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Molecular characterization of tick-virus interactions

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1. ABSTRACT

All viruses infecting ticks (with one possible exception) are arboviruses; their survival depends on infection and replication in both tick and vertebrate host cells. Little is known of arbovirus-tick molecular interactions even though tick-borne viruses spend most of their existence in ticks. Initial interactions occur in the midgut, where bloodmeal digestion is intracellular in contrast to hematophagous insects. The contrast may explain differences in surface architecture of tick-borne and insect-borne orbiviruses. Other indications of molecular interactions can be extrapolated from vertebrate cells, such as utilization of aggresome pathways. Although many tick-borne viruses exploit the immunomodulatory effects of tick saliva, there is no evidence they interact directly with saliva molecules. However, the most fundamental unanswered question concerns the benign infection of arboviruses in tick cells compared with their cytopathic effect in vertebrate cells. As the tick proteome is unraveled, its interaction with the viral proteome should shed new light on the molecular interface between ticks and the many important viruses they transmit.

2. INTRODUCTION

2.1. Tick viruses

With one possible exception, no recognized viruses survive solely in ticks (1) St Croix River virus (SCRV), an orbivirus isolated from the IDE2 cell line derived from the eggs of *Ixodes scapularis,* may be the exception. The eggs were from a tick removed from a deer during hunting by the St Croix river, Wisconsin, USA (2) The tick cells appear to be persistently infected with the virus but with no detectable cytopathic effect. SCRV is unrelated to any of the recognized orbivirus species, including the tick-borne *Great Island virus* (3) If SCRV is a true tick virus (rather than a tick-borne virus that replicates in both tick and vertebrate cells), its life cycle is presumably sustained through transovarial transmission from one tick generation to the next.

In contrast to ticks, viruses that infect and replicate only in insects are numerous, belonging to 15 different virus families (1) Some are viruses that infect insect vectors but they are not transmitted to vertebrate hosts, for example, *Aedes aegypti entomopoxvirus*, *A. aegypti densovirus*, and *A. albopictus densovirus*. Several

insect viruses, such as certain baculoviruses, kill their insect hosts and have been exploited as biopesticides to control insect pests of crops (4)

The reason for the apparent lack of tick viruses probably lies in the paucity of studies to find them. Viruses belonging to the *Metaviridae* are retrotransposons, able to integrate into their host's genome. They have been found in all studied lineages of eukaryotes so they should be expected in the tick genome.

2.2. Tick-borne viruses

All recognized viruses that infect ticks belong to a large biological group known as the arboviruses (arthropod-borne viruses) They are distinguished from 'tick viruses' (section 2.1.) in being able to replicate in both invertebrate and vertebrate cells. Tick-borne viruses depend for their survival on transmission between ticks and vertebrate hosts. The tick acts as a vector and supports 'biological transmission' in which the virus infects and replicates in tick tissues (contrast 'mechanical transmission' in which no viral replication occurs) Besides acting as a vector, the tick may also act as a reservoir host in which the virus can survive for prolonged periods that preclude active virus transmission, such as may occur during winter periods.

There are some 33 recognized tick-borne viral species belonging to 9 different viral genera together with 32 probable tick-borne viruses, some of which are uncharacterised (Table 1) In addition, West Nile virus, although most commonly transmitted by *Culex* mosquitoes, can also be transmitted biologically by ticks and has been isolated many times from ticks collected in Asia (5)

Tick-borne viruses are found in six different virus families: *Asfarviridae*, *Reoviridae*, *Rhabdoviridae*, *Orthomyxoviridae*, *Bunyaviridae*, and *Flaviviridae*. Some as yet unassigned tick-borne viruses may belong to a seventh family, the *Arenaviridae*. Curiously, with one exception, all tick-borne viruses (as well as all other arboviruses) have an RNA genome. The only known exception is African swine fever virus (ASFV), a unique virus that has a relatively large DNA genome.

The RNA genome of tick-borne viruses varies considerably. It may comprise a single molecule of RNA or several segments, and may be single-stranded or double-stranded (Table 1) The nature of the viral genome determines the strategy of replication. The vertebrate cell nucleus is required for productive infection with ASFV, while virus DNA replication and assembly of virus particles occurs in perinuclear areas ('virus factories') In contrast, RNA arboviruses generally replicate and are assembled in the cytosol, although nuclear involvement has been identified in members of the *Thogotovirus*, *Alphavirus*, and *Flavivirus* genera. Whether or not these basic replication strategies differ in tick cells, is unknown.

Viruses with segmented genomes can exchange segments (reassort) with closely related viruses. The classic example is influenza A virus (a member of the *Orthomyxoviridae*) for which reassortment can give rise to new antigenic phenotypes that cause pandemics (6) Thogoto virus, a tick-borne virus distantly related to influenza viruses, has been shown experimentally to undergo reassortment in its tick vector, *Rhipicephalus appendiculatus* (section 4.2.)

Although tick-borne viruses generally have no apparent adverse effect on their tick vectors, some tick-borne viruses cause debilitating diseases in humans and other animals (Table 1) The difference can be replicated *in vitro*. For example, tick-borne encephalitis virus (TBEV) causes a cytopathic effect in vertebrate cell cultures (e.g. porcine PS cell culture) but has no visible effect in tick cells (e.g. *Ixodes scapularis* IDE2) (7) The reasons for these differences are unknown.

3. TICK-ARBOVIRUS INTERACTIONS

Less than 10% of the 904 recognized tick species are incriminated as virus vectors, possibly because comparatively few tick species have been screened for tick-borne viruses (8) Among ixodid species, virus vectors mostly are recorded in the genera *Ixodes*, *Haemaphysalis*, *Hyalomma*, *Amblyomma*, *Dermacentor*, and *Rhipicephalus*, and the sub-genus, *Rhipicephalus (Boophilus*) For argasid ticks, vectors are recognized in the genera *Ornithodoros*, *Carios*, and *Argas*.

Isolation of a virus from a tick (particularly one that has fed recently on a host) does not signify that the tick species is a vector of the virus. For example, the virus may simply be present in the bloodmeal. To determine whether a particular tick species is a competent vector of a particular virus requires demonstration that: (i) the virus can be acquired during blood-feeding on an infected host, and (ii) the virus is transmitted to a host by the tick after it has moulted to the next developmental stage and then feeds. The intervening period between virus acquisition and virus transmission is known as the 'extrinsic incubation period.' During this period the tick is unable to transmit the virus it has acquired.

The association between a tick-borne virus and its tick vector species is intimate and often highly specific. Nevertheless, a few tick species transmit several (e.g. *Ixodes ricinus*, *Amblyomma variegatum*) or many (*I. uriae*) different tick-borne viruses. Whether there is a molecular basis for this apparent promiscuity of some tick species for tick-borne viruses, or whether the reason stems from the tick's ecology (including preferred host species), is unknown. Possibly there is a viral trait that determines vector promiscuity. For example, Crimean-Congo hemorrhagic fever virus (CCHFV) is transmitted by many tick species (it has been isolated from at least 31 species/sub-species) whereas the genetically related Nairobi sheep disease virus is transmitted by *R. appendiculatus* and by few other species.

