Journal of General Virology (2003), **84**, 227–235 DOI 10.1099/vir.0.18705-0

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A possible overwintering mechanism for bluetongue virus in the absence of the insect vector

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Received 10 July 2002
Accepted 7 October 2002
Ahead-of-print 15 October 2002

Bluetongue virus **(BTV) and several other** ***Orbivirus*** **species are transmitted between mammalian hosts via bites from adults of certain species of** ***Culicoides*** **midges. However, BTV can survive for 9–12 months (typically during the winter), in the absence of adult vectors, with no detectable cases of viraemia, disease or seroconversion in the host. The survival of the virus from one 'vector season' to the next is called 'overwintering' but the mechanism involved is not fully understood. It is demonstrated that BTV can persistently infect ovine γδ T-cells** ***in vitro*****, a process that may also occur during infection and viraemia in mammalian hosts, thus providing a mechanism for virus persistence. Interaction of persistently BTV-infected γδ T-cells with antibody to the γδ T-cell-specific surface molecule WC-1 resulted in conversion to a lytic infection and increased virus release. Skin fibroblasts induce a similar conversion, indicating that they express a counter ligand for WC-1. Feeding of** ***Culicoides*** **midges induces skin inflammation, which is accompanied by recruitment of large numbers of activated γδ T-cells. The interaction of persistently infected γδ T-cells with skin fibroblasts would result in increased virus production at 'biting sites', favouring transmission to the insect vector. This suggested mechanism might also involve up-regulation of the WC-1 ligand at inflamed sites. It has been shown previously that cleavage of virus surface proteins by protease enzymes (which may also be associated with inflammation) generates infectious subvirus particles that have enhanced infectivity (100 times) for the insect vector.**

INTRODUCTION

Transmission of several virus species belonging to the genus *Orbivirus*, including *Bluetongue virus* (BTV) and *African horse sickness virus* (AHSV), occurs almost exclusively via bites from adults of certain species of *Culicoides* midge (Mellor, 1994b; Mellor & Wittmann, 2002). Adverse climatic

conditions, for example, in winter, will frequently kill the adults of these vector insects. This may result in their absence for periods that are longer than the maximum reported duration of viraemia in the mammalian host [e.g. for BTV, approximately 50 days in sheep (Sellers & Taylor, 1980; Koumbati *et al.*, 1998) or 60–100 days in cattle (Sellers & Taylor, 1980; MacLachlan *et al.*, 1991)]. Conventional models for the life cycle and transmission of BTV therefore suggest that if adverse (vector-free) winter conditions last for more than 100 days, the virus should be unable to survive from one year to the next (Koumbati *et al.*, 1998; Mellor, 1996). However, in certain areas of the world, BTV and AHSV have survived through the winter, despite the absence of adult insect vectors. Examples include the annual recrudescence of BTV during autumn, interspersed with epizoologically 'silent' periods of 8–9 months, which occurred between 1977 and 1981 in western Turkey (Yonguc *et al.*, 1982; Taylor & Mellor, 1994) and during the current outbreaks (2000–2002) of BTV-2 and BTV-9 (respectively) in Corsica/Sardinia and in Calabria (southern mainland Italy) (C. Hamblin, personal communication). In addition, during September 2001, further isolations of BTV-9 were recorded in both Serbia and Kosovo (after an absence of reported disease in eastern Europe for almost 2 years) in locations where winter conditions are severe and adult *Culicoides* are absent for several months each year (N. Nedelchev & G. Georgiev, personal communication). These outbreaks and the subsequent outbreaks of disease caused by BTV-9 in Bosnia Herzegovina during 2002 (Anon, 2002) have occurred further north than the range of *Culicoides imicola*, the major insect vector species for BTV in southern Europe. This indicates the involvement of one or more alternative vector species, probably *C. pulicaris* and/or *C. obsoletus*, both of which are abundant across much of northern Europe, including the UK, and have long been suspect BTV vectors (Mellor & Wittmann, 2002). The involvement of these novel insect vectors, together with an effective overwintering mechanism, could dramatically increase the threat posed by BTV in areas (particularly within northern Europe) that were previously free of the disease.

AHSV has also survived in Spain from 1987 to 1990, where the silent periods ranged from 8 to 12 months (Mellor, 1994a). Although very small numbers of adult vector insects were present in some locations in southern Spain during these inter-epizootic periods, no disease or evidence of seroconversion was seen in any equids, despite intensive surveillance by the national veterinary authorities (Mellor, 1993; Mellor & Boorman, 1995). These observations strongly suggest that transmission of AHSV did not occur during these inter-epizootic periods.

This pattern of annual episodes of disease in the late summer or autumn, separated by periods of quiescence, is a feature common to many orbivirus diseases that are transmitted by *Culicoides*. Reappearance of the disease coincides with major increases in the abundance of adults of the vector species (Mellor, 1996). The survival of virus from one 'vector season' to the next is popularly termed 'overwintering' (Yonguc *et al.*, 1982; Taylor & Mellor, 1994; Mellor & Boorman, 1980; Mellor, 1998). It

is evident that a reservoir of the virus must exist through the winter but its location and the mechanism by which it re-establishes overt infection of either the vector insect or the mammalian host has not been satisfactorily explained or demonstrated.

