Anatomical Basis of Thogoto Virus Infection in BHK Cell Culture and in the Ixodid Tick Vector, Rhipicephalus appendiculatus

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SUMMARY

Infection by Thogoto (THO) virus, a tick-borne virus related to the orthomyxoviruses, has been compared in vertebrate cell culture and in Rhipicephalus appendiculatus ticks using infectivity titrations, immunofluorescence, and immune electron microscopy with colloidal gold markers to detect cell surface and intracellular antigens. Morphogenesis of THO virus in cell culture was similar to that of influenza virus, with polymorphic virus particles budding at the plasma membrane. In the tick, THO viral infection caused no obvious pathology; virions or budding profiles were not observed in electron micrographs, although replication, trans-stadial persistence and transmission to a susceptible host occur. THO virus was not detected in the salivary glands of trans-stadially infected ticks until about 7 days after the commencement of feeding on a host. The synganglion (brain) appears to be the major organ involved in trans-stadial persistence of the virus; viral antigens were detected in the neural cortex (cell bodies) but not in nerve fibres and axons. The detection of THO viral antigen in basement membranes and connective tissue, but its absence from nerve fibres, suggests that dissemination occurs via the haemolymph rather than a neural route.

INTRODUCTION

Bloodsucking ticks are the most economically important vectors of livestock diseases (Arthur, 1962). Among the diseases transmitted by ticks, those due to arboviruses present a serious health risk to both domestic animals and humans (Hoogstraal, 1973). Despite this, little is known of the interactions between viruses and ticks. Most work on the localization of arboviruses within their vectors has been carried out with mosquitoes (Mitchell, 1982). However, ixodid ticks differ fundamentally from the haematophagous insects in their life history, feeding behaviour and digestive physiology (Obenchain & Galun, 1982), and hence pose unique questions regarding virus–vector associations, e.g. how do tick-borne viruses survive trans-stadially during the extensive tissue histolysis associated with tick metamorphosis (Burgdorfer & Varma, 1967)?

Electron microscopy (EM) has been used to localize arboviruses in ticks using ultrastructural criteria (Nosek et al., 1984; Chastel et al., 1984). Unfortunately, this technique only provides limited information since virus particles are usually difficult to distinguish. Studies relying on organ dissection and titration are hampered by problems of contamination from infected tick haemolymph. Therefore, we have used a combination of immunocytochemistry and virus titration to localize Thogoto (THO) virus in ticks.

Although THO virus is an arthropod-borne virus (Davies et al., 1986), it shows structural and morphogenetic similarities with members of the Orthomyxoviridae (Clerx et al., 1983). THO virus has been isolated from a variety of ixodid ticks and mammals in Africa and southern Europe, and is considered to be of veterinary and medical significance (reviewed by Davies et al., 1986). A laboratory transmission cycle has been established with the tick vector Rhipicephalus appendiculatus (Davies et al., 1986). THO virus persists trans-stadially in this species and can be transmitted by bite to susceptible hosts. This system was used to investigate...
the dynamics of infection, replication, and transmission of a tick-borne virus after first establishing the requisite techniques in cell culture.

**METHODS**

*Cells, virus and antisera.* The SiAr isolate of THO virus (Albanese et al., 1972) was obtained from Dr R. E. Shope (Yale Arbovirus Research Unit, New Haven, Conn., U.S.A.) as an infected suckling mouse brain extract. Virus was grown in BHK-21 cells, maintained in Eagle's minimum essential medium (EMEM) and supplemented with 3% newborn calf serum (NBCS). Plaque assays were performed in Vero cells as described by Davies et al. (1986). Purified virus for negative stain EM was prepared by the method of Clerx et al. (1983). Antisera to THO virus were raised in guinea-pigs (Davies et al., 1986), and hyperimmune ascitic fluid was raised in mice (Shope & Sather, 1979).

