1 **Uukuniemi virus as a tick-borne virus model**

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12 Running title: Uukuniemi virus in tick cells

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Abstract

15

16 In the last decade, novel tick-borne pathogenic phleboviruses in the family *Bunyaviridae*, all

17 closely related to Uukuniemi virus (UUKV), have emerged in different continents. To reproduce

18 the tick-mammal switch *in vitro*, we first established a reverse genetics system to rescue UUKV

19 with a genome the closest to that of the authentic virus isolated from the *Ixodes ricinus* tick

20 reservoir. The IRE/CTVM19 and IRE/CTVM20 cell lines, both derived from *I. ricinus*, were

21 susceptible to the virus rescued from plasmid DNAs and supported production of the virus over

22 many weeks, indicating that infection is persistent. The glycoprotein G_C was mainly highly-

23 mannosylated on tick cell-derived viral progeny. The second envelope viral protein, G_N, carried

24 mostly *N*-glycans not recognized by the classical glycosidases PNGase F and Endo H. Treatment

25 with β-mercaptoethanol did not impact the apparent molecular weight of G_N. On viruses

26 originating from mammalian BHK-21 cells, G_N glycosylations were exclusively sensitive to

27 PNGase F and the electrophoresis mobility of the protein was substantially slower after the

28 reduction of disulfide bonds. Furthermore, the amount of viral nucleoprotein per foci forming

29 units differed markedly whether viruses were produced in tick or BHK-21 cells, suggesting a

30 higher infectivity for tick cell-derived viruses. Together, our results indicate that UUKV particles

31 derived from vector tick cells have glycosylation and structural specificities that may influence

32 the initial infection in mammalian hosts. This study also highlights the importance of working

33 with viruses originating from arthropod vector cells when investigating the cell biology of

34 arbovirus transmission and entry into mammalian hosts.

Importance

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36 Tick-borne phleboviruses represent a growing threat to humans globally. Although ticks are

37 important vectors of infectious emerging diseases, previous studies have mainly involved virus

38 stocks produced in mammalian cells. This limitation tends to minimize the importance of host

39 alternation in virus transmission to humans and initial infection at the molecular level. With this

40 study, we have developed an *in vitro* tick cell-based model that allows production of the tick-

41 borne Uukuniemi virus to high titers. Using this system, we found that virions derived from tick

42 cells have specific structural properties and *N*-glycans that may enhance virus infectivity for

43 mammalian cells. By shedding light on molecular aspects of tick-derived viral particles, our data

44 illustrate the importance of considering the host switch in studying early virus-mammalian

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45   receptor/cell interactions. The information gained here lays the basis for future research, not only

46   on tick-borne phleboviruses, but on all viruses and other pathogens transmitted by ticks.

Introduction

The *Bunyaviridae* is the largest family of RNA viruses. With over 350 members distributed worldwide and classified into five genera (*Tospovirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, and *Hantavirus*), these viruses represent a global threat to public health, livestock, and agricultural productivity (1). Hantaviruses apart, bunyaviruses are mainly transmitted by arthropods including sandflies, mosquitoes, and ticks. In the past five years, a number of emerging tick-borne phleboviruses have been reported in different continents. Severe fever with thrombocytopenia syndrome virus (SFTSV) in Asia and Heartland virus (HRTV) in North America are recent examples of new tick-borne phleboviruses causing severe and often fatal disease in humans (2-5). Other phleboviruses genetically related to SFTSV and HRTV have also been recently isolated from ticks in different parts of the world (6). The increasing number of outbreaks and the apparent wide distribution in tick reservoirs demonstrate that these viruses must be taken seriously as emerging agents of disease. Currently, no vaccines or treatments are approved for human use.