Most tick-borne viruses appear to be transmitted by either ixodid or argasid species, but rarely by both. For example, both CCHFV and TBEV can be transmitted by

Table 1. Classification of tick-borne viruses[1]

Family[2]	**Genus**[3]	**Species**[4]**/Group**
Asfarviridae (dsDNA, single molecule; lipid envelope)	*Asfivirus*	***African swine fever virus***
Orthomyxoviridae (ssRNA, segmented, negative-sense; envelope)	*Thogotovirus* (6 or 7 segments)	***Thogoto virus***
		Dhori virus
Rhabdoviridae (ssRNA, single molecule, negative-sense)	*Vesiculovirus*	*Isfahan virus*
	Unassigned family members	Kern Canyon group
		Sawgrass virus group
Reoviridae (dsRNA, segmented)	*Orbivirus (*10 segments)	*Chenuda virus*
		Chobar Gorge virus
		Great Island virus
		Kemerovo virus[5]
		Mono Lake virus[6]
		St. Croix River virus[7]
		Wad Medani virus
		Lake Clarendon virus[8]
		Matucare virus[8]
	Coltivirus (12 segments)	***Colorado tick fever virus***
		Eyach virus
Bunyaviridae (ssRNA, segmented, negative-sense; envelope)	*Orthobunyavirus* (3 segments)	*Estero Real virus*
		Tete virus
	Nairovirus (3 segments)	***Crimean-Congo hemorrhagic fever virus***
		Dera Ghazi Khan virus
		Dugbe virus[9]
		Hughes virus
		Qalyub virus
		Sakhalin virus
	*Phlebovirus (*3 segments)	*Uukuniemi virus*
	Unassigned family members	Bhanja group
		Kaisodi group
		Upolu group
	Ungrouped viruses	Chim virus
		Issyk-Kul virus (Keterah virus)
		Lone Star virus
		Razdan virus
		Sunday Canyon virus
		Tamdy
		Wanowrie virus
Flaviviridae (ssRNA, single molecule, positive sense; envelope)	*Flavivirus*	*Gadgets Gully virus*
		Kadam virus
		Karshi virus[10]
		Kyasanur Forest disease virus[11]
		Langat virus
		Meaban virus
		Omsk hemorrhagic fever virus
		Powassan virus
		Royal Farm virus
		Saumarez Reef virus
		Tick-borne encephalitis virus[12]
		Tyuleniy virus
	Unassigned family member	Ngoye virus
Unassigned tick-borne viruses	Nyaminini virus group	Hirota virus
		Midway virus
		Nyaminini virus
	Quaranfil virus group[13]	Johnston Atoll virus
		Quaranfil virus
	Ungrouped	Aride virus
		Caspiy virus
		Jos virus
		Mayes virus
		Røst Islands virus
		Runde virus
		Slovakia virus

[1]See (8) for viral strain/type, tick vector species, and geographical distribution. [2]Distinguishing properties of family members. ss, single-stranded; ds, double-stranded. Positive-sense RNA = plus strand, message strand (the strand that contains the coding triplets that are translated by ribosomes); negative-sense RNA = minus strand (the strand with base sequence complementary to the positive-sense strand) Envelope = outer (bounding) lipoprotein bilayer membrane. [3]Distinguishing properties of members of the genus. [4]A virus species is defined as 'a polythetic class of viruses that constitute a replicating lineage and occupy a particular ecological niche' (1) [5]Listed under *Great Island virus* by (1) [6]Listed under *Chenuda virus* by (1) [7]Possibly a tick virus rather than a tick-borne virus (section 2.1.) [8]Tentative species. [9]**Nairobi sheep disease virus** is classed as a strain of *Dugbe virus.* [10]New species (103); listed under *Royal Farm virus* by (1) [11]**Alkhurma virus** is considered a subtype of *Kyasanur Forest disease virus (*125) [12]**Louping ill virus** is considered a type of *Tick-borne encephalitis virus (*103) [13]Possible members of the *Arenaviridae (*126) **In bold** = viruses that cause disease in humans and/or other animals

Table 2. Cellular components involved in tick-borne virus infection and replication

Virus	Cellular component[1]	Process	Section[2]
African swine fever virus	aggresome pathway	virus assembly	4.1.
Tick-borne orbivirus[3]	clathrin	virus internalization by clathrin-dependent endocytosis	4.3.
	casein kinase II	formation of virus assembly factories	
	vimentin	virus egress from infected cells	
	calpactin	virus release from infected cells	
	ESCRT 1 protein Tsg101	virus release from infected cells	
	NEDD4-like ubiquitin ligases	virus release from infected cells	
Crimean-Congo hemorrhagic fever virus	signal peptidase	M segment polyprotein processing	4.4.
	SKI-1 and SKI-1-like proteases	M segment polyprotein processing	
	furin/PC	M segment polyprotein processing	
	subtilisin kexin isozyme-1/site-1 protease	processing of viral glycoprotein Gn	
Tick-borne encephalitis virus	signal peptidase	processing of the viral polyprotein	4.5.
	furin or furin-like	cleavage of preM	

[1] Requirement for components recognised in vertebrate cells; similar components may be required for infection and replication in tick cells. [2] See indicated section for further details, [3] Based on defined or putative requirements for an insect-borne orbivirus (Bluetongue virus)

many different ixodid ticks but not by argasid ticks (sections 4.4. and 4.5.) This is particularly remarkable for CCHFV given its ability to infect both mammals and birds besides a diversity of ixodid species. The restriction in vector competencies suggests there are major differences in tick-virus interactions between the two major tick families. Given that the major evolutionary division into the argasid and ixodid tick families occurred some 100 Myr ago, such differences are perhaps not surprising. Clearly, a complete molecular characterization of tick-virus interactions will need to consider both argasid-virus and ixodid-virus interactions.

Like ticks, viruses are obligate parasites. While ticks need a bloodmeal from their hosts to survive, viruses require the transcription, translation, and post-translation processing machinery of their hosts to propagate. None of these molecular components has as yet been identified in ticks; even the molecular interactions within vertebrate host cells are not fully understood. Generally, infection is initiated when a virus binds to a specific host receptor, following which a complex cascade of intracellular signalling leads to virus internalization. Internalization can be achieved by the virus exploiting different cellular systems (9) The best characterized system is receptor-mediated, clathrin-dependent endocytosis, by which many viruses enter mammalian cells through invaginations of the plasma membrane that are coated with clathrin molecules (10) Whether this occurs in ticks is as yet unknown but evidence from studies on *Rickettsia*-infected ixodid ticks indicates that clathrin-coated vesicles may be involved in Rocky Mountain spotted fever rickettsial infections of the tick vector (11) Many of the cellular components required for replication within tick cells are likely to be similar to those utilized by tick-borne viruses in vertebrate cells, such as when host proteolytic enzymes are required to process viral polyproteins/protein precursors (Table 2) The growing data on tick proteomics indicate that orthologues of vertebrate components required for arbovirus replication exist. For example, TBEV requires cell-derived furin for processing a viral precursor protein to produce new virions (infectious virus particles) (section 4.5.); furin-like proteases have been identified in ixodid tick transciptomes (J.M.C. Ribeiro, *pers com*) As the tick proteome is unravelled, its interaction with the viral proteome will unlock the secret of why tick-borne virus infections have such seemingly little effect on tick cells.

While there is an absolute requirement for tick-borne viruses to interact at the molecular level with tick cells, there is no evidence of any direct interactions with the molecular constituents of tick saliva. All interactions between tick-borne viruses and saliva molecules (so-called 'saliva-assisted transmission') are indirect; the virus exploits the pharmacological effects that saliva components have on the vertebrate host (section 5.2.) However, the physico-chemical conditions in tick saliva may have profound effects on tick-borne viruses. Virus particles are not rigid structures. In particular, their outer layer (which includes a host cell-derived lipid membrane in the case of many tick-borne viruses) is sensitive to environmental conditions. Conformational changes in response to different conditions have been studied extensively with TBEV. The comparatively smooth icosahedral glycoprotein cage of mature TBEV particles is stable at a limited pH range, and opens up when exposed to either acidic or alkaline conditions (12) (section 4.5.) Although it is difficult to measure the pH of tick saliva, studies with *Ixodes scapularis* indicate a pH of 9.0-9.5 (E. Fikrig, *pers com*) Further studies are needed to determine the pH of tick saliva during feeding, and whether or not it affects the conformation of tick-borne viruses as they are transmitted to the vertebrate host via the saliva.

