Arch Virol (1994) 136: 287–298

Archives of Virology

The pathogenesis of bluetongue virus infection of bovine blood cells in vitro: ultrastructural characterization

A. W. Brewer and **N. J. MacLachlan**

Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, California, U.S.A.

Accepted January 29, 1994

Summary. Cattle are proposed to be reservoir hosts of bluetongue virus (BTV) because infected animals typically have a prolonged cell-associated viremia. Enriched populations of bovine monocytes, erythrocytes and lymphocytes were inoculated with BTV serotype 10 (BTV 10) and the infected cells then were examined by transmission electron microscopy to characterize the interaction of BTV with bovine blood cells. Replication of BTV 10 in monocytes and stimulated (replicating) lymphocytes was morphologically similar to that which occured in Vero cells, with formation of viral inclusion bodies and virus-specific tubules. In contrast, BTV 10 infection of unstimulated (non-replicating) lymphocytes and erythrocytes did not progress beyond adsorption, after which virus particles persisted in invaginations of the cell membrane. Studies with core particles and neutralizing monoclonal antibodies established that outer capsid protein VP2 is necessary for attachment of BTV 10 to erythrocytes. These in vitro virus-cell interactions provide a cogent explanation for the pathogenesis of BTV infection of cattle, especially the prolonged cell associated viremia that occurs in BTV-infected cattle.

Introduction

Bluetongue is an arthropod-transmitted viral disease of sheep and some wild ruminant species [3, 32]. Bluetongue virus (BTV) infection of cattle is common throughout tropical and temperate areas of the world, however disease is rare in infected animals. Cattle are proposed to be reservoir hosts of BTV because infected cattle usually have a prolonged viremia and so provide a source of virus that is available for transmission by hematophagous gnats (*Culicoides spp*) to other ruminant species [12, 26, 30]. Although viremia is prolonged in BTV-infected cattle it is not persistent, and recovered animals are immune to reinfection with the homologous serotype of BTV [25, 26].

It is proposed that prolonged viremia in cattle is due to association of BTV with circulating blood cells, and not to antigenic drift of the virus or to an

inadequate anti-viral response by the infected host [17, 21–26]. Viremia typically persists several weeks as determined by virus isolation but viral nucleic acid can be detected in blood cells for a considerably longer period (several months) with a BTV-specific polymerase chain reaction (PCR) detection system [27]. Virus is highly cell-associated throughout viremia but whereas BTV may be isolated from all blood cell fractions soon after infection, late in the course of viremia it is exclusively associated with erythrocytes [2, 25, 26]. Little is known regarding the interaction of BTV with erythrocytes, other than that the virus apparently binds to a glycophorin receptor and that it resides in invaginations of the cell membrane soon after in vitro infection of bovine erythrocytes [4, 13].

Infection of blood cells in BTV-infected cattle is unlikely to be a consequence of infection of hemopoietic precursor cells in bone marrow [2, 25], which is the mechanism responsible for the prolonged cell-associated viremia that occurs in mammals infected with Colorado tick fever virus [16]. BTV replicates in lymphoid tissues of infected cattle, first in the regional lymph node draining the site of virus inoculation and later in tissues such as spleen and other lymph nodes [2, 26]. Efforts to determine the phenotype of cells which support replication of BTV, using immunohistochemical staining, in-situ hybridization, and electron microscopy, have been frustrated by the extremely low numbers of infected cells in the blood and tissues of infected cattle [8–10, 15, 25, 29, 35]. In vitro studies utilizing analytical flow cytometry indicate that bovine monocytes and replicating CD4+ T lymphocytes support replication of BTV whereas resting lymphocytes do not, regardless of their phenotype [1, 33, 35].

BTV infection of cells in lymphoid tissues and blood clearly is critical to the replication, survival and eventual vector transmission of BTV after infection of cattle, thus the goal of this study was to further characterize the interaction of BTV with different bovine blood cell types. Results of these ultrastructural studies indicate that BTV replicates within monocytes and lymphocytes undergoing blastogenesis, whereas virus replication does not proceed beyond attachment of the virus to the cell membrane of infected erythrocytes and unstimulated lymphocytes. These virus-cell interactions likely are critical to the pathogenesis of BTV infection of cattle.

Materials and methods

Virus

Replication of BTV serotype 10 (BTV 10) was characterized in cultures of Vero cells, adherent bovine peripheral blood mononuclear (PBM) cells, stimulated (with interleukin 2 [IL-2] and Concanavalin A [ConA]) and unstimulated non-adherent bovine PBM cells, and suspensions of bovine and murine erythrocytes. The passage history of the strain of BTV 10 used to inoculate the various cells has previously been described [2, 24]. All cell cultures and suspensions were inoculated with BTV 10 at a multiplicity of infection (MOI) of one, and cells were harvested at intervals thereafter for examination by transmission electron microscopy (TEM). Core particles of BTV 10 were prepared by centrifugation (70 000 × **g** for 14 h) on a 29 to 44% cesium chloride density gradient. This procedure stripped the viral outer protein coat of VP2 and VP5 as determined by the ultrastructural appearance

and size of the particles harvested from the gradient [28], and the greatly diminished infectivity of this fraction for Vero cells.