*Ticks.* Larvae, nymphs and adults of uninfected *R. appendiculatus* were initially supplied by Dr M. Mathewsson (Coopers Animal Health, Berkhamsted, U.K.) from a colonized stock that has been maintained for 5 years. Ticks were fed on female Dunkin–Hartley guinea-pigs (average weight 400 g) by confining them in Neoprene cells as described by Jones et al. (1988). Engorgement times were 4 to 5 days for larvae, 6 to 9 days for nymphs and 8 to 14 days for adults. After engorgement, ticks were maintained at 28 °C in perforated tubes held within desiccators at a relative humidity of 80%.

Ticks were infected by allowing them to feed on 6- to 8-week-old Syrian hamsters (supplied by the Department of Physiology, University of Oxford) in which viraemia was induced by subcutaneous inoculation of 500 p.f.u. of THO virus, as described by Davies et al. (1986). Larvae and nymphs infected orally, and trans-stadially infected nymphs and adults (infected as larvae), were examined for virus infection. Trans-stadially infected adults, after feeding for 7 days on guinea-pigs, were dissected and the various organs were assayed for virus and examined by immunocytochemistry. Dissected organs from infected ticks were washed in culture medium to remove infected haemolymph before homogenization individually in a microtissue grinder in 1 ml EMEM with 10% NBCS and antibiotics. The resulting virus suspensions were titrated by plaque assay in Vero cell monolayers.

*Light and electron microscope immunocytochemistry.* Confluent cell monolayers inoculated with approximately 5 p.f.u./cell THO virus were incubated at 37 °C for 4, 6, 8, 12, 24, 30 or 40 h. Both infected and mock-infected cells were acetone-fixed and stained for immunofluorescence, or glutaraldehyde-fixed, washed in 0.1 M-glycine buffer pH 7.5 and immunogold-labelled. For plasma membrane-associated THO antigens, immunolabelling preceded post-fixation with osmium tetroxide, staining in-block with uranyl acetate and embedding in epoxy resin (Emix, Emscope Laboratories) for ultrathin sectioning and staining.

Ticks were rapidly frozen, cryostat-sectioned at 6 μm, and acetone-fixed for indirect immunofluorescent antibody staining. For EM, both whole ticks and dissected organs were fixed for 1 h at 20 °C with 1% glutaraldehyde in 0.1 M-phosphate buffer pH 7.2, dehydrated in an ethanol series and embedded in LR White resin (London Resin) as described by Newman et al. (1983). Ultrathin sections were cut and mounted on uncoated nickel grids and screened for THO virus antigen by immunogold labelling.

Fixed salivary glands from feeding adults were freeze fracture-labelled for EM by the method of Pinto da Silva et al. (1981). Cell cultures and dissected tick salivary glands were fixed and infused with 2:3 M-sucrose, and frozen for cryo-ultramicrotomy and on-grid immunogold labelling according to the methods of Griffiths et al. (1984). For scanning electron microscopy (SEM), cells grown on Therma-Max plastic coverslips were glutaraldehyde-fixed, and coated by the method of Kelly et al. (1973).

For gold labelling experiments, grids were preincubated in 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 10 min followed by 2 to 3 h with antibody diluted in PBS containing 0.1% BSA. The dilution of antibody that gave the maximum specific labelling with the minimum of non-specific labelled background was determined. After washing three times by floating grids on PBS, followed by another 5 min blocking step with PBS-BSA, the site of antibody binding was detected by incubating with 10 nm colloidal gold particles conjugated to either Protein A, goat anti-mouse IgG or goat anti-guinea-pig IgG. Controls included omitting the primary antiserum or substituting an irrelevant one, and uninfected cells or tissue.

**RESULTS**

*Thogoto virus infection of BHK-21 cell cultures*

*Course of infection.* It can be seen from the growth curves of THO virus replication in BHK-21 cells (Fig. 1) that virus release into the supernatant began about 8 h post-infection (p.i.) at an m.o.i. of 0.05 and 0.5 p.f.u./cell. At an m.o.i. of 5 p.f.u./cell, virus release began earlier (about 6 h p.i.) and increased more rapidly. A maximum virus titre of about 7–0 log_{10} p.f.u./ml was reached 40 h p.i.
Immunofluorescent staining of infected cells showed a strong perinuclear cytoplasmic reaction by 18 h p.i. (Fig. 2a) with about 90% of cells infected.