3.1. Tick barriers to arbovirus infection

Molecular interactions between virus and tick determine whether the virus survives the extrinsic incubation period and is successfully transmitted by the tick. Studies of arbovirus infections in insects have identified four barriers, at the gross level, that must be overcome during the extrinsic incubation period: (i) the midgut infection barrier, (ii) the midgut escape barrier, (iii) the salivary gland infection barrier, and (iv) the salivary gland escape barrier. At the cellular level, infection barriers may include the inability of a virus to pass through the cell membrane into the cell cytoplasm or, having infected a cell, a virus may replicate but it may be unable to exit the cell. A permissive infection occurs when a virus is able to infect a cell, replicate within it, and then disseminate from the cell.

These processes of cell infection and dissemination in the midgut may be completed during the prolonged feeding of ixodid tick species, as arboviruses generally take <24 h to complete a replication cycle in tick cells. However, argasid ticks generally take <1h to engorge. Hence, infection of the midgut and dissemination from the midgut to the salivary glands, most likely occurs during moulting in argasid ticks. This is probably also the case for ixodid tick infections because most of the bloodmeal of ixodid species is taken up during the last day of feeding (13) There is no evidence that viruses are retained in the midgut, and pass from the midgut to the salivary glands once the tick moults and starts feeding in the succeeding instar, as is the case for the bacterium, *Borrelia burgdorferi* sl, the agent of Lyme disease (Chapter VIII) There is also no information on the role of the peritrophic membrane as a barrier to virus infection, if indeed ticks produce one.

The route from the midgut to the salivary glands is largely unknown. Viruses may pass from the hemocoel via hemolymph to the salivary glands, either as free virions or infected hemocytes. African swine fever virus (ASFV) has been observed in hemocytes of its argasid tick vector, *Ornithodoros porcinus*, and Dugbe virus in hemocytes of its ixodid vector, *Amblyomma variegatum (*14, 15) Alternatively (or additionally), tick-borne viruses may pass along nerves to the salivary glands. Thogoto virus was detected in the neural cortex of the synganglion, indicating that this virus can infect tick nervous tissue (16) Thus, tick-borne viruses appear to show different tissue tropisms in ticks, suggesting different specificities in the molecular interactions between tick cells and tick-borne viruses. Once a virus reaches the salivary glands, similar barriers to infection must be overcome (cell penetration, replication, and virus release) The ultimate goal is for the virus to be secreted in saliva once the tick has found a host and initiated feeding.

Evidence of virus infection barriers within ticks has been reported. For example, *Rhipicephalus appendiculatus* and *A. variegatum* are competent vectors of Thogoto virus. When fed on virus-infected hamsters, the virus infects and replicates within the ticks, is maintained trans-stadially, and subsequently is transmitted when the succeeding instar feeds. By contrast, both tick species are refractory to infection by Dhori virus when they feed on hamsters infected with this virus. However, when Dhori virus was inoculated into the hemocoel of engorged nymphs, the virus persisted trans-stadially and was transmitted by the infected ticks during feeding. Thus, the midgut of *R. appendiculatus* and *A. variegatum* appears to be a barrier to infection by Dhori virus but not to Thogoto virus (17) Thogoto virus and Dhori virus are members of the same genus (Table 1) The significance of this difference in virus-tick interactions, at the molecular level, is discussed in section 4.2.

Interestingly, Dhori virus survived <4 days in *R. appendiculatus* nymphs fed on Dhori virus-infected hamsters. By contrast, Dugbe virus survived at least 21 days following oral infection but was unable to survive the moulting period and was not transmitted by the adult stage. Like Dhori virus, Dugbe virus can replicate in and be transmitted by *R. appendiculatus* if the virus is inoculated directly into the hemocoel, indicating that (like Dhori virus) there are no barriers to Dugbe virus infection of the salivary glands of *R. appendiculatus* (18) The difference in survival dynamics suggests that *R. appendiculatus* demonstrates a midgut infection barrier to Dhori virus and a midgut escape barrier to Dugbe virus. The molecular reason (s) for their different survival dynamics in *R. appendiculatus* is unknown.

3.2. Bloodmeal digestion and virus infection

The initial stages of arbovirus infection in ticks have not been elucidated. Nevertheless, they are likely to differ markedly from insects, and may be the principal reason why tick-borne viruses are rarely, if ever, transmitted by insects. This is because bloodmeal digestion in ticks involves a process known as heterophagy - bloodmeal digestion is intracellular (19) The lumen of the tick midgut is crowded with digest cells that degrade blood components intracellularly (20, 21); endocytosis of blood proteins occurs by pinocytosis (22) (23) By contrast, the bloodmeal of insect vectors is digested extracellularly, within the gut lumen; numerous proteases are secreted into the midgut lumen. Thus, viruses entering the tick midgut are exposed to environmental conditions that differ greatly from those existing in, for example, the mosquito midgut. Indeed, some arboviruses transmitted by insects behave like enteric viruses, requiring proteolytic processing for infectivity. For example, cleavage of the Gn glycoprotein of La Crosse virus (*Bunyaviridae*, *Bunyavirus*) within the midgut initiates infection of the vector mosquito (24) Similar processing of Bluetongue virus (*Reoviridae*, *Orbivirus*) is required for infection of its *Culicoides* vector. This may explain differences in the surface topography between insect- and tick-borne orbiviruses (section 4.3.)

The state of tick-borne viruses in the bloodmeal is also likely to differ from that of insect-borne viruses. Generally, tick-borne viruses do not produce the high levels of viremia typical of insect-borne viruses. Indeed, they may most commonly be transmitted in the absence of a patent viremia (non-viremic transmission), facilitated by the pharmacological activities of tick saliva (saliva-assisted transmission; section 5.2.) The ability of feeding ticks to attract virus to their feeding site suggests that tick-borne viruses are translocated in the vertebrate host as infected mobile cells (25-27) Thus, the bloodmeal may contain a few infected leukocytes, rather than extracellular virions. Ticks secrete hemolysin (28) which presumably ruptures infected leukocytes (besides erythrocytes), releasing hundreds or thousands of virus particles. Hence, infection via ingestion of infected cells would provide a viral bolus that may act as a highly efficient and effective inoculum for ticks. For those tick-borne viruses that infect monocytes (ASFV, CCHFV) or erythrocytes (Colorado tick fever virus), viremic transmission may similarly involve ingestion of infected cells. The enormous blood volume (>100-fold the unfed body weight) processed by ixodid tick species (and the larval stage of some argasid species) must also play a role in virus acquisition.

3.3. Tick-borne virus conformational changes

Most tick-borne viruses have an envelope (Table 1) comprising viral proteins and a cell-derived lipid bilayer that serves to protect the viral capsid and genome, and operates (at least in mammalian cells) as a 'transport vesicle' during cell-to-cell transmission. Entry of these enveloped viruses into cells typically involves fusion of the viral membrane (the envelope) with a cell membrane. This process is tightly regulated by the viral fusion protein (29, 30) Viral fusion proteins are present at the surface of mature virions in a metastable conformation that, when exposed to a trigger (receptor interactions and/or acidic pH), undergo structural rearrangements to drive the merger of the viral membrane with a membrane of the target cell (29) During these conformational changes, a segment of the fusion protein ('fusion peptide') is exposed and inserts into the cellular membrane, initiating the fusion process (30, 31) Distinct structural classes of viral fusion proteins are recognized that display different architecture and organization on the virion. The fusion peptide (a cluster of hydrophobic and aromatic amino acids) of class I fusion proteins is located at or near the N-terminus, whereas fusion peptides of class II fusion proteins are internal. Class I fusion proteins are found in members of the *Orthomyxoviridae*; the archetypal class II protein is that of TBEV, a member of the *Flaviviridae.* Despite the differences between class I and class II fusion proteins, a common feature is the formation of a hairpin-like trimeric post-fusion structure in which the C-terminal anchor region of the fusion protein is brought into contact with the target membrane inserted fusion peptide (30, 31) Because of the heterophagous nature of tick bloodmeal digestion (section 3.2.), an acidic environment is likely to be first encountered by a tick-borne virus within endosomes of the midgut digest cells. Here, the enveloped tick-borne viruses (Table 1) must undergo profound conformational changes in order to expose their genome to the replicative machinery of the host cell. Conformational changes may also occur in tick saliva (section 3.)

