A potential overwintering mechanism for bluetongue virus – recent findings

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Summary

Bluetongue virus (BTV) is transmitted between its mammalian hosts almost exclusively via bites from the adults of certain species of *Culicoides* biting midges. Theoretically, the spread of BTV into the more northerly areas of Europe should therefore be terminated by the harsh winters experienced in these regions, when adult midges disappear for extended periods of time. However, it has been shown that BTV can survive for periods as long as 9 to 12 months in such locations in the absence of adult insect vectors, with no detectable cases of viraemia, overt disease or seroconversion in the host species. Virus survival in this manner throughout the winter is called 'overwintering' but the mechanism involved has not been satisfactorily explained. With knowledge currently available and results from a series of preliminary experiments, the authors discuss a possible overwintering mechanism.

Keywords

African horse sickness – Bluetongue virus – *Culicoides* – Growth arrest – *Orbivirus* – Overwintering – Persistent infection – Gamma delta T-cells.

Overwintering of bluetongue virus

It has been established that the members of several virus species that belong to the genus *Orbivirus*, including bluetongue virus (BTV) and African horse sickness (AHS) virus (AHSV), are transmitted in the field almost entirely by certain species of *Culicoides* biting midges. Adult midges can only become infected by ingestion of a blood-meal from an infected mammalian host and are only capable of transmitting the virus to a new host when they take a subsequent blood-meal. If adult vectors are absent for a period of time that is longer than the maximum duration of viraemia in the mammalian host, newly emerging insects should not become infected. The life-cycle of these viruses should therefore be broken and they would be unable to survive. Indeed, epidemiological studies have shown that in many areas of the world where adult vector insects are effectively absent during winter periods, viruses (such as BTV and AHSV) and the diseases they cause also 'disappear'. However, these viruses and diseases frequently also recrudesce annually, after quiescent periods that can last as long as 8 to 12 months (30, 31). These periods are significantly longer than the maximum published duration of viraemia, namely: <50 days for BTV in sheep (12, 25), <100 days for BTV cattle (16, 25), 18 days for AHSV in horses (18), and 40 days in zebra (20), confirming that a mechanism must exist that allows the virus to 'overwinter' in these locations.

The re-introduction of either infected adult midges or viraemic vertebrate hosts from other enzootic areas could 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 the available epidemiological data. For example, during the outbreak of AHS in Spain from 1987 to 1990, annual disease episodes were caused by serotype 4, which had never previously been recorded outside southern Africa. At the time

of the outbreaks there was no evidence of AHSV of any serotype within 2 000 miles of Spain (20). Under these circumstances, the possibility of annual re-introduction of the same serotype (AHSV-4) over four consecutive years, is remote in the extreme. Similar situations also prevailed during the BTV-10 outbreak in Spain and Portugal from 1955 to 1960, the BTV-4 outbreak in western Turkey from 1977 to 1981 and during the current series of outbreaks of BTV serotypes 1, 2, 4, 9 and 16 in the Mediterranean and Balkan regions. 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 serotype(s) over periods of years, with no evidence of virus transmission during the quiet periods.

From these observations, we consider it likely that in many such situations BTV is overwintering and that the mechanism involved is likely to play a primary role in the long-term survival of the virus in temperate zones. Elucidation of the virus survival mechanism may be essential to gain a full understanding of the epidemiology of BT and to enable effective control strategies to be developed.

Survival of bluetongue virus in the winter

One possible overwintering mechanism could involve the long-term survival of infected adult vector insects. However, the life-span of an adult midge is usually less than 10 days. In exceptional conditions, some midges can survive for several weeks but there is no evidence to suggest that adult midges can survive for an entire intra-epizootic period of 9 to 12 months (17). The survival of BTV in this way is therefore considered to be exceedingly unlikely. *Culicoides* midges usually survive winter periods as 4th instar larvae but there is no evidence of transovarial or vertical transmission of BTV, AHSV or indeed any other arbovirus in these insects. Persistence of virus in the immature insect stages is therefore also considered to be highly improbable (17). A further 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 the adults of known BTV and AHSV vector species of *Culicoides*, will have precisely the same effect on other less abundant and less efficient vectors (24). In respect of unknown vertebrate reservoirs, attempts to infect a wide range of animals (mice, rats, hamsters, guinea-pigs, rabbits, ferrets, dogs, other carnivores, camel and elephant) have been made at one time or another with either AHSV or BTV. In all cases these species are not considered to play a significant role in the epidemiology of either disease (1, 2, 6). 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 overwintering mechanism – a hypothesis