Tick-borne phleboviruses are enveloped, roughly spherical with a diameter of about 100 nm, and have a tripartite, single-stranded RNA genome that exclusively replicates in the cytosol (7). The large (L) RNA segment codes for the viral RNA-dependent RNA polymerase L, and the medium (M) segment for the glycoproteins G_N and G_C, both in a negative-sense orientation in the viral genomic RNA (vRNA) (7, 8). In addition to the vector, one of the main distinctions between tick-borne phleboviruses and the phleboviruses vectored by dipterans or mosquitoes is the absence of a sequence encoding a non-structural protein NSm in the M segment (7, 8). The small RNA segment (S) codes for the nucleoprotein N and the non-structural protein NSs in an ambisense manner (7, 8). The proteins N and NSs are translated from subgenomic mRNAs transcribed from the vRNA and the antigenomic, replicative-intermediate RNA (cRNA) respectively (7, 8). In the virions, the protein N is associated with the virus RNA genome and, together with the polymerase L, constitutes the ribonucleoproteins (RNPs) (7). In the virus envelope, the glycoproteins G_N and G_C form spike-like projections responsible for virus attachment to host cells and for acid-activated penetration by membrane fusion from late endosomal compartments (7, 9).

During natural transmission to vertebrates, tick-borne phleboviruses are introduced into the skin dermis of hosts following bites by infected ticks. Although ticks are both harmful parasites

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78   and vectors of several emerging diseases, not only those caused by phleboviruses, little
79   information on the cell biology of ticks is available (10-14). As a consequence, composition and
80   structure of tick cell-derived phleboviral particles remain largely undefined. Our knowledge of
81   the initial infection of vertebrate hosts, cellular receptors and cell entry is mainly based on the
82   use of virus stocks produced in mammalian cells. SFTSV has been shown to subvert the non-
83   muscle myosin heavy chain IIA for early steps of infection (15). Rhabdoviral particles
84   pseudotyped with the glycoproteins of SFTSV (SFTSV-pp) have been found to infect
85   macrophages (16). Human dendritic cells (DCs) are productively infected by many bunyaviruses,
86   including Uukuniemi virus (UUKV), a phlebovirus originally isolated from the tick *Ixodes*
87   *ricinus* in the 1960s (17-21). To enter and infect DCs, SFTSV-pp and UUKV have been shown
88   to exploit DC-SIGN, a C-type (calcium-dependent) lectin that binds high-mannose *N*-glycans on
89   the viral glycoproteins through its C-terminal carbohydrate recognition domain (CRD) (16, 17).
90   Interactions with L-SIGN, a C-type lectin closely related to DC-SIGN but expressed in liver
91   sinusoidal endothelial cells, have also been recently documented for SFTSV-pp and UUKV (16,
92   22). After binding, SFTSV-pp and UUKV depend on endosomal acidification for penetration and
93   infection (16, 23). UUKV is a late-penetrating virus, belonging to a large group of viruses that
94   depend on late endosomal maturation for productive entry (9, 23).

95   We focus here on UUKV, a virus that shares high sequence homology with SFTSV and
96   HRTV (8, 24, 25). However, UUKV is not associated with any disease in humans and is a
97   validated BSL2 surrogate for arthropod-borne bunyaviruses of higher biosafety classification
98   (26). Major advances into various aspects of the phlebovirus life cycle, including virion
99   structure, receptors, cell entry and assembly, have been achieved through the use of UUKV (9,
100   23, 27-32). To determine whether tick cells support phlebovirus productive infection *in vitro*, and
101   whether tick cell-derived phleboviral particles infect human or other mammalian cells, we
102   rescued UUKV from cDNAs with a genome the closest to that of the authentic virus isolated
103   from ticks. Using this system, we examined infection and virus production in tick cells, assessed
104   the progeny virus for interactions with DC-SIGN expressed in mammalian cells, analyzed the
105   glycans and the electrophoresis mobility of the virus glycoproteins G_N and G_C, and tested the
106   infectivity of viral progeny in mammalian cells.