3.4. Virus infections of tick cell cultures

Tick cell cultures have a valuable role to play in defining the molecular interactions between tick-borne viruses and tick cells. Over 40 cell lines exist, derived from 13 ixodid and one argasid tick species (32) Tick-borne viruses do not induce an overt cytopathic effect in tick cell cultures, mimicking the lack of pathology seen in infected ticks. They readily establish persistent infections, and persistently infected tick cell lines can be subcultured indefinitely (33) Tick cell lines can be used to isolate tick-borne viruses from infected ticks, and may be susceptible to infection when mammalian cells are not (33, 34) The molecular basis for such differential susceptibility of tick and mammalian cell cultures is unknown. Similarly, it is not understood why many mosquito-borne viruses can readily infect tick cells whereas few tick-borne viruses will grow in mosquito cell lines (33-35) Again, observations in cell culture mimic those in the vector: tick-borne viruses are rarely, if ever, transmitted by insects.

3.5. Tick immune mechanisms

Tick immune mechanisms (Chapter X) are likely to provide fertile ground for molecular interactions with viruses, but little is known. A lectin, named Dorin M, has been identified in the hemocytes and plasma of *Ornithodoros moubata* and *Ixodes ricinus*, important vectors of ASFV and TBEV, respectively. It belongs to a family of invertebrate molecules containing a fibrinogen domain. Since these lectin types are believed to function as non-self recognizing molecules, Dorin M may play a role in innate immunity but its role, if any, in controlling virus infection is as yet unknown (36)

3.6. Tick-borne virus evolution

As illustrated above, little is known of the molecular interactions between tick-borne viruses and their tick vectors. Most likely, tick-borne viruses survive in ticks as a heterogeneous population ('quasi species') (37) Some mutants within a population will be better adapted to infecting and replicating in tick cells; these variants may adapt rapidly to a tick milieu once they are acquired from an infective bloodmeal. Possibly these 'tick adapted' variants aid reproduction of the less well fitted 'vertebrate adapted' variants (for example, by complementation), or suppress replication of the 'vertebrate variants' (for example, by interference) As a result of the greater productivity of 'tick adapted' variants, the variant ratio may change from that of the bloodmeal viral population (in which the 'vertebrate variant' predominates) Consequently, the tick transmits, to a vertebrate host, a mixed population in which the 'tick variant' predominates. The process is then reversed in the vertebrate host, with the 'vertebrate variant' becoming predominate.

RNA viruses generally have high rates of accumulation of mutations due to the error-prone nature of their polymerases (38) However, arboviruses often show relatively low levels of genome diversity, which has been explained by the 'double-filter' concept. Thus, arbovirus evolution is thought to be constrained by the need to maintain high fitness in both invertebrate and vertebrate hosts (39)

Ticks may place an additional constraint on the rate of arbovirus evolution. Comparison of the phylogenetic trees of mosquito-borne and tick-borne flaviviruses reveals a striking difference in tree topology (40) Tick-borne flaviviruses show an asymmetric (ladder-like) topology of apparent continuous branching through time. By contrast, mosquito-borne flaviviruses show a more balanced (pectinate) structure in which relatively long time periods, presumably when lineages were lost, intersperse periods of intense cladogenesis, the so-called "boom or bust" model of evolutionary change (41) In addition, the relative degree of amino acid divergence between tick-borne and mosquito-borne flaviviruses indicates that mosquito-borne flaviviruses have evolved 2.5 times faster than tick-borne flaviviruses (42) This has been explained by the relatively long and slow life cycle of ticks in which the virus may spend years in the tick without being transmitted to a vertebrate host. In contrast, the turnover of mosquito-borne flaviviruses between vector and vertebrate host is much more rapid and provides greater opportunity for genetic variation in the vertebrate host population (40)

The seemingly slow rate of evolution of tick-borne flaviviruses compared with mosquito-borne flaviviruses appears at odds with the genetic diversity of nairoviruses (none of which are insect-borne) (section 4.4.) Possibly the difference results from the greater genome plasticity of a segmented RNA virus (nairovirus) compared with a non-segmented arbovirus (flavivirus) Alternatively, nairoviruses may be 'ancient' tick-borne viruses that carry the genetic evidence of a much longer period evolving with their tick hosts compared with tick-borne flaviviruses (section 4.4.)

The following section describes the small number of tick-borne viruses for which there are some data on molecular interactions between the virus and its tick vector.

4. MOLECULAR CHARACTERISTICS OF TICK-BORNE VIRUSES

4.1. African swine fever virus

Only a single genus, *Asfivirus*, is currently recognized within the *Asfarviridae* family and there is a single species, *African swine fever virus* (ASFV) (Table 1) The virus causes severe disease in domestic pigs (43) ASFV is the only known arbovirus with a DNA genome (44) It is a comparatively large virus with a diameter of 200 nm, containing more than 50 proteins. ASFV resembles members of the *Poxviridae* in genomic organization and the *Iridoviridae* in icosahedral symmetry; neither family includes arboviruses. The viral genome comprises a single molecule of linear, covalently closed, double-stranded DNA, 170–190 kbp in size with about 150 open reading frames, which are read from both DNA strands. It contains a central conserved region and variable terminal regions. The terminal variable regions contain at least five multigene families (MGFs)

The complex ASFV virion comprises a DNA-containing nucleoid coated by a thick protein layer designated the core shell. This coat is wrapped in an inner envelope and an outer icosahedral capsid. The extracellular virion has an additional external membrane acquired by budding through the plasma membrane. Virus assembly occurs within discrete pericentriolar areas designated viral factories, which are close to the microtubule organizing center in mammalian cells. They resemble aggresomes, accumulations of misfolded proteins that form potentially toxic aggregates (45) Such aggregates are transported along microtubules to aggresomes for immobilization and subsequent degradation. The similarity between ASFV viral factories and aggresomes suggests that ASFV may use aggresome pathways to concentrate viral proteins to facilitate replication and assembly (46) Viral factories have been observed in midgut digest cells of ASFV-infected *O. porcinus* indicating that exploitation of the aggresome pathway may occur in ticks (47)

In domestic pigs, ASFV infects cells of the mononuclear-phagocytic system, resulting in tissue damage the severity of which depends on the virulence of the viral strain (48) To facilitate infection, the ASFV genome encodes a plethora of proteins that interfere with host defense mechanisms (49) Whether ASFV similarly suppresses tick host response to infection has not been determined.