**Morphogenesis**

Immunogold labelling of ultrathin cryosections demonstrated THO viral antigens on the cell surface and in cytoplasmic foci as early as 8 h p.i. (Fig. 3a, b). Binding was absent from the nucleus and from mock-infected cells. In specimens labelled before embedding, gold labelling was associated with plasma membrane bulges which appeared to be an initial stage in the budding process of the virus (Fig. 3c). Labelled virions released from the cell surface were visible by 24 h p.i. Budding virions were usually spherical, approximately 100 nm in diameter, but some filamentous budding profiles were observed (Fig. 3f). In the early stages of infection, the distribution of budding was uneven, with little around the central nuclear region (Fig. 3e); later in infection, budding profiles were present all over the cell surface (Fig. 3d, f). Budding profiles were absent from uninfected cells. Irregular intracellular vesicles of approx. 50 nm to 200 nm in diameter were seen in the cytoplasm of infected cells near areas of plasma membrane where budding occurred (Fig. 3c). In infected cell cultures, 8 to 18 h p.i., only cells undergoing budding displayed surface antigen (Fig. 4f). By 24 to 30 h p.i., many cells appeared rounded and vacuolated (Fig. 4f). Some cells in the final stages of infection showed high levels of THO viral antigen on the cell surface but no longer had budding profiles (not shown). Most cells had degenerated by 40 h p.i.

**THO virus infection of R. appendiculatus ticks**

**Course of infection**

A total of 32 ticks were examined by immunofluorescence (Table 1). In larvae infected by feeding on a viraemic host, specific cytoplasmic fluorescence was observed in the cortex of the synganglion (brain) and in the periganglionic sheath, but not in the central fibrous neuropile consisting of axons (Fig. 2b). The microscopic anatomy of the synganglion is explained in Fig. 4. In the salivary glands, trace fluorescent labelling was not observed until day 25 post-engorgement when small areas of specific peripheral fluorescence were found near the basal surface of some acini (data not shown). Specific cytoplasmic fluorescence was also observed in...
Fig. 2. THO viral antigens labelled by the immunofluorescent antibody technique. (a) BHK-21 cells infected at an m.o.i. of 1 and fixed at 18 h p.i. demonstrate specific labelling in the cytoplasm and perinuclear region. (b) Cryostat section of the synganglion (brain) of infected, unfed adult R. appendiculatus showing intense fluorescence in the outer layer (cortex), perineurium and periganglionic sheath but none in the central neuropile (NP). Bar markers represent 10 μm (a) and 100 μm (b).

the neural cortex of the synganglion of nymphs, 6 days after a viraemic blood meal, and in trans-stadially infected unfed adults approximately 150 days following oral infection in the larval stage. Specific fluorescence in adults was not observed in any other organ (midgut, muscles, Malpighian tubules, ovaries, seminal receptacle, rectum, salivary glands, trachea). Fluorescence was not observed in the salivary glands of unfed adults.

Organs dissected from 38 adult ticks were assayed for virus. All ticks had been infected orally in the nymphal stage, 20 to 50 days before dissection. Adult ticks (seven male, eight female) were examined unfed, and the rest (nine male, 19 female) were examined after feeding for 7 days on a

Fig. 3. (a, b) Ultrathin cryosections of BHK-21 cells 8 h p.i., labelled with THO antiserum and 10 nm immunogold. Labelling (arrows) is present on the cell surface (a), and in cytoplasmic foci, but is absent from the nucleus (N). Bar marker represents 100 nm. (c) Epoxy resin-embedded sections of BHK-21 cells fixed 18 h p.i. and surface-labelled with immunogold before embedding. (d) Scanning electron micrograph showing THO virus particles (arrows) budding from the surface of a BHK-21 cell 24 h p.i. (e) Budding of virus also occurs from the spindle-like processes of cells (S), which contain a longitudinal cytoskeleton. Cell fixed 12 h p.i. Budding profiles of virus are gold-labelled (arrows). Bar marker represents 200 nm. (f) Section of a cell culture fixed 24 h p.i. showing labelled (L) and unlabelled (UL) cells adjacent to each other. Only the labelled cell shows an extensive 'rounded' shape and plasma membrane budding profiles which are usually spherical but also tubular or filamentous (arrows). Large vacuoles (V) are also characteristic of cells undergoing viral budding. Bar marker represents 200 nm.
Thogoto virus infection