Here, we demonstrate that ovine γδ T-cells that are persistently infected with BTV can be converted to lytic infection through interaction with skin fibroblasts or an antibody to the γδ T-cell-restricted surface protein WC-1. On the basis of these observations we suggest a novel mechanism by which overwintering of BTV could occur in the absence of adult vector insects.

METHODS

Viruses. BTV-1 and -3 from South Africa were obtained originally from the Onderstepoort Veterinary Institute (South Africa) and passaged several times in BHK cells (Jeggo & Wardley, 1982; Takamatsu & Jeggo, 1989). All of the experimental cell lines were tested initially with BTV-3 but in later experiments BTV-1 was used. Sheep were infected with BTV-1, purified as described by Burroughs *et al*. (1994). Virus titres were determined in 96-well plates using BHK cells (Mellor & Boorman, 1980) and expressed as $TCID_{50}$ ml^{-1}.

Animals. Eight 6-month-old female Dorset Horn sheep were obtained from the Institute for Animal Health (Compton Laboratory, Reading, UK) and were held in insect-proof, high-security accommodation at the Pirbright Laboratory (Surrey, UK) throughout the experiments. At 18 days prior to BTV infection, six of the sheep were exposed to the bites of *Culicoides* midges (see below). Four of these and the two untreated control sheep were inoculated intradermally with 10^6 $TCID_{50}$ of BTV-1 (Takamatsu & Jeggo, 1989). The two remaining 'midge-bitten' sheep were used as uninfected controls. Viraemia was monitored by virus isolation from peripheral blood (see below). At 9 weeks post-infection (p.i.), the sheep were exposed to feeding midges (400 midges per animal per exposure) three times within a 24 h period (7:00 am, 3:00 pm and 7:00 am). Peripheral blood, skin biopsy tissues, fed midges, tissue fluid and blood from skin biopsy sites were all collected at 0, 8, 24 and 32 h after the first midge feeding point. All animal experiments were carried out humanely and in accordance with the requirements of the Animal (Science Procedure) Act 1986, under project licence number PPL90/00866. Isolations of BTV from these samples were performed as described below.

Insects. Adult *C. sonorensis* (previously named *C. variipennis*) (a known vector of BTV) from a colony maintained at IAH Pirbright (Boorman, 1974) were used in these experiments.

Virus isolation. Heparinized whole blood, homogenized skin biopsy samples, tissue fluids and blood collected from skin biopsy sites were inoculated into BHK cell cultures and chick embryos (Mellor *et al.*,

1984) in attempts to isolate BTV. Similarly, the supernatants from skin biopsy tissue cells (collected after culturing for 1 week at 37 °C) and lymphocyte cultures (established from tissue fluids and bloods recovered from skin biopsy sites and cultured with IL-2 for 1 week) were also assayed for BTV. Midges that had been fed on infected sheep were incubated for 10 days at 25 °C and were then homogenized in ground glass grinders before assay for infectious virus (Mellor *et al.*, 1984).

Cell lines and cultures. Ovine (S-59 and S-96) γδ T-cell lines, a bovine (Th-67) γδ T-cell line (Takamatsu *et al.*, 1997), a short-term cultured ovine (S-40) γδ T-cell line [γδ T-cell receptor $(TCR)^+$, $WC1^+$, $CD2^-$ and $CD8^-$], a bovine (TC-6) $CD4^+$ αβ T-cell line (Takamatsu *et al.*, 1990) and an ovine (TS-47) αβ T-cell line (γδ TCR^-, $WC\text{-}1^-$, $CD2^+$ and $CD4^+$) were used in these experiments. All of the T-cell lines used were IL-2 dependent and were cultured with Iscove's modified MEM (IMEM) supplemented with recombinant human IL-2 (10 U ml^{-1}), 10 % heat-inactivated FCS and antibiotics, as described previously (Takamatsu *et al.*, 1997). Primary sheep skin fibroblast cell lines (skin 59, skin 59-3, skin 73, skin 82, skin 83, skin 85 and skin 96) were prepared from skin biopsy samples and were maintained as described previously (Takamatsu & Jeggo, 1989). A sheep endothelial cell line was prepared from mechanically harvested large vessel endothelium from sheep aorta and cultured as described by Ryan *et al*. (1978). The γδ T-cell line S-59, as well as skin fibroblast cell lines skin 59 and skin 59-3, were all derived from the same animal.