Argasid ticks of the genus *Ornithodoros* are the main vectors of ASFV. In most parts of sub-Saharan Africa, where the disease is enzootic, ASFV persists in nature by a sylvatic cycle of transmission between wild suids (mainly the warthog, *Phacochoerus aethiopicus*) and *O. moubata*, which infest warthog burrows (43) In southern Europe, the vector is *O. erraticus (*50) The infection in ticks is typically long-term and persistent with relatively high levels of viral replication occurring in various tissues and organs (15, 51, 52) However, the ability of ASFV to infect *Ornithodoros* ticks varies widely, depending on the virus isolate and origin of the ticks (53) The virus can be transmitted in ticks trans-stadially, transovarially, and sexually, and is excreted in coxal fluid (43) ASFV is one of the few arboviruses that can kill its tick vector (53, 54) Studies on ASFV isolates from *O. moubata* collected in warthog burrows revealed considerable genetic diversity, the significance of which is unknown (55)

Using gene deletion mutants, studies have begun to identify the genetic determinants of ASFV infection and replication in ticks. Interestingly, MGF360, which promotes survival of infected suid macrophage cells (56), also appears to play a role in early virus replication in tick midgut cells. Deletion of MGF360 genes *3HL*, *3IL*, and *3LL* impairs virus replication (57) In *O. moubata* infected orally with the deletion mutant, a comparatively small number of digest cells contained virus, and there was no evidence of infection in undifferentiated midgut epithelial cells at 21 days after infection. Previous studies showed that, following oral uptake of an infected bloodmeal, the initial infection and viral replication occurs in the midgut. Furthermore, the generation of comparatively high viral titers in the midgut appears to be required to ensure viral dissemination from the midgut to other tissues and organs (51, 58) Thus MGF360 genes *3HL*, *3IL*, and *3LL*, which are transcribed within the first 10 days after infection, appear to be critical for generating the high titer infection within the midgut that is essential for establishing a generalized infection necessary for the natural transmission of virus from tick to suids (57) As yet, MGF360 genes show no similarity to other genes or motifs in current databases; how they function in tick midgut infections is unknown.

4.2. Thogoto virus and Dhori virus

The *Thogotovirus* genus is unusual in that it belongs to the same virus family, the *Orthomyxoviridae*, as the influenza viruses. Nevertheless, the two species within the genus, *Thogoto virus (*THOV) and *Dhori virus (*DHOV), are undoubtedly arboviruses (17, 59)

The type species, THOV, contains six single-stranded RNA segments. RNA segments 1 to 3 encode gene products that correspond to the viral polymerases (PB2, PB1 and PA) and segment 5 to the nucleocapsid protein (NP) of influenza viruses (Weber *et al.* 1998)

However, the fourth largest segment encodes the single glycoprotein (GP) that is unrelated to any influenza viral protein but instead shows striking sequence homology to the glycoprotein (gp64) of baculoviruses (60) The same is true for DHOV (61) This unique glycoprotein is probably the key to the ability of members of the *Thogotovirus* genus to infect ticks (62) Influenza viruses use sialic acid residues on the surface of vertebrate cell membranes as receptors for infecting cells. Sialylation of invertebrates is somewhat controversial although sialylated glycoconjugates have been detected in the salivary glands of female *I. ricinus (*63) Clearly *Thogotovirus* members have circumvented this problem by evolving a different mechanism of cell infection from that of influenza viruses.

Although *R. appendiculatus* and *A. variegatum* are competent vectors of THOV, they are refractory to oral infection with DHOV (section 3.) The barrier to infection appears to be at the level of the midgut because, when DHOV is inoculated into the hemocoel, it infects the salivary glands and can be transmitted by the tick during feeding (17) The sequence diversity of 31% in GP of THOV and DHOV may contain the reason for their difference in vector species specificity (61)

THOV has been used extensively in experimental studies of saliva-assisted transmission in which THOV has been shown to exploit the pharmacological properties of tick saliva (section 5.2.) THOV has also been used to investigate reassortment using temperature-sensitive mutants to follow the exchange of genomic segments between viruses. The ability of THOV to reassort has been demonstrated in both ticks and a vertebrate host (Davies *et al.* 1987; Jones *et al.* 1987) However, the significance of such genetic exchange in virus-tick interactions is unknown.

4.3. Tick-borne orbiviruses

Members of the *Orbivirus* genus are all arboviruses, with the possible exception of St Croix River virus (section 2.1.) The type species is *Bluetongue virus (*BTV), a *Culicoides*-transmitted pathogen of ruminants. Five tick-borne viral species are recognised by the International Committee on Taxonomy of Viruses (ICTV) whereas 7 species are shown in Table 1 (Fauquet *et al.* 2005) The two additional species, *Kemerovo virus* and *Mono Lake virus*, are consistent with the definition of a virus species (see Table 1 legend) Kemerovo viruses, classified by the ICTV as serotypes of *Great Island virus*, are maintained in the Palearctic region among small mammals and birds by two related species of ixodid tick, *I. persulcatus* and *I. ricinus*. Their ecology is distinct from that of Great Island viruses (GIV) that circulate in seabird colonies in circumpolar regions transmitted by *I. uriae*, which is phylogenetically distinct from *I. persulcatus* and *I. ricinus* (64) Moreover, Kemerovo virus shows limited genome segment reassortment with three serotypes of *Great Island virus*, and none with Chenuda virus, Essaouira virus or Mono Lake virus (65) Speciation of *Kemerovo virus* and *Great Island virus* may be at a transitional stage in which ancestral links can be detected under highly selective experimental conditions. Presumably genetic exchange between these two species does not occur in nature, particularly given their different ecologies. Mono Lake viruses are classified by the ICTV as serotypes of *Chenuda virus.* Again, like Kemerovo viruses, *Mono Lake virus* can be considered a distinct species based on its inability to demonstrate genome segment reassortment when tested experimentally with representatives of *Chenuda virus*, *Great Island virus*, and *Kemerovo virus* (65)

More than 30 GIV have been recorded (8) They are distinguished serologically and by the electrophoretic gel profile of their 10 genomic segments of dsRNA. The diversity of RNA profiles indicates they frequently reassort their segments during mixed infections. Given the high prevalence of neutralizing antibodies to GIV in seabird colonies, and the ability of the viruses to be transmitted non-viremically between co-feeding ticks (section 5.1.), it seems likely that reassortment occurs more frequently within ticks than in seabirds (66, 67)

As the *Orbivirus* genus comprises both insect-borne and tick-borne members, structural comparisons can test the hypothesis that different methods of bloodmeal digestion exert a strong selective pressure on the surface structure of arboviruses (section 3.2.) The insect-borne BTV has an outer capsid comprising 'spike-like' VP2 protein arranged as 'triskelion' structures and an interdispersed and underlying VP5 protein. Both proteins interact with the N-terminus of VP7 protein, which forms the core particle (68, 69) Interestingly, in the VP7 homologue of Broadhaven virus (*Great Island virus*), the N-terminus differs markedly from the conserved N-terminus of insect-borne orbiviruses (70)

Comparison of three-dimensional models of BTV and Broadhaven virus indicate remarkable similarity except for differences in accessibility of the outer capsid proteins, VP2 and VP5 (Schoehn *et al.* 1997) The VP2 equivalent of the tick-borne Broadhaven virus is approximately half the molecular weight of BTV VP2 and relatively compact. VP2 of BTV is readily cleaved by proteases. The marked difference in accessibility of the outer surface proteins of BTV compared with Broadhaven virus reflects the perceived need to access and cleave VP2 of BTV within the midgut of its *Culicoides* vector (71) Cleavage of VP2 exposes the core protein, VP7, that bears the Arg–Gly–Asp (RGD) motif, which may be involved in insect cell infection. In contrast to insects, bloodmeal digestion in ticks occurs intracellularly (section 3.2.) Furthermore, none of the viral proteins of tick-borne orbiviruses has been reported to carry a RGD motif. The differences are consistent with tick-borne viruses having evolved a mechanism of tick cell infection that does not rely on proteolysis in the midgut, in contrast to their insect-borne relatives. However, VP2-mediated attachment of BTV to a mammalian cell, and the membrane permeabilising activity of VP5, are believed to trigger the induction of an apoptotic response in mammalian cells (72) Possibly cleavage of BTV VP2 in the insect midgut prevents this happening (which would help explain why the virus has no apparent detrimental effect on its vector) If a similar apoptotic

response is initiated by Broadhaven virus surface proteins in vertebrate cells, some mechanism must exist that prevents such induction of apoptosis in tick cells.