(a) N

(b) N

(c)

(d)

(e)

(f) L, V, UL
Fig. 4. (a) Diagram showing synganglion anatomy in the tick as seen by the light microscope. PSH, periganglionic sheath. (b) Diagram showing interpretation of the fine structure of the synganglion comprising cortex and axons, as seen by transmission EM. HC, haemocoel; PSH, periganglionic sheath; BL, basal laminae; PS, periganglionic sinus; NL, neural lamella; PN, perineurial cells; IS, intercellular space; NS, neurosecretory cell; GC, glial cell.

Table 1. Course of Thogoto virus infection in ticks examined by immunofluorescence

<table>
<thead>
<tr>
<th>Stage infected</th>
<th>Stage sectioned</th>
<th>Time p.i. (days)</th>
<th>Number examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larva</td>
<td>Larva</td>
<td>0</td>
<td>Infected 2   Control 2</td>
</tr>
<tr>
<td>Larva</td>
<td>Larva</td>
<td>3</td>
<td>Infected 2   Control 2</td>
</tr>
<tr>
<td>Larva</td>
<td>Larva</td>
<td>6</td>
<td>Infected 1   Control 2</td>
</tr>
<tr>
<td>Larva</td>
<td>Larva</td>
<td>7</td>
<td>Infected 1   Control 7</td>
</tr>
<tr>
<td>Larva</td>
<td>Larva</td>
<td>8</td>
<td>Infected 3   Control 1</td>
</tr>
<tr>
<td>Larva</td>
<td>Larva</td>
<td>12</td>
<td>Infected 1   Control 1</td>
</tr>
<tr>
<td>Larva</td>
<td>Nymph</td>
<td>13</td>
<td>Infected –   Control 1</td>
</tr>
<tr>
<td>Larva</td>
<td>Nymph</td>
<td>16</td>
<td>Infected 1   Control 2</td>
</tr>
<tr>
<td>Larva</td>
<td>Nymph</td>
<td>25</td>
<td>Infected 4   Control 1</td>
</tr>
<tr>
<td>Nymph</td>
<td>Adult</td>
<td>150</td>
<td>Infected 2   Control 2</td>
</tr>
<tr>
<td>Nymph</td>
<td>Nymph</td>
<td>6</td>
<td>Infected 1   Control 1</td>
</tr>
</tbody>
</table>

* Location of THO viral antigen in infected ticks as detected by fluorescent antibody staining.

guinea-pig. Virus was detected, in descending order of recovery rate, in synganglion, salivary gland, ovaries, seminal receptacle, midgut, trachea and rectum, but not in the Malpighian tubules, testes and male sex accessory gland (Table 2). After feeding for 7 days, there was a significant increase in the virus recovery rate and the mean virus titre of salivary glands. Relatively high levels of virus were recovered from nine of the infected synganglia; there was no significant change in the virus recovery rate and level of virus following feeding. Virus was detected in a relatively high proportion of female sex glands after feeding, but in none of the male sex glands.

The faeces (mostly undigested blood) of nymphs and adults were collected immediately after they had fed on viraemic hamsters and assayed for THO virus. A mean virus titre of 3·0 log_{10}
Table 2. *Titres of Thogoto virus in organs of trans-stadially infected adult ticks (day 20 to 40 post-nymphal engorgement)*