The γδ T-cells from BTV-infected sheep were cultured as follows: peripheral blood lymphocytes (PBLs) were isolated from heparinized blood by gradient centrifugation on Nycoprep Animal (Nycomed) and plastic-adherent cells were removed by repeated incubation at 37 °C for 30 min in plastic tissue culture flasks. Non-adherent PBLs were then incubated with anti-sheep CD2 (mAb 36F, IgG2a), anti-sheep CD8 (mAb 7C2, IgG2a) and polyclonal rabbit anti-sheep immunoglobulin (Dacopatts) at 4 °C for 30 min. After washing twice with IMEM, the antibody-coated αβ T-cells and B-cells were lysed by rabbit complement (Cedarlene Laboratories) by incubating at 37 °C for 30 min. Live cells were isolated by gradient centrifugation on Nycoprep Animal and cultured with IL-2-containing IMEM. The purity of the γδ T-cells in the cultures was assessed by FACS analysis (see below).

FACS analysis. FACS analysis was performed with FACScan (Becton Dickinson) using the LYSIS II program and the following mouse mAbs: anti-bovine WC-1, which cross-reacts with sheep; mAb CC 15 (IgG2a), anti-sheep WC-1; mAb SC-29 (IgG1), anti-sheep γδ TCR; mAb 86D (IgG1), anti-sheep CD4; mAb 17D (IgG1), anti-sheep CD8; mAb 7C2 (IgG2a), anti-sheep CD45; mAb 1D (IgG1) and goat anti-sheep Ig conjugated with FITC. Secondary antibodies, conjugated with either FITC or R-phycoerythrin (PE), were obtained from Southern Biotechnology. For BTV antigen staining, cells were fixed with 1 % paraformaldehyde, permeabilized with 0.3 % saponin/PBS (containing 0.25 % BSA) and stained with

mAb 3-17-3A (mouse anti-BTV VP7) in 0.3 % saponin/PBS, followed by anti-mouse PE-conjugated immunoglobulin in saponin/PBS. mAbs 124 (anti-human Bcl-2, which cross-reacts with sheep cells) and ILA-30 (anti-bovine IgM, which does not cross-react with sheep cells) were used as positive and negative controls, respectively.

Immunohistology. Skin biopsy samples were frozen in Tissue-Tek OCT (Miles) by floating in isopropanol cooled with dry ice. Cryostat sections (5 μm) were prepared using a Jung Frigocut cryostat (Reichert–Jung) and standard immunochemical staining techniques were used following the protocol of the Vectastain ABC kit (Vector Laboratories).

RESULTS

All of the established ovine and bovine T-cell lines were tested and found to be susceptible to infection with BTV *in vitro*. Each of these cell cultures continued to grow after infection, with no obvious CPE. The bovine cell line TC-6 was cultured for 6 weeks, while other cell lines, including Th-67 (bovine), S-40 (ovine) and TS-47 (ovine), were cultured for 2–3 weeks before culture termination, with no sign of CPE. Some of the ovine γδ T-cell lines (S-59 and S-96) were cultured for 10 weeks p.i. and remained viable (Fig. 1C). The virus titre in these cultures was generally low (<1 infectious unit produced per cell), although the amount of virus recovered from the cell supernatant gradually increased during the first 5 days of culture (Fig. 1A).

To confirm that all of the cultured cells were infected with BTV, the γδ T-cell line (S-59) was fixed at 4 weeks p.i., permeabilized and examined by FACS analysis for intracellular expression of the BTV VP7(T13) protein. The results (Fig. 1B) show that all of the cells were positively stained by an anti-VP7(T13) mAb (mAb 3-17-3A), confirming that they were infected. The number of cells present continued to increase over time, demonstrating continued growth despite BTV infection (Fig. 2A). This was confirmed by cellular uptake of [^{3}H]thymidine (TdR) (Fig. 2C). Therefore, BTV can infect ovine γδ T-cells without causing shut-off of host cell protein synthesis.

In order to assess the effect of host cell shut-off on persistent infection of γδ T-cells with BTV, growth arrest was induced in BTV-infected S-59 cells by treatment with a mAb to the γδ T-cell-specific molecule WC-1 (mAb SC-29) (Takamatsu *et al.*, 1997). The persistent nature of the infection was changed immediately, resulting in growth arrest (Fig. 2A) and a progressive reduction in cell viability (Fig. 2B), so that at 4 days post-treatment, the culture consisted largely of dead cells (Fig. 2B). Treatment with the anti-WC1 mAb SC-29 also resulted in >100-fold increase in the amount of infectious BTV released into the cell culture supernatant (Fig. 2D).