Cattle and other ruminants were indeed incriminated as possible hosts for overwintering as early as towards the end of the 1960s (4). However, after the publication and retraction of controversial work indicating both latent BTV infection and a 'showering' phenomenon in cattle (13), the overwintering mechanism is a subject that has been largely avoided. For successful BTV transmission and recrudescence of the disease to occur, the BTV overwintering mechanism must make infectious virus particles available to feeding midges at a time when sufficient adult insects are available for successful transmission of the disease. A possible overwintering mechanism was recently suggested (29) based on the following observations:

a) ovine and bovine lymphocyte cultures (including γδ T-cells) can be persistently infected with BTV *in vitro* (26, 27, 29) without apparent cytopathogenic effect (CPE), or host cell protein shut-off, as has also been observed in persistently BTV-infected *Culicoides* cells (15)

b) BTV-infected γδ T-cells can be isolated from experimentally infected sheep, at least during viraemic periods (29)

c) persistently BTV-infected γδ T-cells can be converted to a more productive, lytic infection *in vitro* by co-culturing with anti-white collar-1 (WC-1) antibody, or with certain skin fibroblasts (29) (note: orthoreovirus structural protein σ-1 is required for cell lysis and CPE. Orthoreovirus σ-1 blocks the host cell cycle at the G1 phase (23). Anti-WC-1 antibody induces G1 cell growth arrest of proliferating γδ T-cells) (9, 10, 11, 28)

d) feeding of *Culicoides* midges induces skin inflammation in both ruminants and horses (19, 29; P.S. Mellor & M.H. Jeggo, unpublished observation)

e) skin inflammation recruits activated γδ T-cells into the inflamed areas (3, 7, 29)

f) BTV can be isolated for at least 9 weeks after the termination of detectable viraemia from cultures of activated γδ T-cells that were derived from skin biopsy sites on previously infected sheep, then cultured with interleukin-2 (IL-2) (29)

g) proteases released by inflammation of the skin (8) may cleave the outer capsid protein of BTV to form infectious subviral particles (ISVP) (5, 14, 21, 22), which are ~100 times more infectious to midge cells and adult midges than intact BTV particles (22).

Thus we hypothesise that the inflammation induced at the site of midge feeding recruits γδ T-cells that are persistently infected with BTV, where interaction with skin fibroblasts converts the persistent infection to a lytic form. This results in an increase in BTV replication and virus production. The released BTV is then modified by inflammatory proteases, generating ISVP, the more infectious particle type (for insects), thereby increasing the likelihood that an infection will be established in the vector midges. The transmission of BTV from a fully infected adult midge to a susceptible mammalian host is very efficient, requiring only a single bite. The infection of even a single midge from a host in which the virus has successfully survived the winter may therefore be sufficient to reinitiate an outbreak of disease (Fig. 1).

Questions to be answered

We believe this hypothesis is the most logical and reasonable explanation of BTV overwintering mechanism based on currently available knowledge. A number of the component steps in the proposed mechanism remain to be explored and confirmed, so this still remains as only a hypothesis. These include:

1. Where are persistently infected γδ T-cells localised in the host during vector free periods?
2. How do persistently infected γδ T-cells (and possibly other infected cell types) remain undetected by immune surveillance?
3. Is there any role in this mechanism for other lymphocyte subsets, such as CD4+ and CD8+ T-cells, which can be persistently infected with BTV *in vitro*?
4. Are some species/breeds of ruminant more likely to support overwintering of BTV than others?
5. Are some BTV types/strains better adapted to overwintering than others?
6. What is the molecular mechanism by which BTV fails to shut-down persistently infected γδ T-cells, and how does WC-1 signalling enhance BTV replication?
7. Are sufficient numbers of infectious BTV particles released at the biting site to initiate infection in an adult vector insect?

It appears likely that in the absence of cell lysis BTV would exit persistently infected lymphocytes (e.g. γδ T-cells) by budding through the cell