<table>
<thead>
<tr>
<th>Tick organ*</th>
<th>Unfed adult</th>
<th>Partially fed adult</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. infected/no. titrated</td>
<td>Mean titre†</td>
</tr>
<tr>
<td>SG</td>
<td>1/16</td>
<td>1.3</td>
</tr>
<tr>
<td>S</td>
<td>6/14</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>MG</td>
<td>1/10</td>
<td>1.5</td>
</tr>
<tr>
<td>R</td>
<td>0/4</td>
<td>-</td>
</tr>
<tr>
<td>MT</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>Tr</td>
<td>0/3</td>
<td>-</td>
</tr>
<tr>
<td>Te</td>
<td>NT‡</td>
<td>-</td>
</tr>
<tr>
<td>AG</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>O</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>SR</td>
<td>0/4</td>
<td>-</td>
</tr>
</tbody>
</table>

* SG, salivary gland; S, synganglion; MG, midgut; R, rectum; MT, Malpighian tubule; Tr, trachea; Te, testes; AG, accessory gland (male); O, ovaries; SR, seminal receptacle.
† log10 p.f.u./organ.
‡ NT, Not titrated.

p.f.u./tick was recovered from the faeces of 500 nymphs immediately following engorgement on five viraemic hamsters, and a mean titre of 2.4 log10 p.f.u./tick was recovered from 24 adults partially fed for 7 days on two viraemic hamsters. However, no virus was recovered from the excreted material (mostly guanine crystals) from the Malpighian tubules of about 200 trans-stadially infected adults following moulting. This is not surprising as virus was not detected in Malpighian tubules.

**Morphogenesis**

Intracellular THO viral antigens were detected in the brain or synganglion (Fig. 4) of infected non-feeding adult females by immunogold labelling. Labelling was apparent on the perineural sheath, especially the basal laminae, and in the cytoplasm of some cells (Fig. 5). Gold labelling was also present on the neural lamella along the innermost layer near the perineurial cells, and in small foci in the neural lamella (Fig. 5). Labelling was absent from the neuropile and from uninfected controls.

Salivary glands of infected female ticks were examined for the presence of THO antigen on days 1, 2 and 7 after the start of feeding. No antigen was detected until day 7 of feeding. Gold labelling was localized in the basement membranes of all three types of acini (Fig. 6a, b). Some labelling was present in the apical microvillar border of the secretory lumen of type II and III acini (Fig. 6c, d). Only trace labelling of the cytoplasm was seen. A similar labelling pattern on the basement membrane was seen in immunogold-labelled ultrathin cryosections (Fig. 6a) compared with that of plastic-embedded sections (Fig. 6b). The basement membranes of peripheral nerves and trachea also labelled with THO antiserum (data not shown).

**DISCUSSION**

The results of the *in vitro* study showed that THO virus, like the influenza viruses, undergoes morphogenesis by budding at the plasma membrane (Bachi et al., 1969; Compans & Dimmock, 1969). The envelope of THO virus appears less electron-dense and the glycoprotein spikes are smaller and less distinct than in influenza virus (Compans & Choppin, 1975).

Cytoplasmic vesicles, seen adjacent to regions where THO virus particles were budding, are also a feature of influenza virus morphogenesis (Compans & Dimmock, 1969). They may originate from the Golgi apparatus and be involved in transport of viral glycoproteins (Simons & Garoff, 1980). Areas of the plasma membrane labelled with THO virus antibodies had an electron-dense layer on the cytoplasmic side of the membrane, similar to that observed for influenza virus (Bachi et al., 1969; Compans & Dimmock, 1969), although glycoprotein spikes
on the outside of the membrane bulges were not prominent. The binding of the immunogold is thus a way of positively identifying sites of viral morphogenesis and, therefore, provides a method for examining the anatomical basis of THO virus infection in ticks.

Within 6 days following an infective blood meal, THO virus disseminated from the gut to the synganglion of the tick. The results are consistent with the overall dynamics of THO virus infection in orally infected *R. appendiculatus* (Davies *et al.*, 1986). There was no sign of infection in the salivary glands until at least 25 days following larval engorgement on a viraemic host, and virus was demonstrated in only a few small foci of the salivary glands. In trans-stadially infected adults, the prevalence of infection in the salivary glands increased following a blood meal. THO virus was thus not detected in salivary glands until after moultng, i.e. until the salivary glands had undergone necrosis and resorption during moultng and then subsequently had redeveloped and increased significantly in size. A similar timing of salivary gland infection has been described for Powassan virus in *Dermacentor andersoni* (Chernesky & McLean, 1969).