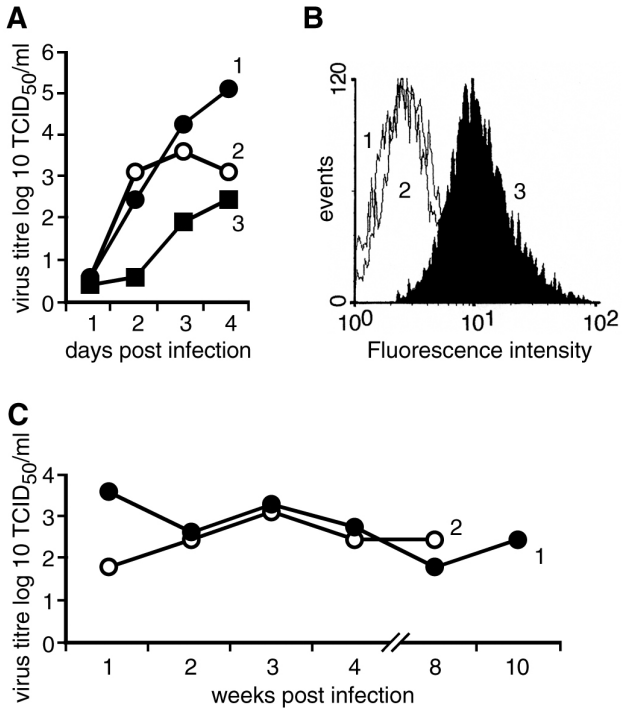

Fig. 1. Establishment of BTV persistence in γδ T-cells *in vitro*. (A) The amount of infectious BTV released from γδ T-cell cultures between days 2 and 5 p.i. was determined by titration of samples of cell culture supernatant. Established bovine Th-67 cell line (marked as 1) or ovine S-59 (marked as 2) and S-96 (marked as 3) γδ T-cell lines were used. (B) Expression of the BTV protein VP7(T13) in infected γδ T-cells (cell line S-59) was detected at 4 weeks p.i. by FACS analysis. Cells were fixed, permeabilized and stained with the anti-BTV-VP7(T13) mAb 3-17-3A (marked as 3) or an isotype control mAb (marked as 2) or without antibodies (marked as 1). (C) Titres of infectious virus released for up to 10 weeks p.i. were also determined for ovine γδ T-cell lines S-59 (marked as 1) and S-96 (marked as 2) cultures that were infected persistently with BTV.

A total of seven sheep skin fibroblast cell lines and a sheep endothelial cell line were also examined for their ability to induce growth arrest of the γδ T-cell line S-59. Three of the skin fibroblast lines [skin 82 (Fig. 3A), skin 59-3 and skin 96 (data not shown)] did induce growth arrest, a process that was therefore not MHC-restricted. However, two of these cell lines (skin 59-3 and skin 96) later lost this ability. The absence of growth arrest by two further cell lines (skin 59 and skin 73) provides MHC-matched and MHC-mismatched negative controls for the induction of growth arrest by skin 82 (Fig. 3A).

The endothelial cell line also had no effect on γδ T-cell proliferation (data not shown). Co-cultivation of infected γδ T-cells with sheep skin fibroblast cells skin 82 also induced conversion to the lytic and more productive form of BTV infection, in a manner that appeared to be similar to that induced by treatment with the anti-WC1 mAb SC-29 (Fig. 3B).

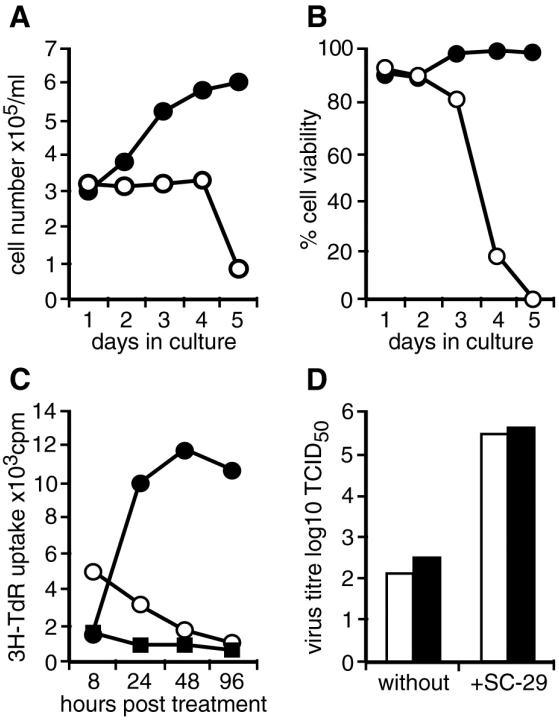

Fig. 2. Anti-WC-1 antibody induces γδ T-cell growth arrest and converts persistently BTV-infected γδ T-cells to lytic infection. Inhibition of the growth of γδ T-cells (cell line S-59) that were persistently infected with BTV by treatment with the anti-WC-1 mAb SC-29. (A) Counts of total cell numbers and (B) the percentage of viable cells in mAb SC-29-treated (○) and untreated cells (●). (C) Measurement of [^{3}H]TdR uptake by infected and untreated cells (●), BTV-infected cells treated with mAb SC-29 (■) and uninfected cells treated with mAb SC-29 (○). (D) Enhanced production of infectious virus as a result of treatment with the anti-WC-1 mAb SC-29. Open bars indicate virus recovery from culture supernatants and solid bars indicate virus recovery from total cell lysate.