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Materials and methods

Cells and viruses. All products used for cell culture were obtained from Life Technologies or Sigma Aldrich. Baby Hamster Kidney cells (BHK-21) were grown in Glasgow's minimal essential medium (GMEM) supplemented with 10% tryptose phosphate broth, 5% fetal bovine serum (FBS), 1% glutaMAX, 100 units.mL^{-1} penicillin, and 100 μg.mL^{-1} streptomycin (33). Human B (Raji) and epithelial (HeLa) cells that stably express DC-SIGN were cultured according to ATCC recommendations (17, 34). All mammalian cell lines were grown in an atmosphere of 5% CO_2 in air at 37°C. The tick cell lines IRE/CTVM19 and IRE/CTVM20 were cultured in L-15-based medium in sealed, flat-sided tubes (Nunc) in ambient air at 28°C as reported elsewhere (35-37). The prototype UUKV strain 23 (UUKV S23) was originally isolated from the tick *I. ricinus* in the 1960s (i.e. the virus in tick suspension) (21). The UUKV strain used in this study results from five successive plaque purifications of UUKV S23 in chicken embryo fibroblasts (CEFs) and subsequent passages in BHK-21 cells (38, 39). Virus multiplicity of infection is given according to the titer determined in BHK-21 cells.

Antibodies and reagents. The mouse monoclonal antibodies 8B11A3, 6G9E5 and 3D8B3 are directed against the UUKV nucleoprotein N and the glycoproteins G_N and G_C respectively (40). The rabbit polyclonal antibodies K1224 and K5 are directed against the UUKV glycoproteins G_N and G_C respectively (41). All of these antibodies were a kind gift from Anna Överby and the Ludwig Institute for Cancer Research (Stockholm, Sweden). The rabbit polyclonal antibody U2 has been described previously, and recognizes the UUKV proteins N, G_N, and G_C (17). The neutralizing anti-DC-SIGN mouse monoclonal antibody IgG2a (mAb1621) was purchased from R&D Systems. NH_4Cl and EDTA were obtained from Sigma Aldrich and dissolved in deionized water.

Plasmids. The expression plasmids pUUK-N and pUUK-L were a kind gift from Anna Överby and code for, respectively, the UUKV nucleoprotein N and polymerase L (39). The cDNAs corresponding to the S, M, and L segments of UUKV were synthesized by RT-PCR from vRNA extracts of purified-virus stock using the reverse transcriptase Superscript III (Life Technologies). Their amplification as a single PCR product was carried out using the Herculase II fusion DNA polymerase (Agilent). The PCR products were then cloned between the murine Pol I RNA polymerase promoter and terminator sequences in the pRF108 vector (a generous gift

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137   from Ramon Flick, Bioprotection Systems Corporation) (30). The resulting Pol I-driven plasmids

138   (pRF108-S, pRF108-M, and pRF108-L) encoded each of the antigenomic UUKV RNA

139   molecules (i.e. S, M, and L segments). The point mutation G2386A in the M segment was

140   obtained with the QuikChange XL site-directed mutagenesis kit (Agilent) using the plasmid

141   pRF108-M as a template. The complete list of primers and restriction enzymes used for cloning

142   and mutagenesis is shown in **Table 1**.

143   **Rescue of UUKV from plasmid DNAs.** UUKV was rescued by transfecting BHK-21 cells (0.6

144    $\times 10^6$) with the expression plasmids pUUK-L (1 µg) and pUUK-N (0.3 µg) together with 0.5 µg

145   each of pRF108-S, pRF108-M, and pRF108-L. Transfection was performed in the presence of

146   Lipofectamine 2000 (Life Technologies) using a ratio of 3.8 µL to 1 µg of plasmids in 400 µL of

147   OptiMEM (Life Technologies). One hour post-transfection, complete GMEM medium

148   containing 2% FBS was added to the cells. After 5 days, supernatants were collected, clarified,

149   and titrated as described below. Rescued viruses were passaged a minimum of five times in

150   BHK-21 cells.

151   **Virus titration by focus-forming assay.** Following infection of confluent monolayers with 10-

152   fold dilutions of virus in FBS-free medium, cells were grown in the presence of medium

153   containing 5% serum and supplemented with 0.8% carboxymethyl-cellulose to prevent virus

154   spread. Foci were revealed with a diaminobenzidine solution kit (Vector Laboratories) after a

155   two-step immunostaining with the antibody U2 (1:1,000) and an anti-rabbit horseradish

156   peroxidase-conjugated secondary antibodies (Vector Laboratories) (23).