Cells

The ultrastructural features of the replication of BTV 10 were first characterized using Vero cells (American Type Culture Collection, Rockville, MD) grown in supplemented RPMI 1640 medium. Replication of BTV 10 in bovine PBM cells was then characterized by TEM. Venous blood was collected in acid citrate dextrose solution from a colostrum deprived calf reared in insect-secure isolation facilities. PBM cells were separated from the buffy coat fraction by centrifugation through Ficoll and sodium diatrizoate (Histopaque; Sigma Chemical Co., St. Louis, MO) and cultures of adherent and non-adherent PBM cells were prepared as previously described [1]. The non-adherent PBM cells were divided into two fractions; lymphocytes in one fraction were stimulated to undergo blastogenesis by addition of recombinant human IL-2 (100 units/ml; generously supplied by Cetus Corporation, Emeryville, CA) and ConA (1 μg/ml; Sigma Chemical Co., St. Louis, MO). The identity of cells in the various cultures of PBM cells was determined by analytical flow cytometry using monoclonal antibodies (MAbs) to bovine leukocyte differentiation antigens, as previously described [1]. The ultrastructural morphology of individual PBM cells in the various cultures was compared with published descriptions of the ultrastructural appearance of different PBM cells [5, 29]. Erythrocytes were isolated from the blood of the donor calf as previously described [2], and then were washed in calcium-free MEM and resuspended in conditioned Dulbecco's MEM prior to inoculation with BTV 10. Suspensions of murine erythrocytes were prepared in the same manner. The titer of BTV present in suspensions of bovine erythrocytes was determined at intervals up to 48 h after the cells suspension was inoculated with BTV 10. Aliquots of erythrocytes taken from the suspensions were washed three times in PBS and then lysed in water prior to plaque titration of BTV 10 on Vero cells [23].

Transmission electron microscopy and immunogold labelling

Cells for examination by TEM were pelleted by centrifugation at 4000 × **g**, and then fixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.5). Cells were post-fixed in osmium tetroxide and stained with uranyl acetate followed by dehydration in graded alcohols and embedding in Epon-Araldite. Ultra thin sections were viewed with a Zeiss 10 electron microscope. Vero cell cultures inoculated with BTV 10 processed for immunogold labelling of the non-structural BTV protein NS-1 were fixed for 30 min in 0.5% glutaraldehyde/2% paraformaldehyde, washed in PBS and resuspended in PBS containing 100 mM NH_4Cl to quench unreacted aldehydes. Cells then were incubated for 1 h with MAb 047 specific for NS-1 [35] and then incubated for 1 h with 5 nm gold-labelled goat anti-mouse lgG (Ted Pella, Redding, CA) and processed for TEM.

Results

BTV 10 infection of Vero cells

The sequence of events observed in BTV 10 infected Vero cells was comparable to that previously described [7, 14, 20]. Virus particles were present in invaginations of the cell membrane within 15 min after infection. Viral inclusion bodies were identified in infected Vero cells by 4 h after infection, and manifest