Six sheep were infected with BTV by intradermal inoculation. All of them developed mild but typical clinical signs of bluetongue, including fever, lymphopenia and swollen muzzles. Viraemia was detected in each case from 4 or 5 days until 21 to 28 days p.i. The γδ T-cells recovered from peripheral blood samples taken on days 3–13 p.i. from each of the infected sheep were shown to be infected with BTV. BTV was rarely detected in the freshly isolated and purified γδ T-cells but the virus was readily recovered when the cells were cultured with IL-2. Few, if any, γδ T-cells were recovered from blood samples taken between 14 and 34 days p.i. due to lymphopenia, which reduced very significantly the number of these cells in circulation. Virus was not recovered from the γδ T-cells that were isolated from peripheral blood samples taken between 35 and 63 days p.i., even when they were cultured with IL-2.

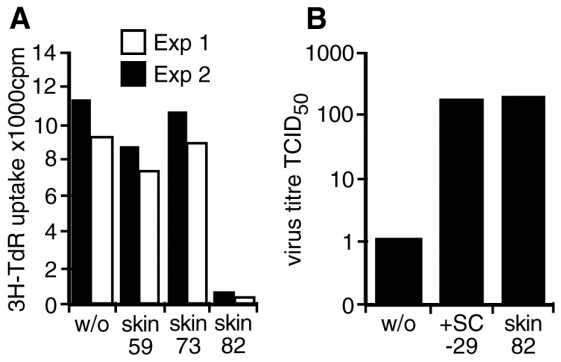

Fig. 3. Skin fibroblasts induce γδ T-cell growth arrest (A) and convert persistently BTV-infected γδ T-cells to lytic infection. (A) γδ T-cells S-59 (2×10^5 cells per well) were cultured for 24 h in 96-well plates in the absence (w/o) or presence of γ-irradiated (2500 rad) monolayers of skin fibroblast cells. Results for [^{3}H]TdR uptake (c.p.m.) in the presence of different fibroblast cell lines (skin 59, skin 73 and skin 82, as indicated) are shown as a mean (triplicate). The results of two similar experiments (1 and 2) are shown. (B) γδ T-cells (S-59), persistently infected with BTV, were treated with mAb SC-29, co-cultured with γ-irradiated skin 82 fibroblast cells or left without treatment for 3 days. Supernatants from each culture were collected and virus production was assessed by titration in BHK cells.

The γδ T-cells isolated from the blood of BTV-infected sheep (3–13 days p.i.) were cultured for up to 6 weeks and it was confirmed by recovery of infectious virus that they were persistently infected. At 4 weeks into the culturing period, five of the γδ T-cell cultures were co-cultured with γ-irradiated skin 82 cells. As observed previously with established ovine γδ T-cell lines, this treatment resulted in conversion to a lytic form of BTV infection. The treatment also increased the amount of virus recovered from these cultures (Table 1), although the degree of enhancement (between log 0.2 and 2.6) was less dramatic than that observed with the established S-59 cell line (Fig. 3B).

Table 1. Enhanced recovery of BTV from cultured PBL γδ T-cells isolated from infected sheep and co-cultured with skin fibroblasts

	Virus titre (log $TCID_{50}$ ml^{-1})	
Culture	**Without skin 82**	**With skin 82**
A30	4.7	5.0
A31	3.1	5.6
A36	4.1	5.3
A37	4.0	4.3
B37	3.8	4.0

At 9 weeks after the initial BTV infection and at least 35 days after termination of any detectable viraemia, adult female *C. sonorensis* were fed on the BTV-infected sheep. 'Longitudinal' samples of blood-fed midges, sheep blood and skin biopsies of the insect biting area were taken and analysed for infectious virus. No virus was recovered from any of the engorged midges, nor, initially, from the sheep skin biopsies or blood samples. However, infectious BTV was subsequently recovered (in tissue culture

supernatant) by culturing cells from skin biopsy sites for 7 days in the presence of IL-2 (Table 2). The virus was recovered in this way from samples taken 8 h post-midge feeding from all six sheep. Virus was also recovered from four of six samples taken just prior to midge feeding (time 0) and from half of the samples taken at 24 and 32 h post-midge feeding (Table 2).

Table 2. Isolation of BTV from skin biopsies of midge-biting areas from sheep infected previously with BTV

	Isolation of BTV from biopsy (h after feeding midges)			
Sheep	**0**	**8**	**24**	**32**
TE30	+	+	–	+
TE31	+	+	–	–
TE33*	–	+	+	–
TE34*	–	+	+	+
TE36	+	+	+	+
TE37	+	+	–	–

*All sheep, except TE33 and TE34, were midge sensitized.

Of the 24 lymphocyte cultures originating from skin biopsy sites, three were examined by FACS and 11.7±2.4 % of the cells present were identified as γδ T-cells. No virus was recovered from skin biopsies taken from uninfected control sheep.

Severe skin inflammation was caused by feeding *Culicoides* midges on the sheep, which can be seen in Fig. 4(A), extending across the whole diameter (6 cm) of the cup used previously to keep the insects in place. In addition, immunohistological studies of skin biopsy tissues taken at 8 h post-feeding from three of the 'midge-sensitized' and two untreated sheep demonstrated a massive accumulation of γδ T-cells in the midge feeding areas (more than 350 γδ T-cells per field at ×400 magnification) (Fig. 4B). In samples from untreated sheep, or from non-inflamed areas of the sensitized sheep, an average of 20–30 γδ T-cells per field were detected. These cells were spread evenly and not clustered as in the inflamed tissue samples. Both $CD4^+$ (120–210 cells per field at ×400 magnification) and $CD8^+$ T-cells (65–77 cells per field at ×400 magnification) were observed in samples from midge feeding areas but in smaller numbers than γδ T-cells (Fig. 4C, D).