The female *R. appendiculatus* salivary gland consists of three types of acinar glands (Fawcett *et al.*, 1981). The two granular acini each have several cell types arranged around a central secretory lumen which connects with the salivary duct. During feeding, the basal region of the glands develops into an extensive labyrinth associated with water transport. THO viral antigen was present in the basement membranes of all three types of salivary gland acini. This suggests that the haemolymph may act as a route of dissemination of virus to the salivary glands, since the basement membrane of arthropods forms a barrier between the haemolymph and the extracellular spaces which is permeable to small molecules (Kefalides, 1979).

The major organ involved in the persistence of THO virus in ticks appears to be the synganglion. Virus titres in the synganglion are especially significant when the small size of this organ is considered. Localization of THO virus in the nervous system allows the virus to persist trans-stadially as this organ does not undergo extensive histolysis during metamorphosis, unlike other organs such as the salivary glands.

The apparently incomplete blood–brain barrier of ticks (Hart *et al.*, 1980) may permit transport of THO virus between the nervous system and haemolymph via the periganglionic sinus. Such a route of infection would explain the presence of THO viral antigen in the perineurial cell cytoplasm, basement membranes, and neural lamellae. The latter is known to be the major site of release of neurosecretions into tick haemolymph (Binnington, 1983). Alternatively (or additionally), THO virus may pass from the synganglion to the salivary glands via the interconnecting nerves. The apparently neurotropic nature of THO virus in its tick vector has not been observed in previous studies of tick-borne viruses, although a number of arboviruses are neurotropic in mosquitoes (Kuberski & Rosen, 1977).

When tick organs dissected from infected *R. appendiculatus* were examined by EM, no clearly defined virus particles were observed. This suggests that the infection is at a low level and/or that THO virus morphogenesis in ticks differs from that in vertebrate cell culture. With mosquito-borne alphaviruses virus assembly in vertebrate cells occurs at the plasma membrane, but in arthropod cells envelopment occurs at vacuoles which are exocytosed (e.g. Enzmann, 1987). Further studies, using more sensitive high resolution EM labelling techniques, are required to resolve the question of THO virus morphogenesis in ticks.

We thank Barry Martin of the Department of Zoology, Oxford for arranging the use of the ultrathin cryosectioning facility provided by the E.P.A. Cephalosporin Fund, Peter Lackie for instruction in its use, and Professor D. H. L. Bishop for reviewing the manuscript.

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**Fig. 5.** Immunogold labelling of the perineurium in trans-stadially infected female *R. appendiculatus*. Gold labelling localizes antigens to the basement membranes (BM), arrows; cell cytoplasm of perineurial cells and neural lamella (NL). Some concentrated foci of antigen are present in the neural lamella (b) and along its edges (a). Labelling is absent from nuclei (N) and the cytoplasm of perineurial sheath cells (PSH); H, haemolymph space. Bar markers represent 300 nm (a), 200 nm (b), 300 nm (c).


Fig. 6. (a) Ultrathin cryosection of a type III acinus from a salivary gland of a trans-stadially infected female *R. appendiculatus* after 7 days of feeding. Immunogold label is present along the basal lamina (arrow), adjacent to the haemolymph space (H). ES, extracellular space forming basal labyrinth. M, mitochondrion. Bar marker represents 400 nm. (b) Gold labelling of basement membranes of two salivary gland cells from type II acini from an infected female after feeding for 7 days. Label is present on the haemolymph (H) side of the membranes (arrows). Bar marker represents 400 nm. (c) The fracture-labelled luminal surface of an infected female *R. appendiculatus* (fed for 7 days) type III acinus labels with immunogold on profiles which may represent budding (arrows). A patch of antigen is present on the apical surface near some cytoplasmic vesicles (V). L, lumen; G, salivary gland granule. Bar marker represents 100 nm. (d) Section of a 7 day infected salivary gland shows gold particles on the apical microvilli (arrows) which line the secretory lumen (L). Bar marker represents 100 nm.


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