The dsRNA genome imposes constraints on the replication cycle of orbiviruses. RNA interference (RNAi) is an evolutionarily conserved mechanism by which dsRNA initiates post-transcriptional silencing of homologous genes. Since the dsRNA genome would trigger the host's RNAi defence mechanisms if released into the cell cytoplasm, the orbivirus must be retained within the viral capsid. In addition, the absence of host transcriptases that can use dsRNA as a template for mRNA synthesis means that, if the orbivirus genome was released into the cytoplasm, it would be inert. Thus it is very likely that transcription of tick-borne orbivirus RNA occurs within core viral particles, and that the raw material derived from the tick (nucleotide triphosphates) is translocated through pores in the core particles (73)

Once inside the cytosol, tick-borne orbiviruses form virus assembly factories or viral inclusion bodies (VIBs) characteristic of many animal and plant viruses (74) A non-structural protein, NS2, plays a key role in VIB formation in BTV infected cells (69) In its phosphorylated form, NS2 forms homomultimers in which phosphorylation by a cellular protein kinase, casein kinase II, is involved in the global folding of the protein that appears to stabilize its multimerization (75) Again, by extrapolation it is likely that the NS2 homologue of tick-borne orbiviruses is phosphorylated by a cellular enzyme if tick-borne viruses produce VIBs in tick cells.

Besides its role in cell receptor binding, VP2 of BTV also mediates viral egress from infected mammalian cells through its interaction with vimentin, a protein found in certain intermediate filaments that contribute to the cytoskeletal structure of eukaryotic cells (76, 77) Possibly, the interaction with vimentin also occurs in tick-borne orbivirus infections of tick cells.

Ordered mechanisms appear to traffic newly formed orbivirus particles out of infected cells. For BTV, non-structural proteins NS3 (and its shorter form, NS3A) and NS1 are implicated in the exit process. Release of BTV from most mammalian cells follows cell lysis whereas release is nonlytic from insect cells. In mammalian cells, NS3/NS3A are expressed at relatively low levels whereas levels are much higher in infected insect cells (78) High levels of NS3/NS3a correlate with nonlytic virus release. The N-terminal 13 amino acids of NS3 (absent in NS3A) interact with calpactin light chain (p11) of cellular annexin II complex (79) The complex is implicated in trafficking of vesicles. A model has been proposed in which NS3 in association with p11 forms a bridge with newly assembled virus particles through a secondary interaction with VP2, and these interactions facilitate trafficking of the virus particle to the cell membrane (69) At the cell membrane, a PSAP motif in NS3 is then proposed to interact with the cellular release factor Tsg101 resulting in pinching off of vesicles containing virus particles and consequence exiting of the virus from the cell (80) In addition, a PPxY motif in NS3 may bind NEDD4-like ubiquitin ligases and also facilitate trafficking and budding of virions. Mature virions are then released from vesicles by an undescribed mechanism. Although the model is consistent with the nonlytic BTV infection of insect cells, and the overexpression of NS3/NS3a in insect cells, the mechanism is based on studies using mammalian cells in which orbiviruses, although lacking a lipid envelope, appear to usurp the vacuolar protein sorting pathway as do many enveloped viruses (81) However, NS3 was shown to bind to mammalian Tsg101 and its *Drosophila* orthologue with similar strengths. As most proteins that function in the formation of multivesicular bodies (MVB) are conserved from yeast to mammals, it seems likely that orbiviruses utilize the MVB pathway in their insect or tick vectors. Unlike insect-borne orbiviruses, the NS3 homologue of tick-borne Broadhaven virus carries overlapping motifs (**PTAP**PAY) Overlapping motifs (PTAP and PPEY) are found within the Ebola virus VP40 protein and function independently as late budding domains (82)

4.4. Crimean-Congo hemorrhagic fever virus

Crimean-Congo hemorrhagic fever virus (CCHFV) is the most notorious of tick-borne viruses, requiring biosafety level 4 handling and included on the Select Agent list of potential bioterrorism agents. In humans, it causes hemorrhagic fever with severe typhoid-like symptoms and mortality rates up to 30% or even higher if untreated (83, 84) It has one of the widest geographical distributions of the medically important arboviruses (85)

Structurally and genetically, CCHFV and other nairoviruses have some notable features that set them apart from other members of the *Bunyaviridae*. In particular, the L RNA genome segment and encoded L polymerase protein of CCHFV are approximately twice the size of those found in viruses of the *Bunyaviridae* family (86, 87) Similarly, the M segment is approximately twice the size, and encodes a polyprotein that undergoes a complex proteolytic cascade to generate the mature structural glycoproteins, Gn and Gc (88) The reasons why nairoviruses differ so strikingly from other members of the *Bunyaviridae* is unknown. All members of the genus are tick-borne (with the possible exception of *Thiafora virus*, for which a vector has not been identified), suggesting a long-term association with ticks (see below)

Although CCHFV has been isolated from at least 31 different tick species and subspecies (including two argasid species), the principal vectors are ixodid ticks of the genus *Hyalomma (*such as *H. marginatum*, *H. rufipes*, *H. anatolicum* and *H. asiaticum*) The isolates from argasid ticks probably represent virus survival in the recently ingested bloodmeal as experimental studies indicate that CCHFV cannot be transmitted by argasid species (89-91) Remarkably, CCHFV did not replicate in 3 argasid species (*Argas walkerae*, *Ornithodoros porcinus*, and *O. savignyi*) following intracoelomic inoculation of the virus, and could not be reisolated from the ticks >24h after inoculation, suggesting these species may have a mechanism of clearing the virus. The apparent inability of CCHFV to replicate in argasid ticks is consistent with conclusions deduced from

phylogenetic analysis of a conserved region of the L gene of CCHFV and other nairoviruses (92) Two monophyletic groups were distinguished, one associated with ixodid tick species (CCHFV, Dugbe virus, Nairobi sheep disease virus, Hazara virus, and Tillamook virus) and the second associated with argasid species (Bandia virus, Qalyub virus, Raza virus, Punta Salinas virus, Farallon virus, Abu Mina virus, and Abu Hammad virus) If the evolution of nairoviruses has tracked their evolving tick vectors, this apparent division between ixodid tick-borne and argasid tick-borne nairoviruses occurred some 100 Myr ago (93, 94)

For an arbovirus, the genome plasticity of CCHFV is considered high compared with the comparatively low levels of genetic diversity typical of arboviruses (39) In a comparative study of 15 CCHFV complete genomes and additional partial genome sequences, nucleotide differences of 20, 31, and 22% for the S, M, and L RNA segments, and deduced amino acid differences of 8, 27, and 10% for the nucleocapsid, glycoprotein precursor, and polymerase, respectively, were found (95) In addition, phylogenetic analysis revealed evidence of both genome reassortment and intragenomic recombination. The low genome diversity of arboviruses, compared with most other RNA viruses, is explained by the 'double-filter' concept in which arbovirus evolution is constrained by the demands of maintaining fitness for both tick and vertebrate systems (39) However, CCHFV is notable for the number of tick species from which it has been isolated (see above), and in utilising both avian and mammalian hosts. In fact, nairoviruses as a genus show comparatively high genetic variation (92) Given that tick-borne viruses probably evolve relatively slowly (section 3.6.), the simplest explanation for the apparent genome plasticity of CCHFV is that it is an ancient virus whose roots have been maintained over many millions of years.