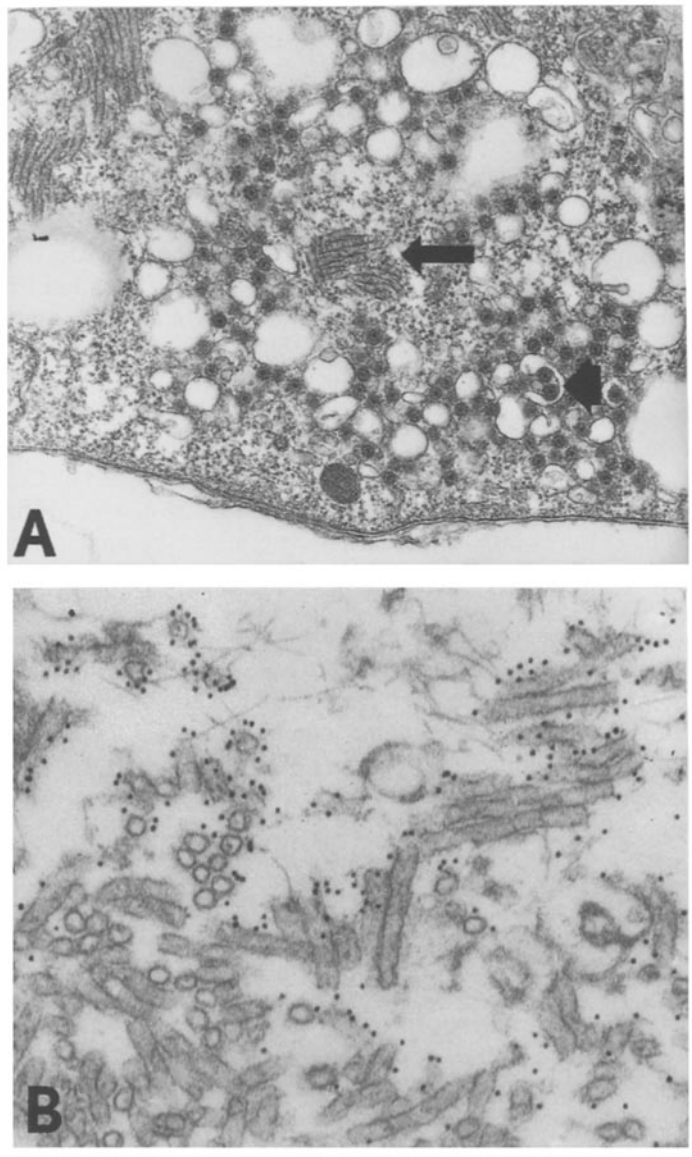

Fig. 1. Ultrastructural characterization of replication of BTV 10 in Vero cells. **A** Cytoplasm of an infected cell at 36 h after inoculation is filled with tubules (arrow) and virus particles which surround and sometimes appear to bud (arrowhead) into cytoplasmic vesicles. Magnification × 31 000. **B** Immunogold labelling of tubules with monoclonal antibody 047 specific for non-structural BTV protein NS-1. Magnification × 35 000

as granular or fibrillar electron dense aggregates, often with virus particles at the periphery. Apparent budding of virions into cytoplasmic vesicles occurred late in the course of infection (Fig. 1). Virus-specific tubules, which are long, cylindrical structures 68 nm in diameter, were present in the cytoplasm of infected cells by 4 h. Immunogold labelling confirmed that tubules in infected cells were composed of the non-structural BTV protein NS-1 (Fig. 1).

BTV 10 infection of bovine PBM cells

The cultures of adherent PBM cells consisted predominantly of monocytes and lesser numbers of B cells. The vast majority of cultured non-adherent PBM cells stimulated with IL-2 and ConA were T cell blasts that expressed CD4, whereas the unstimulated non-adherent cells contained a mixture of B cells, monocytes, and CD4+, CD8+ and gamma/delta T cells. The ultrastructural

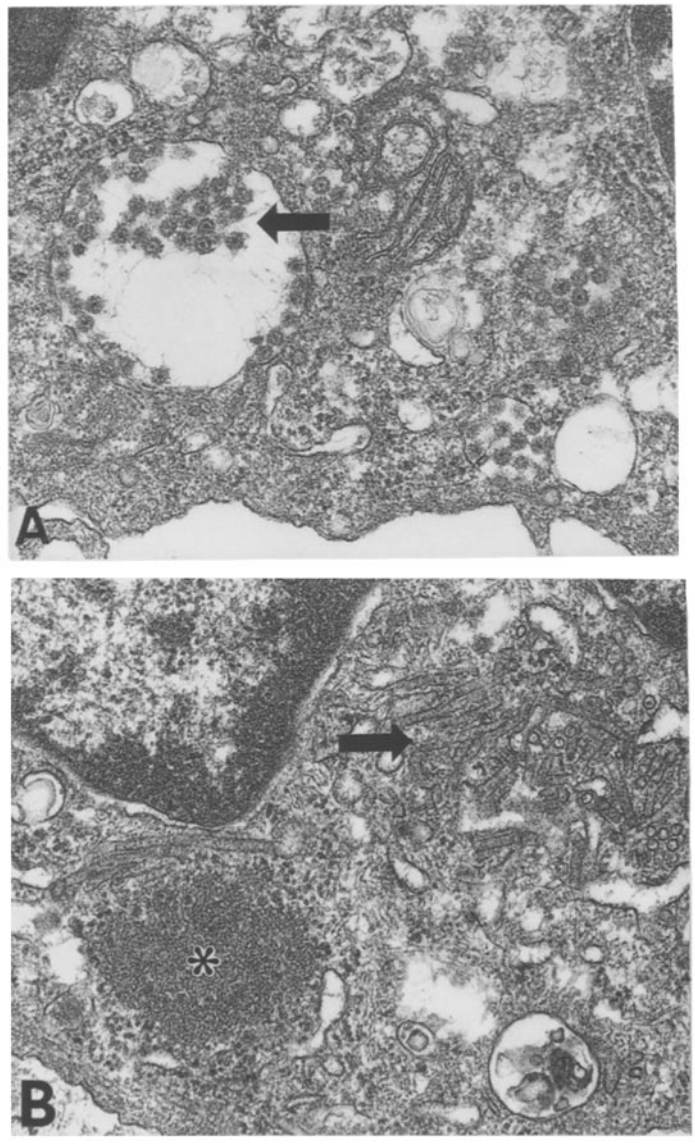

Fig. 2. Ultrastructural characterization of replication of BTV 10 in cultured bovine monocytes. **A** BTV-infected monocyte at 30 min after infection. Note the presence of virus particles in phagocytic vesicles (arrow). **B** BTV-infected monocyte at 8 h after inoculation. Note viral inclusion body (asterisk) and tubules (arrow) in the cytoplasm. Magnification × 31 000

