *J. Clin. Microbiol.*, **26**, 2324-2327.
14. Wechsler S.J., Austin K.J. & Wilson W.C. (1990). – Limits of detection of bluetongue virus with different assay systems. *J. Vet. Diag. Invest.*, **2**, 103-106.
15. Wellman M.L., Krakowka S., Jacobs R.M. & Kociba G.J. (1988). – A macrophage-monocyte cell line from a dog with malignant histiocytosis. *In vitro Cell Devel. Biol.*, **24** (3), 223-229.
16. Whetter L.E., MacLachlan N.J., Gerhard D.H., Heidner H.W. & Moore P.F. (1989). – Bluetongue virus infection of bovine monocytes. *J. Gen. Virol.*, **70**, 1663-1676.
17. Zweygarth E. & Josemans A.I. (2001). – Continuous *in vitro* propagation of *Cowdria ruminantium* (Welgevonden stock) in a canine macrophage-monocyte cell line. *Onderstepoort J. Vet. Res.*, **68**, 155-157.