157   **Deglycosylation.** To assess the glycosylation pattern of the UUKV glycoproteins, virus stocks

158   purified through a 25%-sucrose cushion were denatured and exposed to one of the five

159   following treatments: 1,000 units of Endo H (Promega), 5 units of PNGase F, 0.005 units of α-

160   2(3,6,8,9)-neuraminidase, 0.003 units of β-1,4-galactosidase, and 0.05 units of β-N-

161   acetylglucosaminidase (all enzymes from Merck Millipore) according to the manufacturer's

162   recommendations, and then analyzed by SDS-PAGE on a 4-12% or 10% Bis-Tris Nu-PAGE

163   Novex gel (Life Technologies) and immunobloting.

164   **Protein analysis.** Viral protein extracts from virus stocks purified through a 25% sucrose

165   cushion were analyzed by SDS-PAGE (Nu-PAGE Novex 4-12% Bis-Tris gel, Life

166   Technologies) and transferred to a PVDF membrane (iBlot transfer Stacks, Life Technologies)

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167 (17, 23). When indicated, purified viruses were pre-treated with glycosidases or β-
168 mercaptoethanol (40%). For western blot analysis, the PVDF membranes were first incubated
169 with primary mouse monoclonal antibodies 8B11A3 (1:2,000), 3D8B3 (1:100) or 6G9E5
170 (1:100), or rabbit polyclonal antibodies K5 (1:250-1,000), 1224 (1:500-1,000) or U2 (1:2,000),
171 all diluted in TBS containing 0.1% Tween and 5% milk, and then with an anti-mouse or anti-
172 rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000, Santa Cruz),
173 respectively. Bound antibodies were detected by exposure to enhanced chemiluminescence
174 reagents (ECL, GE Healthcare or Life Technologies). For quantitative detection of viral proteins,
175 membranes were first incubated with the rabbit polyclonal antibody U2 (1:1,000) and then with
176 an anti-rabbit infrared fluorescence (IRDye) secondary antibody and analyzed with the Odyssey
177 Imaging Systems and the software Image Studio (Li-Cor Biosciences).

178 **Infection assays.** Mammalian cells were infected with virus at different MOIs in medium
179 without FBS at 37°C for one hour. Virus supernatant was then replaced by complete culture
180 medium and cultures were incubated for up to 64 hours before fixation. Tick cells were exposed
181 to viruses at different MOIs in culture medium containing FBS at 28°C for up to 48 hours. When
182 used for microscopy, tick cells were seeded on poly-L-Lysin (0.01%)-coated coverslips at 28°C
183 on the day before infection. For inhibition assays, cells were pretreated with inhibitors at
184 different concentrations for 30 min and exposed to UUKV in the continuous presence of the
185 inhibitors. The infection was monitored by either wide-field fluorescence microscopy or flow
186 cytometry.

187 **Flow cytometry.** The flow cytometry-based infection assay has been described previously (23).
188 Briefly, after fixation and permeabilization with 0.1% saponin, infected cells were incubated
189 with the mouse monoclonal antibodies 8B11A3 (1:400), 6G9E5 (1:400) or 3D8B3 (1:500) at
190 room temperature for one hour, washed, and subsequently exposed to Alexa Fluor (AF) 647-
191 conjugated secondary anti-mouse antibodies (1:500, Life Technologies) at room temperature for
192 one hour. When the mouse monoclonal antibody 1621 (25 μg.mL^{-1}) was used in infection assays
193 to neutralize DC-SIGN, UUKV-infected cells were immuno-stained with the rabbit polyclonal
194 antibody U2 (1:400) and AF647-conjugated secondary anti-rabbit antibody (1:500, Life
195 Technologies). Flow cytometry-based analysis involved the use of a FACS Calibur cytometer
196 (Becton Dickinson) and Flowjo software (Treestar).