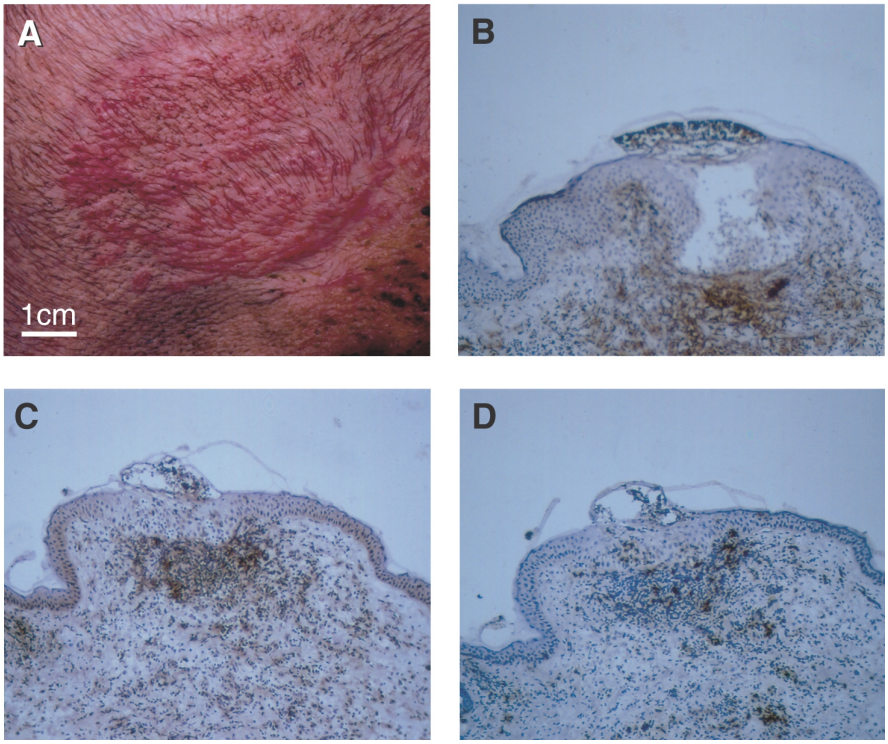

Fig. 4. (A) Inflammation of sheep skin induced by the biting of *Culicoides* midges. Immunohistochemical staining of the inflamed region of the skin with (B) antibody CC-15 to detect accumulation of γδ T-cells, (C) antibody 17D to detect accumulation of $CD4^+$ T-cells and (D) antibody 7C2 to detect $CD8^+$ T-cells. Large numbers of γδ T-cells were detected (darkly stained regions, B). $CD4^+$ and $CD8^+$ T-cells (C, D) were also observed but in smaller numbers. Histological photographs, magnification ×100.

DISCUSSION

The overwintering mechanisms of BTV and AHSV are poorly understood. However, it is well known that both viruses are transmitted in the field almost entirely by certain species of *Culicoides* biting midge. Adult midges only become infected by ingestion of a blood meal from a viraemic mammalian host and are only capable of transmitting the virus when taking a subsequent blood meal. Therefore, if the vector is absent for a period of time longer than the maximum duration of viraemia in the mammalian host, the life cycle of the viruses should be broken and they would be unable to survive. Epidemiological studies have shown that in many areas of the world, the numbers of adult vector insects declines dramatically as a result of adverse weather conditions and they are effectively absent, usually during winter periods. Under these circumstances, the viruses (BTV and AHSV) also 'disappear' and cases of clinical disease and seroconversion in susceptible ruminants or equids cease abruptly with the onset of severe weather. However, in such locations the viruses (and the diseases they cause) frequently recrudesce annually after quiescent periods that can last as long as 8–12 months (Yonguc *et al.*, 1982; Taylor & Mellor, 1994). These periods are significantly longer than the maximum duration of detectable viraemia in the mammalian hosts: <50 days for BTV in sheep (Sellers & Taylor, 1980; Koumbati *et al.*, 1998), <100 days

for BTV in cattle (Sellers & Taylor, 1980; MacLachlan *et al.*, 1991), 18 days for AHSV in horses (Mellor, 1993) and 40 days in zebra (Mellor & Boorman, 1995), indicating that an overwintering mechanism exists, allowing these viruses to survive throughout vector-free periods. The existence and nature of this mechanism plays a vitally important role in virus survival and in the epidemiology of the diseases that they cause.