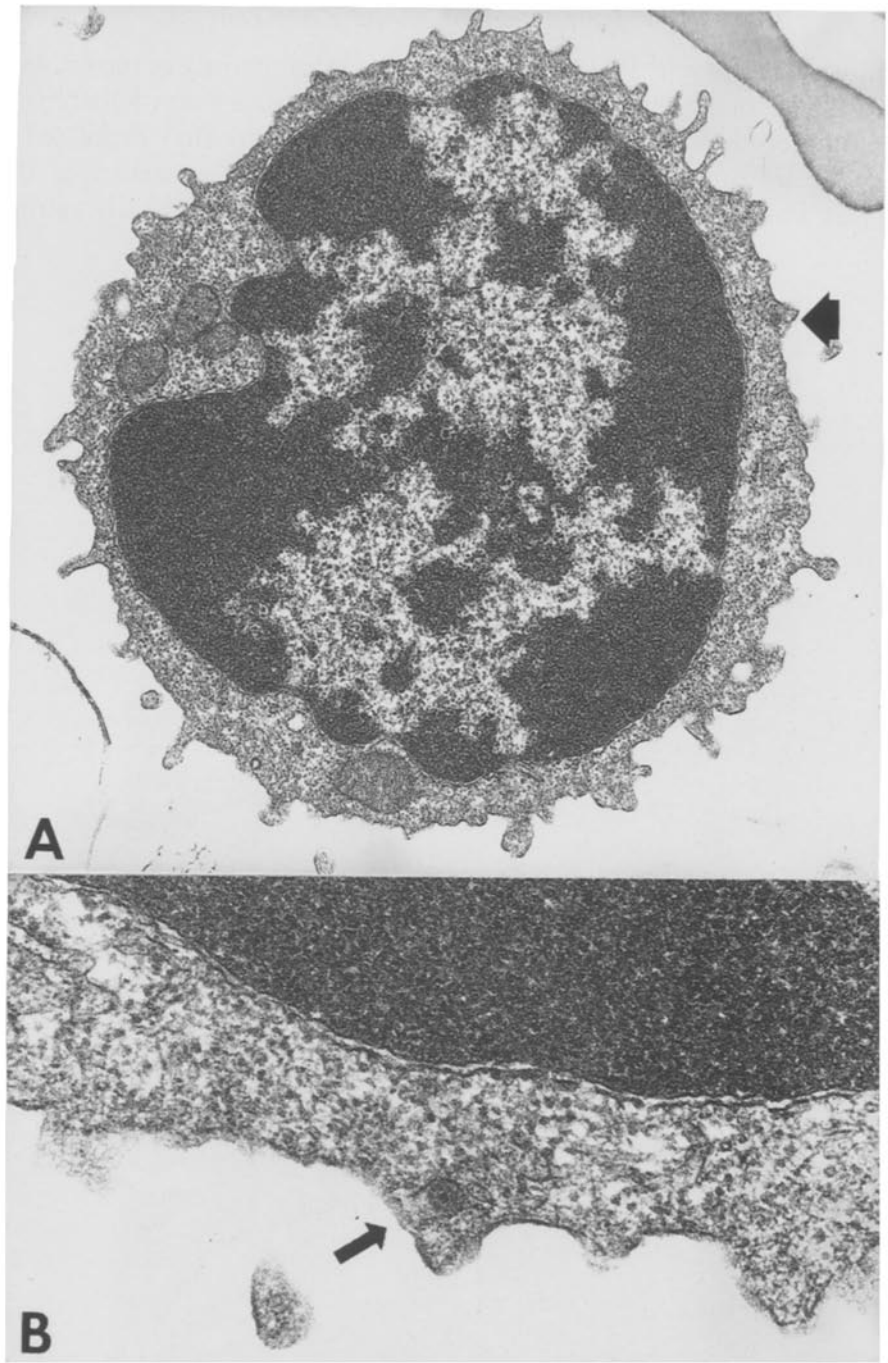

Fig. 3. Ultrastructural characterization of replication of BTV 10 in an unstimulated bovine lymphocyte. **A** The unstimulated lymphocyte contains a virus particle within an indentation of the cell membrane (arrowhead). Magnification × 15 000. **B** Higher magnification of the structure identified in **A** (arrow). Virus particles were present in these pits throughout the 48 h duration of the study, whereas replicative forms such as inclusion bodies and tubules were never observed in unstimulated lymphocytes infected with BTV 10. Magnification × 77 000