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197   **Fluorescence microscopy.** Infected cells were fixed and permeabilized with PBS containing

198   0.1% Triton X-100 (Merck Millipore), incubated with the mouse monoclonal antibody 8B11A3

199   (1:1,000) at room temperature for one hour, washed, and then exposed to AF488-conjugated

200   secondary anti-mouse (1:800, Life Technologies) at room temperature for one hour. Nuclei were

201   subsequently stained with Hoechst 33258 (0.5 μg.mL^{-1}, Life Technologies). Infection was

202   quantified by counting cells in three independent fields and cells were imaged with an Olympus

203   IX81 microscope.

204   **Statistical Analysis.** The data shown are representative of at least three independent

205   experiments. Values are given as the mean of triplicates ± standard deviation (SD).

206   **Sequencing of the full-length M segment isolated from UUKV-infected ticks.** Questing

207   nymphs of the tick *I. ricinus* were collected in the region of Ramsvik and Hindens Rev (Sweden,

208   2013). Pools of 25 nymphs were homogenized and the total RNA was extracted with a magnetic

209   bead-based protocol as described elsewhere (kind gift of Janne Chirico, National Veterinary

210   Institute, Uppsala, Sweden and Sara Moutailler, ANSES, Maisons-Alfort, France) (42). The

211   cDNA corresponding to the M segment of UUKV was synthesized by RT-PCR with the reverse

212   transcriptase Superscript III (Life Technologies) and the specific primer RT-M (**Table 1**) before

213   amplification as a single PCR product using the pfu DNA polymerase (Promega) and the primers

214   UUKV-M-5NC and UUKV-M-3NC (**Table 1**). PCR products were analyzed with a capillary

215   sequencer by ABI (Eurofins Scientific).

216   **GenBank accession numbers.** The GenBank accession numbers for the nucleotide sequence of

217   the M segment of the tick isolates RVS and HRS are KX219593 and KX219594 respectively.

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218   **Results**

219   **Recovery of UUKV S23 from RNA polymerase I (Pol I)-driven plasmid DNAs.** The UUKV

220   lab strain that we used in this study as a template for cloning purposes results from the adaptation

221   of the prototype tick isolate strain 23 (UUKV S23) to BHK-21 cells after successive plaque-

222   purifications in CEFs (21, 38, 39). Compared with the S, M, and L nucleotide sequences

223   published for the original UUKV S23 plaque-purified five times in CEFs (GenBank accession

224   numbers NC_005221.1, NC_005220.1, and NC_005214.1 respectively), we identified only a few

225   mutations in our UUKV lab strain (**Figure 1A**). Most were in the M segment. Over the entire

226   virus genome, only one mutation was conserved, a non-silent substitution (A2386G) in the M

227   transcript (43-45) (**Figure 1A**). In all further experiments, for convenience, UUKV will refer to

228   our current mammalian cell-cultured adapted lab strain, rUUKV to the same virus but rescued

229   from cDNAs, UUKV S23 to the original tick isolate plaque-purified five times in CEFs, and

230   rUUKV S23 to the viral particles produced from plasmids encoding genome sequences identical

231   to those published for UUKV S23 in the late 1980s and early 1990s (43-45).

232   Using a reverse genetics system that relies on the cellular Pol I promoter for the synthesis of

233   viral transcripts, the anti-genomic full-length segments S, M, and L from UUKV were cloned

234   into the vector pRF108, which contains the Pol I promoter and terminator. This system has been

235   successfully employed to synthetize chimeric transcripts of the UUKV segment M (30) and to

236   recover infectious particles of the mosquito-borne phlebovirus Rift Valley fever virus (RVFV)

237   from plasmid DNAs (46, 47). The complete system is depicted in **Figure 1B**. From the plasmids

238   coding for the genome of UUKV, it was possible to obtain all the segments encoding UUKV S23

239   with only one site-directed mutagenesis (G2386A in the M segment). This reversion results in

240   the addition of an arginine instead of a glutamine at position 276 in the sequence of the

241   glycoprotein G_C.