One possible overwintering mechanism could involve survival of infected adult vector insects. The lifespan of an adult midge is usually less than 10 days. However, in exceptional conditions, some individuals can survive for several weeks, but there is no evidence to suggest that they could survive for an entire intra-epizootic period of 9–12 months (Mellor, 1990). Survival of the virus in persistently infected, long-lived adult *Culicoides* is therefore considered to be very unlikely indeed. *Culicoides* midges usually survive winter periods as larvae but there is no evidence of transovarial transmission of either BTV or AHSV in these insects. Persistence of virus in larvae is therefore also considered to be highly improbable (Mellor, 1990).

The re-introduction of either infected adult midges or viraemic vertebrate hosts from other enzootic areas could also give the appearance of overwintering. Although this possibility is often difficult to exclude with certainty, in at least some cases it cannot be reconciled with epidemiological data. For example, during the outbreak of African horse sickness (AHS) in Spain in 1987–1990, annual disease episodes were each caused by AHSV-4, a serotype that had never been recorded previously outside southern Africa. At the time of the outbreaks there was no other evidence of AHSV, of any serotype, within 2000 miles of Spain (Mellor & Boorman, 1995). Under these circumstances, the possibility of annual re-introduction of AHSV-4 over four consecutive years is remote in the extreme. Precisely the same situation prevailed during the BTV-10 outbreaks in Spain and Portugal from 1955 to 1960, the BTV-4 outbreaks in western Turkey from 1977 to 1981 and the current outbreaks of BTV-2, -4, -9 and -16 in the Mediterranean region. In each case there was an initial introduction of virus to a location that was geographically remote from the nearest enzootic zones, followed by recrudescences of the same virus type(s) over a period of years.

A third possibility is that an unknown vector or vertebrate host species could be involved, providing a natural reservoir in which the virus could persist. This is also most unlikely, as the ‘winter’ conditions that result in the absence of known BTV and AHSV vector species of *Culicoides* will have precisely the same effect on other less abundant and less efficient vector species of *Culicoides* and other biting insects (Ortega *et al.*, 1998). Attempts to infect a wide range of animals, including mice, rats, hamsters, guinea pigs, rabbits, ferrets, dogs, other carnivores, camel and elephant, have been carried out with either AHSV and/or BTV. In all cases these species are not considered to play a significant role in the epidemiology of either disease (Coetzer & Erasmus, 1994; Alexander *et al.*, 1994; Akita *et al.*, 1994). In the absence of an

identifiable overwintering reservoir, an alternative vector or mammalian host species, we have considered the possibility that orbiviruses might persist via some unidentified mechanism in their usual vertebrate hosts.

The experiments described here demonstrate that infectious BTV can be recovered from ovine skin biopsies for more than 9 weeks p.i. (i.e. for the entire duration of the experiment), although the viraemic phase had ended after only 3–4 weeks. It appears likely that the source of the virus is γδ T-cells that became persistently infected during the initial stages of virus replication and viraemia in the host. BTV association with leukocytes and virus replication in lymphocytes has been demonstrated previously by many authors (Morrill & McConnell, 1985; Whetter *et al.*, 1989; Stott *et al.*, 1990). Stott *et al.* (1990, 1992) also observed a persistent BTV infection in bovine $CD8^+$ T-cells and in null cells *in vitro*. The null cells were so described due to an absence of expression of CD2, CD4, CD6, CD8 or WC-1 molecules, as recognized by mAb IL-A29. Although the authors started with γδ T-cell-enriched cultures, IL-A29-positive cells were gradually lost from the culture (Stott *et al.*, 1992). Activated γδ T-cells isolated from ruminants are not only negative for CD2, CD4 and CD8 expression, they also show low or zero expression of CD6 and loss of the WC-1 epitope recognized by mAb T-19 or IL-A29 (Lund *et al.*, 1993; H. Takamatsu unpublished data). However, they do show increased expression of the WC-1 epitope recognized by mAb SC-29 (H. Takamatsu, unpublished data). The null cells identified by Stott *et al.* (1992) are therefore likely to be activated γδ T-cells. In this study we have confirmed persistent BTV infection of IL-2-dependent ovine and bovine γδ T-cells cultures *in vitro*. These BTV-infected γδ T-cell cultures grow continuously, with no obvious CPE. A similar persistent infection of BTV has been demonstrated in some insect cell cultures without shut-off of host cell DNA or protein synthesis (Jennings & Boorman, 1979; Wechsler *et al.*, 1989; Fu *et al.*, 1999) where there was also little or no sign of CPE.

BTV and AHSV possess many characteristics in common with other members of the family *Reoviridae* and the model for BTV replication is partly based on orthoreovirus replication (Eaton *et al.*, 1990). Orthoreovirus non-structural protein σ 1 is essential for cell lysis and CPE (reviewed by Oberhause *et al.*, 1998). The protein σ 1 inhibits host cell DNA synthesis and blocks the host cell from progressing to the S phase so that they remain in the G_1 phase. BTV infection also causes host cell shut-off in lytically infected mammalian cell lines, including BHK cells, but does not induce shut-off in cultured γδ T-cells. It was therefore considered possible that anti-WC-1 antibodies, which also cause growth arrest at the G_1 phase (Takamatsu *et al.*, 1997; Kirkham *et al.*, 1997, 1998), might mimic the processes of host cell shut-down that occur in lytically BTV-infected mammalian cells, thereby causing the conversion to a lytic mechanism, and, indeed, this proved to be so. The same conversion from persistent to lytic BTV infection could also be induced in BTV persistent γδ T-cells by co-culture with some, but not all, skin fibroblast cell lines, incidentally suggesting that the latter bear a WC-1 counter receptor.