morphology of BTV 10 replication was similar in monocytes and activated lymphocytes (those stimulated with IL-2 and ConA), and essentially the same as that described in infected Vero cells. Specifically, virus particles were identified in membrane invaginations and endosomes at 15 min after infection, and viral inclusion bodies and tubules were present by 4 h (Fig. 2). Apparent budding of virions into cytoplasmic vesicles occurred late in the course of infection, as described in infected Vero cells. In contrast, infection of resting lymphocytes (those not stimulated with IL-2 and ConA) did not progress beyond the virus-containing invagination of the cell membrane (Fig. 3). Virus particles in invaginations of the lymphocyte cell membrane were first detected at 15 min after virus was inoculated into the cultures, and were present as long as 48 h after inoculation (the final time point evaluated). Structures such as tubules, inclusion bodies or virus-containing endosomes were not detected in unstimulated lymphocytes inoculated with BTV 10.

BTV 10 infection of bovine erythrocytes

The ultrastructural features of BTV 10 infection of erythrocytes were similar to those of infected resting lymphocytes. Specifically, individual virus particles were present in indentations and invaginations of the erythrocyte cell membrane by 15 min after virus was added to the suspended cells, and these structures were present as long as 2 weeks after inoculation (the last time point evaluated). No other virus-specific structures were identified in erythrocytes at any time after inoculation of the cell suspension. Whereas single virus particles were included in discrete invaginations of the cell membrane, aggregated particles were associated with more extensive depressions of the cell membrane in which individual particles sometimes were associated with discrete membrane indentations (Fig. 4). The presence of clusters of virus particles associated with individual erythrocytes, despite an MOI of 1, is consistent with the tendency of BTV virus to form aggregates adherent to cellular material [34]. Such aggregates likely would be present in the unpurified virus inoculum.

Characterization of the interaction of BTV 10 with bovine erythrocytes

Specificity of the interaction of BTV 10 with bovine erythrocytes was established with erythrocytes from mice. Whereas erythrocytes with membrane indentations containing virus particles were very numerous in suspensions of bovine erythrocytes inoculated with BTV 10, no such structures were identified in similarly inoculated suspensions of murine erythrocytes. Viral proteins responsible for attachment to bovine erythrocytes were evaluated using core particles of BTV 10 and BTV-specific MAbs. Viral cores were used to determine if the viral outer capsid is critical for attachment of BTV 10 to bovine erythrocytes. Core particles did not adhere to erythrocytes as determined by TEM, indicating that the viral outer capsid is essential for attachment of BTV 10 to bovine erythrocytes. A MAb (039) that neutralizes BTV 10, and that specifically binds VP2 [11], was used to establish the role of VP2 in attachment