242   As there is no evidence for Pol I promoter activity in tick cells, both rUUKV and rUUKV

243   S23 were first rescued from BHK-21 cells, which are highly permissive to most bunyaviruses.

244   The infectious progenies were detected in the cell culture medium by a focus-forming unit (ffu)

245   assay (**Figure 1C**). Both focus formation and growth properties of the recombinant rUUKV and

246   rUUKV S23 in BHK-21 cells were similar to those of UUKV (**Figure 1C**). Co-transfection of

247   the Pol I-driven full-length S, M, and L plasmids together with plasmids coding for the viral

248   polymerase L and nucleoprotein N, whose expression depends on the cytomegalovirus promoter,

249   was essential for the recovery of infectious particles (data not shown).

250   The level of infectious rUUKV and rUUKV S23 in the cell supernatant remained relatively

251   modest five days after transfection, from thousands to hundreds of thousands tffu.mL^{-1} (**Figure**

252   **1D**). However, the titers significantly increased over subsequent rounds of amplification in

253   BHK-21 cells and reached a plateau of 10^7 ffu.mL^{-1} after 2-3 passages, typical for UUKV (17,

254   23) (**Figure 1E**). To assess the identity of the recombinant virus strains recovered from cDNAs,

255   we used the point reversion G2386A in the M sequence as a genetic marker. Total vRNA from

256   rUUKV or rUUKV S23 was extracted after 5 passages in BHK-21 cells, reverse transcribed, and

257   sequenced with primers spanning the reversion site (**Figure 1F**). The sequencing showed that

258   rUUKV S23 was derived from the transfected plasmids and not from contaminating UUKV.

259   We next analyzed rUUKV particles, infection, replication, and progeny production in BHK-

260   21 cells in comparison to UUKV. When the viruses were subjected to SDS-PAGE and western

261   blotting, all three major structural proteins, namely N, G_N, and G_C, were observed in both

262   rUUKV and UUKV virions (**Figures 2A and 2B**). To monitor infection, we used mouse

263   monoclonal antibodies against each of the newly-synthesized virus nucleoprotein N and

264   glycoproteins G_N and G_C before flow cytometry analysis (**Figure 2C**). Using the N protein

265   expression as readout, we found that the kinetics of rUUKV infection were quite similar to those

266   of UUKV (**Figure 2D**). The increase in the proportion of infected cells over time emphasized

267   that viral replication, and not input virus, was quantified in these assays. A complete cycle, from

268   binding to release of infectious progeny, lasted about 6-7 hours in BHK-21 cells (data not

269   shown) and reached a plateau after 48 hours (**Figure 2E**). Similar results were obtained with

270   rUUKV S23 (data not shown).

271   Taken together, our results show that infectious viruses with genomic RNAs identical to

272   those of the original UUKV S23 can be recovered from cDNAs. In turn, our reverse genetics

273   system can be confidently used to investigate the life cycle of tick-borne phleboviruses in vector

274   tick cells as well as the subsequent transmission and initial infection in mammalian hosts.

275   Because it is the model closest to the authentic tick isolate, we then focused on the recombinant

276   strain rUUKV S23.

277   **Tick cells are persistently infected by UUKV.** rUUKV S23 rescued in BHK-21 cells was then

278   used to infect tick cells and regenerate virions with all the features of the original tick-derived

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279   virus (e.g. lipids, glycosylation, etc.). Two cell lines derived from embryos of *I. ricinus*, the tick

280   species from which UUKV was first isolated, were used: IRE/CTVM19 and IRE/CTVM20 (35),

281   obtained from the Tick Cell Biobank at The Pirbright Institute, UK. To determine whether

282   IRE/CTVM19 and IRE/CTVM20 cells support infection by tick-borne phleboviruses, they were

283   exposed to rUUKV S23 for 48 hours and immuno-stained with mouse monoclonal antibodies

284   against the nucleoprotein N of UUKV. Flow cytometry allowed quantitative detection of infected

285   cells. Independent of the tick cell line, nearly 50% of cells were infected at a multiplicity of

286   infection (MOI) of 5 and about 20% at a MOI as low as 1.25 (**Figure 3A**). The sensitivity of tick

287   cells to rUUKV S23 infection was confirmed by fluorescence microscopy after immuno-staining

288   of the newly synthesized virus nucleoprotein N (**Figure 3B**). These experiments showed that

289   both tick cell lines support infection of rUUKV S23.