Although molecular interactions between CCHFV and its tick vectors are implied in the studies of phylogeny and genetic diversity, no direct evidence has been published. Critical molecular interactions are likely to involve the CCHFV surface glycoproteins, both in receptor binding and virus entry. Proteomic analysis suggests Gc may be a class II viral fusion protein (96) Hence one of the first molecular interactions between CCHFV and its tick vector is likely to occur in tick digest cells, enabling the internal fusion peptide of Gc to interact with tick cell membranes. In mammalian cells, the polyprotein encoded by the M segment undergoes a complex proteolytic cascade to generate several precursor and possible non-structural proteins in addition to the mature structural glycoproteins, Gn and Gc. Proteolytic processing of the precursors involves multiple signalase cleavage events in the endoplasmic reticulum, SKI-1 and SKI-1-like protease cleavage events in the *cis*-Golgi network and furin/PC cleavage in the *trans*-Golgi network (97-100) N-glycosylation of Gn is required for the correct folding, localization, and transport of all CCHFV glycoproteins (101) However, the same dependence on N-linked glycosylation of Gn occurs for other *Bunyaviridae* viral glycoproteins, and yet CCHFV utilizes a highly complex pathway to produce its glycoproteins compared with other family members. The reasons for such complexity are unknown; possibly they lie in the interactions of CCHFV with its tick vector. Curiously, one site within the extracellular domain of Gn, and two sites in the extracellular domain of Gc, are N-glycosylated (101) The motif representing these glycosylation sites is conserved among 32 CCHFV M segments for which the sequence is known, strongly suggesting a functional role. However, in CCHFV-infected mammalian cells, only the N-glycosylation site in Gn was shown to have a function (101) Perhaps the functional role of the other conserved glycosylation sites lies in the infection of ticks, in which the virus spends most of its existence.

4.5. Tick-borne encephalitis virus

Most of what little is known of the molecular interactions between tick-borne flaviviruses and their tick vectors is from studies of *Tick-borne encephalitis virus* (TBEV), one of the most life-threatening neuroinfections in Europe and Asia (102) Four types of TBEV have been recognised: Eastern TBEV, Western TBEV, Turkish sheep encephalitis virus (TSEV), and Louping ill virus (LIV) (103) *Ixodes ricinus* is the principal vector of Western TBEV, LIV, and TSEV, while Eastern TBEV (Far Eastern and Siberian subtypes) is transmitted primarily by *I. persulcatus*. Many ixodid tick species can transmit TBEV under experimental conditions, including species that the virus does not naturally encounter, such as *Rhipicephalus appendiculatus* (the African brown ear tick) (104) However, argasid ticks do not appear to be competent vectors of TBEV, supporting the hypothesis that there are major differences in tick-borne viral interactions between ixodid and argasid tick species (104) (section 3.)

Like the orbiviruses, and unlike the nairoviruses, the *Flavivirus* genus comprises both insect- and tick-borne members ('*flavi*' is derived from the mosquito-borne Yellow fever virus; latin *flavus* = yellow reflecting jaundice) The *Flavivirus* genus also includes members that have no known vector. However, unlike other tick-borne viruses, the genomic RNA of flaviviruses is infectious and represents the only viral messenger RNA in infected cells. The genomic RNA is a single long open reading frame that encodes a polyprotein, comprising all the structural and nonstructural proteins. The coding region is flanked by relatively short untranslated regions (UTRs) at the 5'- and 3'terminal ends; in TBEV the 3'UTR can vary from 450 to ~800 nucleotides and may contain an internal poly (A) tract. The UTRs contain conserved structural elements that are essential for viral replication. Direct repeat sequences located within the 3'UTR may represent RNA-recognition signals that interact with tick proteins, possibly directing viral RNA to the appropriate cellular compartments (105) This hypothesis needs to be tested.

TBEV particles are enveloped, approximately spherical, with a diameter of ~50 nm. The viral envelope comprises two virus-encoded proteins, the envelope glycoprotein (E) and the small membrane protein (M), both of which are anchored in a host-derived lipid bilayer. At the center of the virion resides the nucleocapsid comprising

a single capsid protein (C) in complex with the genomic RNA. Immature, intracellular virions contain a precursor membrane protein (preM), which is proteolytically cleaved during virus maturation. Like the tick-borne orbiviruses, TBEV does not appear to have a spiky surface. Instead, the E protein is situated parallel to the virion surface in the form of head-to-tail homodimeric rods (106) Interestingly, some mosquito-borne flaviviruses carry an integrin-binding motif Arg–Gly–Asp (Glu) which is not found in the E protein of TBEV, again showing an interesting parallel with the orbiviruses (section 4.3.) Conformational changes in the E protein trigger viral membrane fusion, the first step of flavivirus entry into a cell (107) These conformational changes occur at acidic pH, and involve conversion of the E protein dimers into trimers that adopt a hairpin-like structure during the fusion process. In virions exposed to alkaline conditions, the E dimers dissociate into monomers that interact with target membranes via the fusion peptide (a segment of the E protein) without proceeding to fusion of viral and cellular membranes (12) This conformation may be adopted when virions are secreted in tick saliva (section 3.3.)

Infection and replication of TBEV in mammalian cells is similar to mosquito-borne flaviviruses (107) TBEV infects vertebrate cells by receptor-mediated endocytosis; putative host cell receptors include heparin sulfate (108) Specificity for binding to a tick receptor (s) appears to reside in ectodomain III of the E protein (109) Once inside endosomes within the mammalian cell cytoplasm, the acidic environment triggers an irreversible trimerization of the E protein that results in fusion of the viral and cell membranes (110) After fusion has occurred, the nucleocapsid is released into the cytosol where it disassembles releasing the infectious genomic RNA. This positive-sense RNA is translated into a single polyprotein that is processed by viral and host proteases. Replication of genomic RNA occurs on intracellular membranes. Virus assembly occurs on the surface of the endoplasmic reticulum (ER), with structural proteins and newly synthesized RNA budding into the lumen of the ER (111) Immature non-infectious particles (which contain E and preM proteins, lipid membrane and nucleocapsid) collect in the lumen of the ER. They are transported through the trans-Golgi network where prM is cleaved by host protease furin, resulting in mature, infectious particles (112) Mature virions are released from the mammalian host cell by exocytosis.

Although little is known of the TBEV infection cycle in tick cells, striking differences have been observed compared with mammalian cell infection (7) At the gross level, TBEV (Western strain Hypr) induced a cytopathic effect in porcine (PS) cell cultures, characterised by dilatation and hypertrophy of ER. Comparable cytopathology was not observed in tick cell cultures (phagocytic cell lines derived from *Rhipicephalus appendiculatus*, RA-257, or *Ixodes scapularis*, IDE2) The first signs of viral morphogenesis and cytopathology in PS cells were observed 15 h post-infection (p.i.) whereas no signs of infection were observed in the tick cells 24 h p.i. At 4 days p.i., when most of the infected PS cells were dead, the infected tick cells displayed virus particles within vacuoles with electron-dense particles resembling nucleocapsids in proximity and attached to the membranes of these vacuoles. The virus titer was approximately 1000-fold greater in the mammalian compared with the tick cell cultures. Whereas E and non-structural protein, NS1, were believed to colocalize on the ER of PS cells, in infected tick cells E and NS1 were associated with the plasma cell membrane and vacuolar membranes of the infected tick cells.

Assuming that endosomal entry into the cell cytoplasm is similar for TBEV infection of mammalian and tick cell cultures, a key early event in virus-tick cell interactions appears to be the processing of the polyprotein translated from the incoming infectious genomic RNA. The polyprotein is co- and post-translationally cleaved by viral and cellular proteases (113) None of these cellular proteases in tick cells has as yet been identified (section 3.) Interestingly, the NS1 protein (which appears to be functionally linked with E and released from cells with the E protein) was reported to differ in the N-terminal region when produced in an acutely infected compared with a persistently infected mammalian cell line (114) As the N-terminus is generally responsible for protein targeting, this region of NS1 should be examined in infected tick cells to determine whether its modification might help explain differences in TBEV maturation in tick compared with mammalian cells. Clearly, these observations comparing TBEV infection in mammalian and tick cells need to be confirmed. They indicate marked differences in the maturation of TBEV that are consistent with the outcomes of infection: disease in certain vertebrate hosts but no apparent effect in the tick vector.