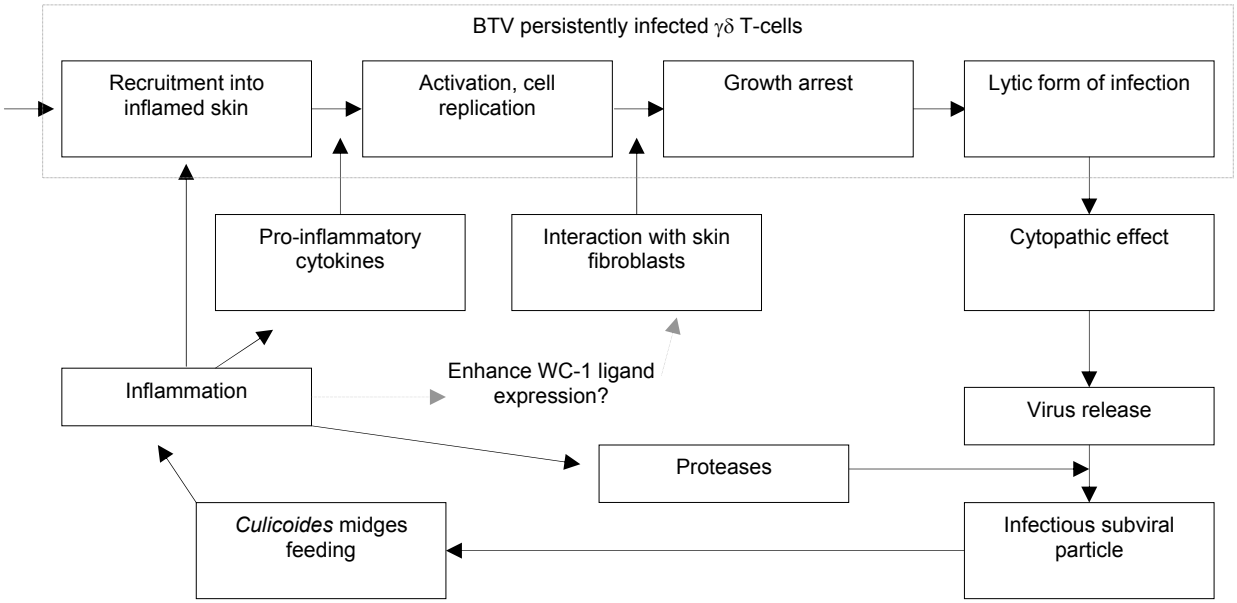

Figure 1
A possible overwintering mechanism for bluetongue virus
BTV can persistently infect ruminant γδ T-cells. Skin inflammation induced by biting midges has been shown to induce a severe inflammatory response in the ruminant host, resulting in recruitment of activated γδ T-cells (some of which may be infected with BTV) into the inflamed areas. Interaction between skin fibroblasts and γδ T-cells appears likely to occur via binding of WC-1 on the γδ T-cell surface, to a WC1 ligand on skin fibroblasts (inflammation may also up-regulate expression of the WC1 ligand). This interaction causes host cell shut-off in the activated γδ T-cells, accompanied by conversion to a lytic and more productive form of BTV-infection. This will result in the release of BTV particles at skin locations where midges are feeding. Inflammation associated proteases may also cleave the BTV outer capsid protein 'VP2', generating infectious subviral particle (ISVP) that are ~100 times more infectious for *Culicoides* midges than intact virus, further increasing the infection rate of the feeding midges

membrane and would become coated by the host lymphocyte membrane in the process – 'membrane enveloped virus particles' (MEVP) (15). This suggests some possible answers to the questions listed above (Fig. 2). For example:

1. Since the BTV outer capsid would be coated with a lymphocyte membrane, neutralising antibodies may be unable to recognise the concealed outer capsid proteins (VP2 and VP5).
2. Lymphocyte surface membranes contain a number of important molecules associated with lymphocyte-lymphocyte interactions. Thus, the lymphocyte-derived MEVP may specifically bind to and infect other lymphocytes generating further persistently infected cells.
3. Interaction between persistently infected and uninfected lymphocytes may also result in transmission of BTV infection via fusion between the cellular and budding membranes (interaction between γδ T-cells and CD4+ T-cells has been reported).
4. MEVP may be less effectively detected by conventional BTV isolation methods (but could be detected by reverse transcriptase-polymerase chain reaction [RT-PCR]).
5. MEVP that may bind to or be taken-up by dendritic cells (DC), may induce peripheral tolerance or anergy, due to 'self-antigen' on the virus surface. Consequently DC could suppress anti-BTV immune responses.
6. It appears likely that host lymphocyte membranes would be removed by proteases associated with inflammation and may not therefore affect the ability of the resulting modified virus particles (ISVP) to infect midges.

A vital component of the suggested mechanism for BTV overwintering is the requirement for persistent or latent BTV-infection of γδ T-cells, which has been demonstrated *in vitro*. However, for this mechanism to be effective, persistently infected γδ T-cells (or possibly other lymphocytes) must exist *in vivo* for periods of up to 9 to 12 months after the termination of viraemia. A demonstration that these cells can exist for long periods in the post-viraemic mammalian host is still required and would strongly support the overwintering hypothesis.