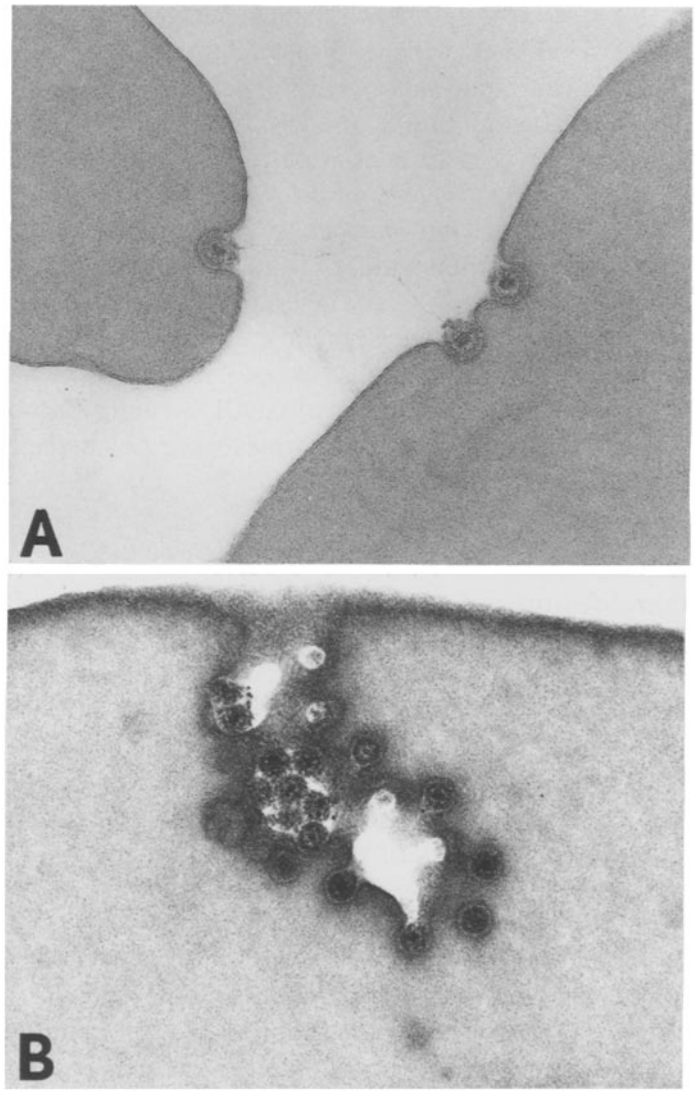

Fig. 4. Ultrastructural characterization of BTV 10 infection of bovine erythrocytes. **A** Erythrocytes 15 min after inoculation with BTV 10. Magnification × 77 000. **B** An extensive depression of the cell membrane of an infected erythrocyte which contains numerous virus particles which are localized within distinct indentations of the cell membrane. Magnification × 62 000. Virus particles associated with the cell membrane of inoculated erythrocytes were readily detected throughout the 14 day duration of the study, whereas replicative forms such as inclusion bodies and tubules were never observed in BTV-inoculated erythrocytes

of BTV 10 to bovine erythrocytes. Aliquots of BTV 10 were incubated with either VP2-specific MAb 039 or MAb 290 (specific for core protein VP7 [35]), prior to inoculation of suspended bovine erythrocytes and subsequent examination by TEM. The VP2-specific MAb prevented binding of virus particles to erythrocytes whereas the MAb to VP7 had no effect on virus attachment, indicating that VP2 solely is responsible for adherence of BTV 10 to bovine

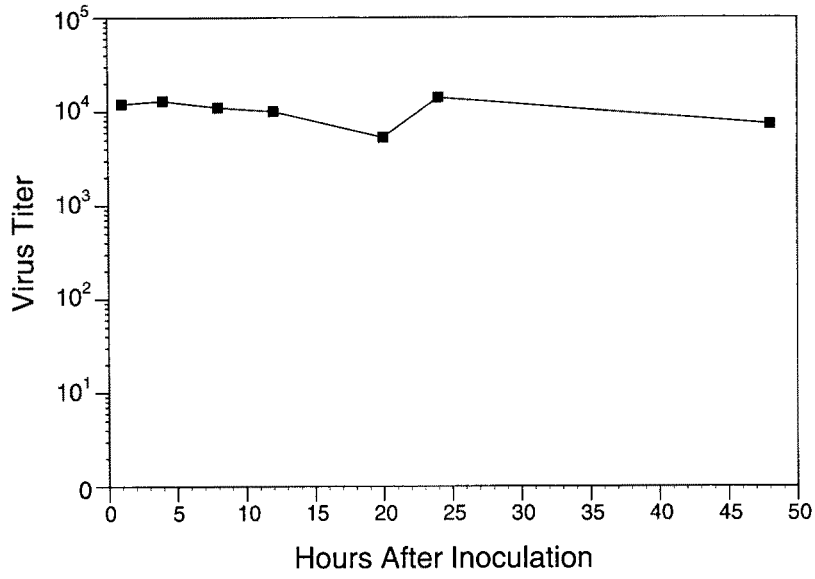

Fig. 5. Virus titer (plaque forming units per 10^6 erythrocytes) of a suspension of bovine erythrocytes at intervals after inoculation with BTV 10

erythrocytes. VP2 also is singularly responsible for adsorption of BTV to BHK cells [18].

Virus particles associated with bovine erythrocytes were infectious for mammalian cells and were not removed from the erythrocytes by washing. There was minimal change in the titer of virus present in the suspension of bovine erythrocytes for 48 h after the suspension was inoculated with BTV 10 (Fig. 5). In contrast, we previously have shown that titers of virus present in enriched populations of either bovine monocytes or stimulated (with IL-2 and ConA) CD4+ T cells increased after inoculation of BTV 10. We also have shown, by immunofluorescence staining and pulse-chase radiolabelling, that BTV proteins were produced in cultured bovine monocytes and stimulated T lymphocytes infected with BTV 10, but not in cultured lymphocytes that were not stimulated with IL-2 and phytomitogens prior to inoculation of BTV 10 [1, 35]. These results indicate that productive replication of BTV 10 occurred in bovine monocytes and replicating lymphocytes but not in unstimulated lymphocytes or erythrocytes.