Passage of different strains of TBEV in different tick and vertebrate species has been used as a means of identifying viral determinants of host range. Most of these studies have detected phenotypic changes. For example, tick-passage experiments resulted in reduced virulence for mice, a small-plaque phenotype in cell culture, and changes in antibody reactivity (reflecting changes in the surface of the E protein) When passaged in mammalian hosts, the tick-adapted phenotypes were lost (115-117) More recent studies have attempted to relate phenotypic changes to genotype. For example, a Siberian strain isolated from *I. persulcatus* and passaged in mouse brain, was subsequently passaged in *Hyalomma marginatum* (by artificial inoculation), and then again through mice (118) Two amino acid substitutions in the E protein, $Glu_{122} \rightarrow Gly$ and $Thr_{426} \rightarrow Ile$, correlated with increased viral yields in *H. marginatum* but other nucleotide substitutions and phenotypic changes were also observed hence cause and effect were unclear. The observed amino acid substitution $Glu_{122} \rightarrow Gly$ in the conserved stem-anchor region of the E protein results in an increased net positive charge of the E protein and increased affinity for glycosoaminoglycans (GAGs) (119) TBEV shows two types of interaction with cells: high affinity and low affinity. High affinity interactions account for >90% virions adsorbed to the vertebrate cell surface (108, 120) Based on the phenotypic changes observed when a Siberian TBEV strain was

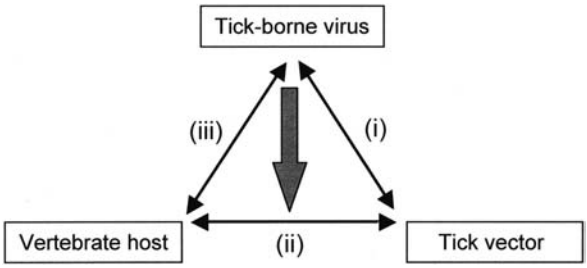

Figure 1. The triangle of tick-virus-host interactions: (i) tick-virus interactions, (ii) tick-vertebrate host interactions, and (iii) vertebrate host-virus interactions. The vertical arrow represents indirect tick-virus interactions occurring at the feeding interface, so-called 'saliva-assisted transmission.'

passaged in *H. marginatum (*including acquisition of a GAG-binding phenotype), it has been suggested that heparin sulfates are the low affinity receptor (118) The possibility that genotypic changes associated with the GAG-binding phenotype are linked with TBEV adaptation to ticks, needs to be tested.

The second amino acid substitution in the E protein, $Thr_{426}\rightarrow Ile$, is in a conserved sequence between two predicted alpha-helical regions in the stem-anchor region, the function of which is unknown. Residue 426 in this conserved region differs in different strains. European subtype strains have Ala at this position whereas strains of Far Eastern and Siberian subtypes have Thr (118) The difference correlates with different principal vectors: *I. ricinus* for European strains and *I. persulcatus* for Far Eastern and Siberian strains. Again, the significance of these observations needs further study. Currently, the genetic determinants that enable TBEV to infect ticks and subsequently to be transmitted naturally, through tick feeding, remain elusive as do the molecular interactions between TBEV and its different tick vectors.

5. TICK-ARBOVIRUS-HOST INTERACTIONS

Direct molecular interactions between tick-borne viruses and their tick vectors occur during the processes of infection, replication, and transmission involving the vector (sections 3. and 4.) These processes are depicted in the triangle of parasitic interactions as shown in Figure 1 (i) In addition, important indirect interactions (arrow) between vector and virus occur at the feeding interface, shown as Figure 1 (ii) Here the skin of the vertebrate host is physically and chemically modified by the tick (Chapter XI) Virus-vector interactions are also affected by events in the vertebrate host, shown as Figure 1 (iii) as they dictate the state of the virus as acquired by the feeding tick (e.g. whether virions carry vertebrate specific post-translational modifications) and whether the imbibed virus is extracellular or acquired as infected blood cells (section 3.2.)

5.1. Non-viremic transmission

The first evidence of indirect interactions between ticks and the viruses they transmit was in the demonstration of non-viremic transmission of Thogoto virus (121) For Thogoto virus, transmission from infected to uninfected ticks co-feeding on non-viremic guinea pigs was more efficient than transmission on hamsters that exhibited high levels of viremia. Non-viremic transmission (NVT) has been shown for several other tick-borne viruses, including CCHFV and TBEV (122) Importantly, NVT of TBEV was demonstrated experimentally using the tick vector (*I. ricinus*) and natural rodent hosts of the virus (123) Estimations of the basic reproduction number of TBEV indicate that non-viremic transmission is critical for survival of the virus in Nature, and invoked the 'Red herring' hypothesis that viremia is a by-product rather than a prerequisite of tick-borne virus transmission (27) An alternative model of tick-borne virus transmission was proposed, based on data for TBEV, involving: (i) tick-induced immunomodulation at the skin site of tick feeding (section 5.2.); (ii) infection of Langerhans cells, which shuttle the virus to the draining lymph nodes; (iii) infection and priming of lymphocytes in lymph nodes; (iv) lymphocyte trafficking that conveys the virus to the skin site of uninfected tick feeding; and finally (v) virus acquisition by uninfected co-feeding ticks. The significance of this model, in terms of direct tick-virus interactions, is that virus-infected cells are attracted to the skin site where uninfected ticks are feeding (25) This model of NVT proposes that uninfected ticks acquire virus in their bloodmeal in the form of infected cells (section 3.2.) .

5.2. Saliva-assisted transmission (SAT)

Non-viremic transmission (section 5.1.) can be reproduced experimentally by needle and syringe inoculation of susceptible vertebrate hosts with a mixture of a tick-borne virus and salivary gland extract (or saliva) obtained from partially fed uninfected ticks. The phenomenon has been named saliva-assisted transmission (122) Extensive studies have shown that SAT results from exploitation by the virus of the pharmacological effects of tick saliva in the skin of the vertebrate host on which the ticks are feeding. In contrast with the Lyme disease spirochete (124), there is no evidence to support the idea that the salivary components act directly on tick-borne viruses. It remains to be determined which of the many pharmacologically active components of tick saliva (Chapter XI) mediates SAT, or indeed if one or several different saliva molecules are involved. Comparison of SAT between TBEV and Thogoto virus indicates that different molecules promote SAT, depending on the virus. Moreover, the SAT factor of Thogoto virus appears to be a protein or peptide.

6. CONCLUSIONS AND PERSPECTIVES

Molecular characterization of the interactions between ticks and tick-borne viruses is an unexplored field. Potentially it is one that could be hugely rewarding as tick-borne viruses spend >95% of their existence in ticks. As ticks are ancient invertebrates, the time in which tick-borne viruses have adapted to ticks may span 100s Myr. Interestingly, the *Nairovirus* genus comprises only tick-borne members; most other genera containing tick-borne viruses also include insect-borne viruses. Phylogenetic

analysis of nairoviruses reveals a split between nairoviruses transmitted by ixodid tick species and those transmitted by argasid species. If nairoviruses have 'specialised,' and can only infect and replicate in ixodid or argasid species, and not both, there must be critical ixodid-virus interactions that are distinct from argasid-virus interactions. Thus a complete molecular characterization of tick-virus interactions will need to consider both argasid-virus and ixodid-virus interactions. As the tick proteome is unravelled, its interaction with the viral proteome hopefully will unlock the secrets of tick-arbovirus interactions, and reveal why tick-borne viruses have such seemingly little effect on the ticks they parasitise.

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