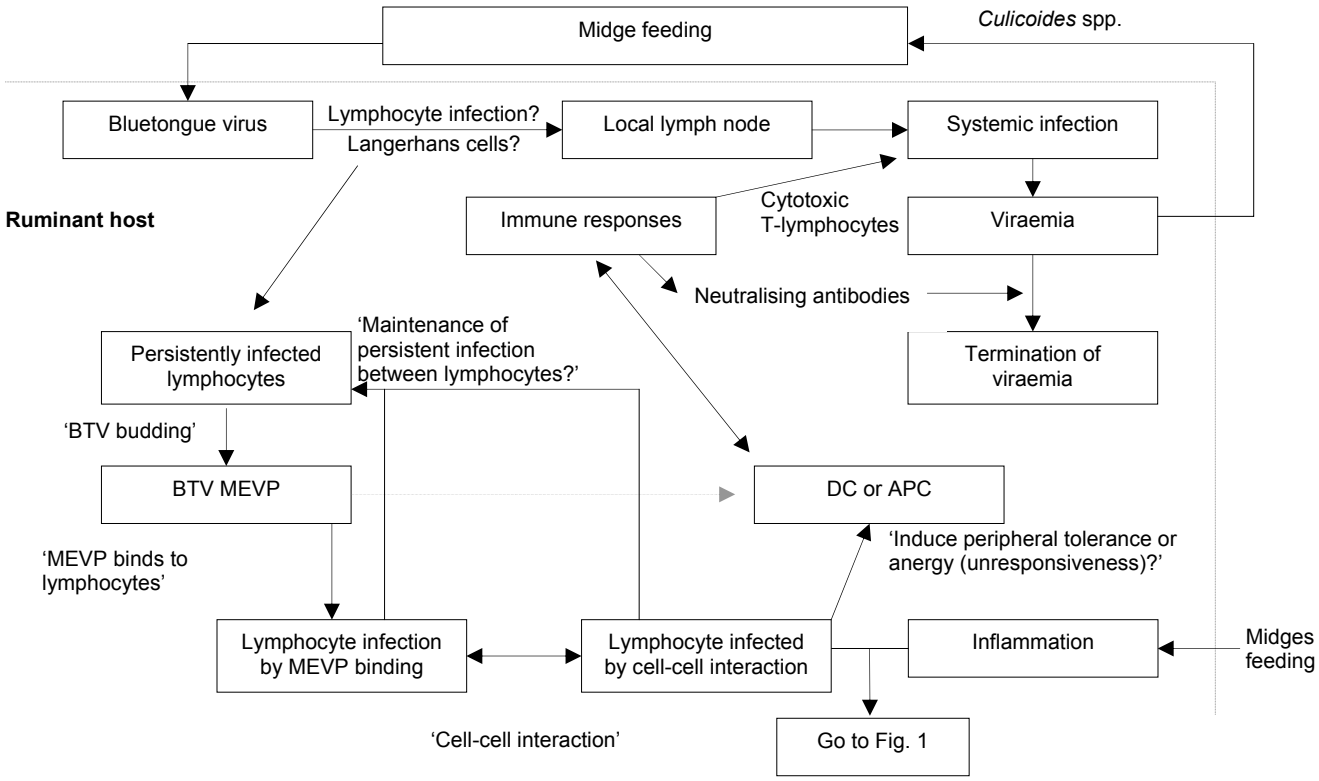

Figure 2
A possible mechanism to maintain persistent infection of bluetongue virus in mammalian host species
Lymphocytes from cattle and sheep can be persistently infected with BTV. Although this persistent infection does not result in host cell shut-off or lysis (so there is little or no sign of CPE) the virus can still escape by budding through the cell membrane, a mechanism that has previously been observed in both mammalian and insect cells. In doing so, the virus particles initially become coated with host cell membranes and have been identified as membrane enveloped virus particles (MEVPs). The BTV outer capsid components (VP2 and VP5) are likely to be masked by this envelope, making MEVPs less likely to be neutralised by antibodies. On the other hand, when taken-up by dendritic cells (DC) or other antigen presenting cells (APC), this could result in peripheral tolerance, or anergy due to 'self-antigen' on the virus surface. Lymphocyte membranes contain a number of important molecules associated with lymphocyte-lymphocyte interactions. As a consequence, MEVP may be able to bind to other lymphocytes, enhancing their infectivity for these cells. Direct interactions between persistently infected and uninfected lymphocytes could also result in BTV transmission

It is recognised that BTV infection in cattle is clinically milder than sheep and longer viraemic periods have been reported. It therefore appears likely that the virus may be better adapted to bovines than ovines. Thus cattle may be a better mammalian host species for further study of BTV persistence and overwintering. Epidemiological studies of specific breeds of sheep, cattle or goats from locations where BTV overwintering has been observed may also identify other species/breeds of ruminant host that are more susceptible to persistent infection and overwintering. Molecular biological studies (sequencing and/or generation of reassortant virus strains) with BTV isolates from locations where overwintering has been observed, may also contribute to our understanding of the mechanism involved. Field isolates from the current outbreaks in Eastern Europe may therefore provide valuable reagents allowing us to study and elucidate the overwintering mechanism in the laboratory. The design of fully effective anti-BTV strategies may only be possible with a full understanding of the mechanisms involved in virus persistence in the field. We therefore hope, by proposing this hypothesis, to trigger an international effort to solve the mystery of BTV overwintering.

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