Discussion

Results of these in vitro studies are consistent with the findings of previous in vivo studies, and together the data provide a cogent explanation for the prolonged cell-associated viremia that occurs in BTV-infected cattle. Replication of BTV principally occurs in lymphoid tissues of infected cattle, likely in monocytes, lymphoblasts and, perhaps, endothelial cells [1, 2, 25, 26]. In contrast, BTV 10 infection of unstimulated lymphocytes does not progress after attachment of virus to the cell membrane. It is possible that unstimulated lymphocytes which

harbor receptor-bound BTV in invaginations of the cell membrane would traffic virus between lymphoid tissues in infected cattle. Virus replication would proceed in unstimulated lymphocytes that harbor BTV only after they undergo blastogenesis, which is consistent with the fact that BTV is more readily isolated from lymphocytes in the blood and lymph of infected cattle after the lymphocytes are stimulated with IL-2 and phytomitogens prior to virus isolation [2]. We propose that virus produced in lymphoid tissues is released into circulation where it non-selectively associates with blood cells [2, 25]. Virus associated with blood cells other than erythrocytes is removed relatively promptly, likely because of the short circulating half-life of some blood cells and cytotoxicity of BTV to other cells such as monocytes and stimulated lymphocytes.

Association of BTV with blood cells, especially erythrocytes, is responsible for the prolonged viremia that occurs in BTV-infected cattle [2, 21, 25, 26], and our in vitro studies illustrate the mechanism that likely is responsible for this phenomenon. Infection of erythrocytes resembles the initial phase of BTV infection of nucleated cells but replication does not progress beyond attachment, after which virus particles persist in invaginations of the erythrocyte cell membrane. Virus particles associated with the erythrocyte cell membrane clearly are infectious to mammalian cells. Outer capsid protein VP2, the viral hemagglutinin [6], is responsible for attachment of BTV to bovine erythrocytes. Virus particles present within invaginations of the erythrocyte cell membrane probably are sequestered from antibodies as BTV and specific neutralizing antibody may co-circulate in the blood of infected cattle for several weeks [2, 12, 17, 21, 24, 25, 31]. Furthermore, BTV nucleic acid can be detected in blood cells of infected cattle by PCR analysis for at least 140 days after infection [27], which is remarkably similar to the lifespan of the bovine erythrocyte [19]. This novel interaction between BTV and the bovine erythrocyte likely is the critical mechanism that allows cattle to serve as natural reservoir hosts of BTV. Specifically, this interaction would protect the virus from prompt immune clearance and erythrocytes harboring virus particles would contaminate blood ingested by vector gnats that feed on BTV-infected cattle. Infection of the gnat vector obviously is critical to completion of the natural cycle of infection. Although BTV infection of cattle is characterized by a prolonged viremia, it is not truly persistent and viremia likely continues only as long as erythrocytes that harbor infectious BTV are present in the circulation.

Acknowledgements

The authors gratefully acknowledge V. Wong and T. Harrington for their excellent technical assistance. These studies were supported by USDA Special Research Grant 89–34116–4447, USDA Competitive Research Grant 92–37204–7999 and funds provided by the Livestock Disease Research Laboratory and the USDA under the Animal Health Act, 1977, Public Law 95–113.

References

1. Barratt-Boyes SM, Rossitto PV, Stott JL, MacLachlan NJ (1992) Flow cytometric analysis of in-vitro bluetongue virus infection of bovine blood mononuclear cells. J Gen Virol 73: 1953–1960
2. Barratt-Boyes SM, MacLachlan NJ (1994) Dynamics of viral spread in bluetongue virus infected calves. Vet Microbiol (in press)
3. Bowne JG (1971) Bluetongue disease. Adv Vet Sci Comp Med 15: 1–46
4. Brewer AW, MacLachlan NJ (1992) Ultrastructural characterization of the interaction of bluetongue virus with bovine erythrocytes in-vitro. Vet Pathol 29: 356–359
5. Cheville NF (1983) Cell pathology. The lowa State University Press, Ames
6. Cowley JA, Gorman BM (1987) Genetic reassortants for identification of the genome segment coding for the bluetongue virus hemagglutinin. J Virol 61: 2304–2306
7. Cromack AS, Blue JL, Gratzek JB (1971) A quantitative ultrastructural study of the development of bluetongue virus in Madin-Darby bovine kidney cells. J Gen Virol 13: 229–224
8. Dangler CA, de la Concha-Bermejillo A, Stott JL, Osburn BI (1990) Limitations of in situ hybridization for the detection of bluetongue virus in blood mononuclear cells. J Vet Diagn Invest 2: 303–307
9. de la Concha-Bermejillo A, Schore CE, Dangler CA, de Mattos CC, de Mattos CA, Osburn BI (1992) Comparison of slot blot nucleic acid hybridization, immunofluorescence, and virus isolation techniqies to detect bluetongue virus in blood mononuclear cells from cattle with experimentally induced infection. Am J Vet Res 53: 2245–2250
10. de la Concha-Bermejillo A, Odeon A, BonDurant RH, Osburn BI (1993) Experimental infection of pregnant cattle with bluetongue virus serotype 11 between postbreeding days 21 and 48. J Vet Diagn Invest 5: 329–335
11. DeMaula CD, Heidner HW, Rossitto PV, Pierce CM, MacLachlan NJ (1993) Neutralization determinants of United States bluetongue virus serotype 10. Virology 195: 292–296
12. Du Toit RM (1962) The role played by bovines in the transmission of bluetongue of sheep. J S Afr Vet Med Assoc 33: 483–490
13. Eaton BT, Crameri GS (1989) The site of bluetongue virus attachment to glycophorins from a number of animal erythrocytes. J Gen Virol 70: 3347–3353
14. Eaton BT, Hyatt AD, Brookes SM (1990) The replication of bluetongue viruses. Curr Topics Microbiol Immunol 162: 89–118
15. Ellis JA, Coen ML, MacLachlan NJ, Wilson WC, Williams ES, Luedke AJ (1993) Low prevalence of bluetongue virus expression in leukocytes from experimentally infected ruminants. Am J Vet Res 54:1452–1456
16. Emmons RW, Oshiro LS, Johnson HN, Lennette EH (1972) Intraerythrocytic location of Colorado tick fever virus. J Gen Virol 17: 185–195
17. Heidner HW, MacLachlan NJ, Fuller FJ, Richards RG (1988) Bluetongue virus genome remains stable during prolonged infection of cattle. J Gen Virol 69: 2629–2636
18. Huismans H, Van der Walt NT, Cloete M, Erasmus BJ (1983) The biochemical and immunological characterization of bluetongue virus outer capsid polypeptides. In: Compans RW, Bishop DHL (eds) Double stranded RNA viruses. Elsevier, New York, pp 165–172
19. Jain NC (1986) The erythrocyte: its morphology, metabolism, and survival. In: Jain NC (ed) Veterinary hematology, 4th edn. Lea & Febiger, Philadelphia, pp 527–562
20. Lecatsas G (1968) Electron microscopic study of the formation of bluetongue virus. Onderstepoort J Vet Res 35: 139–149