If persistent infection of γδ T-cells *in vivo* plays a central role in a BTV overwintering mechanism, then it appears likely that the conversion of persistence to the more productive lytic form of infection would occur at a location and at a time when midges interact with the mammalian host. Recruitment of activated sheep γδ T-cells to inflamed areas of the skin has been observed previously (Bowles *et al.*, 1992; Egan *et al.*, 1996). Biting by *Culicoides* midges has been shown to induce a severe inflammatory response in equid and bovine skin (Mellor & McCaig, 1974; P. S. Mellor & M. H. Jeggo, unpublished data). A similar response was also observed in the sheep used in the study described here. Interactions between skin fibroblasts and the WC-1 molecules on the surface of γδ T-cells may also result in a similar shut-down of those activated γδ T-cells that become involved in lymphoid infiltration to the site of vector biting. If these cells were already persistently infected with BTV, the data presented here indicate that this would be accompanied by conversion to a lytic infection and enhanced virus replication and release. In practice, the release of BTV particles from persistently infected γδ T-cells might be restricted. Low levels of virus production might be masked by the presence of neutralizing antibodies, until sufficient biting insects were present in the environment to induce a sufficiently intense dermal inflammatory response to trigger the release of infectious virus at the site of inflammation and, consequently, of insect biting.

In addition, proteases, which are associated with such an inflammatory response (Harvima *et al.*, 1994), may also play an important role in transmission. Treatment of BTV or AHSV particles with proteases results in cleavage of the outer capsid protein VP2, forming infectious subvirus particles (ISVPs) (Marchi *et al.*, 1995, Mertens *et al.*, 1987, 1996; Burroughs *et al.*, 1994). These ISVPs have a specific infectivity for insect cells or adult *Culicoides* of ~100 times greater than that of intact virus particles and it is considered likely that infection of vector insects is by these protease-modified particles (Mertens *et al.*, 1996).

A vital component of the proposed BTV overwintering mechanism is the requirement for persistent or latent infection of γδ T-cells. At this moment we do not know how persisting BTV escapes detection during immune surveillance. One of the possibilities is that the low level of virus replication in resting lymphocytes (Stott *et al.*, 1992) could result in little or no production of potential cytotoxic T-cell epitopes. Thus BTV-infected, resting γδ T-cells may remain undetected during immune surveillance or attempts at virus isolation. These observations may explain why, in the present study, virus was only recovered when lymphocytes were cultured *in vitro* with IL-2, thus activating γδ T-cells. Confirmation of this proposed BTV overwintering mechanism requires the demonstration of infected ovine γδ T-cells *in vivo* for periods of up to 9–12 months. In relation to this, it is well known that BTV infection in cattle is clinically milder than in sheep and the viraemic period is longer, so that the virus may be more closely adapted to bovines than ovines. Thus, cattle may be a more appropriate mammalian host species in which

to study BTV persistence further. Indeed, although previous reports of 'latent-infection' and 'showering' phenomenon after insect bites in cattle (Luedke *et al.*, 1977) are now largely disregarded, they might be at least partially explained by persistent BTV infection of bovine γδ T-cells.

In the present bluetongue epizootic in Europe and North Africa (1998–2001), penetration of the virus further north than ever before has occurred, particularly in eastern Europe, so that regions of Bulgaria, Serbia and Kosovo have become affected (Mellor & Wittmann, 2002). Astonishingly, the virus also appears to have succeeded in overwintering in this northerly region where adult vectors are absent for several months each year. In these locations, the traditional vector, *C. imicola*, does not occur and a novel vector species with a distribution that extends across central and northern Europe is operating (Mellor & Wittmann, 2002). Should the mechanism outlined in the present study prove to be the method by which BTV is overwintering in the region, then the (seasonal) presence of a more northerly vector could provide the virus with the means to extend its range much further northwards and much more widely across Europe.

ACKNOWLEDGEMENTS

This work was supported by MAFF/DEFRA. P.A.K. was supported by BBSRC grant AT201/439. The European Commission supports P.P.C.M. (Phylogenetic sequence analyses of the family *Reoviridae*, contract no. QLK2-2000-00143, and Bluetongue vaccination, contract no. QLK2-CT-2001-01722) and P.S.M. (Arbovirus vectors and disease, contract no. QLK2-CT-2000-00611, and Bluetongue vaccination, contract no. QLK2-CT-2001-01722). The authors thank Sue Graham and Eric Denison for excellent technical assistance, Mick Denyer for FACS analysis, Brian Taylor and Natasha Smith for animal care, Steven Archibald for photography.

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