21. Luedke AJ (1970) Distribution of virus in blood components during viremia of bluetongue. Proc US Anim Health Assoc 74: 9–21
22. MacLachlan NJ, Thompson J (1985) Bluetongue virus-induced interferon in cattle. Am J Vet Res 46: 1238–1241
23. MacLachlan NJ, Fuller FJ (1986) Genetic stability in calves of a single strain of bluetongue virus. Am J Vet Res 47: 762–764
24. MacLachlan NJ, Heidner HW, Fuller FJ (1987) Humoral immune response of calves to bluetongue virus infection. Am J Vet Res 48: 1031–1035
25. MacLachlan NJ, Jagels G, Rossitto PV, Moore PF, Heidner HW (1990) The pathogenesis of experimental bluetongue virus infection of calves. Vet Pathol 27: 223–229
26. MacLachlan NJ, Barratt-Boyes SM, Brewer AW, Stott JL (1992) Bluetongue virus infection of cattle. In: Walton TE, Osburn BI (eds) Bluetongue, African horse sickness, and related orbiviruses. CRC Press, Boca Raton, pp 527–536
27. MacLachlan NJ, Nunamaker RA, Katz JB, Sawyer MM, Akita GY, Osburn BI, Tabachnick WS (1994) Detection of bluetongue virus in the blood of inoculated calves: Comparison of virus isolation, PCR assay, and in-vitro feeding of *Culicoides variipennis*. Arch Virol 136: 1–8
28. Mertens PPC, Burroughs JN, Anderson J (1987) Purification and properties of virus particles, infectious subviral particles, and cores of bluetongue virus serotypes 1 and 4. Virology 157: 375–386
29. Morrill JC, MacConnell S (1985) An electron microscopic study of blood cells from calves experimentally infected with bluetongue virus. In: Barber TL, Jochim MM (eds) Bluetongue and related orbiviruses. Alan Liss, New York, pp 279–287
30. Nevill EM (1971) Cattle and Culicoides biting midges as possible overwintering hosts of bluetongue virus. Onderstepoort J Vet Res 38: 65–72
31. Richards RG, MacLachlan NJ, Heidner HW, Fuller FJ (1988) Comparison of the virologic and serologic responses of lambs and calves infected with bluetongue virus serotype 10. Vet Microbiol 18: 233–242
32. Spreull J (1905) Malarial catarrhal fever (bluetongue) of sheep in South Africa. J Comp Pathol Therap 18: 233–242
33. Stott JL, Blanchard-Channell M, Scibienski RJ, Stott ML (1990) Interaction of bluetongue virus with bovine lymphocytes. J Gen Virol 71: 363–368
34. Verwoerd DW (1969) Purification and characterization of bluetongue virus. Virology 38: 203–212
35. Whetter LE, MacLachlan NJ, Gebhard DH, Heidner HW, Moore PF (1989) Bluetongue virus infection of bovine monocytes. J Gen Virol 70: 1663–1676

Authors' address: Dr. N. J. MacLachlan, Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA 95616, U.S.A.

Received November 29, 1993