290   We next assessed infected cells for the production of virus progeny. Although cells were

291   infected, titration of cell-free supernatants collected from challenged tick cells indicated that the

292   production of infectious rUUKV S23 particles was almost nonexistent within the first 24 hours

293   of infection (data not shown). After this period, the amount of infectious progeny produced from

294   both tick cell lines increased over time to reach a plateau value within three weeks (**Figure 3C**).

295   Subculturing of infected tick cells 34, 54, and 74 days post-infection, consisting of the removal

296   of half of the cell suspension and replacement with fresh culture medium, did not result in a

297   decrease in viral progeny production (indicated by black arrows in **Figure 3C**). At this time, no

298   cytopathic effects were observed following subculture and the cells grew normally. At 100 days

299   post-infection, high levels of infectious virus were still detectable despite the subculturing of

300   cells. Similar results were obtained when rUUKV was used to infect IRE/CTVM19 and

301   IRE/CTVM20 cells (**Figure 3D**). After several months of amplification in tick cells, the

302   reversion G/A introduced at the position 2386 in the M segment was still present in the genome

303   of rUUKV S23, but not in that of rUUKV (data not shown). Altogether these data indicate that *I.*

304   *ricinus* cells support persistent infection by recombinant UUKV.

305   **Mammalian cells support infection by rUUKV S23 grown in tick cells.** To examine whether

306   rUUKV S23 derived from tick cells remains able to infect mammalian cells, BHK-21 cells were

307   exposed to the viral progeny derived from IRE/CTVM19 cells for 18 hours. Infected cells were

308   analyzed with conformation-dependent mouse monoclonal antibodies in a flow cytometry-based

309   assay. A large proportion of the infected cells were positive for both the virus nucleoprotein N

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310 and glycoproteins G_N and G_C (**Figure 4A**), suggesting that infection leads to viral replication.

311 Therefore our phlebovirus-tick cell system appears to be an excellent model for reproducing

312 transmission of tick-borne viruses to mammalian hosts *in vitro*.

313 **DC-SIGN mediates infection by tick cell-derived rUUKV S23.** Due to their presence at the

314 site of initial infection via tick bite, dermal DCs are among the first cells to encounter the

315 incoming viruses (1, 48). We recently established that DC-SIGN, which is highly expressed on

316 the surface of human dermal DCs, binds UUKV directly via interactions with high-mannose *N*-

317 glycans on the virus glycoproteins (17). The capacity of DC-SIGN to bind tick cell-derived viral

318 particles was therefore evaluated using Raji and HeLa cells stably expressing the lectin after

319 transduction with a TRIPΔU3 lentiviral vector encoding human DC-SIGN (34). Raji and HeLa

320 cells normally have low or no sensitivity to phlebovirus infection. As expected, parental Raji

321 cells were not detectably infected with IRE/CTVM19 cell-derived rUUKV S23 at a MOI of 5 or

322 less (**Figure 4B**). However, when DC-SIGN was expressed, up to 40% of Raji cells became

323 infected at a MOI of 0.1 (**Figure 4B**). Similarly, fluorescence microscopy analysis of HeLa cells

324 exposed to various MOIs of the virus showed that infection was greatly increased when the lectin

325 was expressed (**Figure 4C**).

326 To confirm that the infection was mediated by DC-SIGN, we utilized the neutralizing mouse

327 monoclonal antibody mAb1621 and EDTA, which inhibits the DC-SIGN binding function by

328 extracting the bound calcium (33). The increase in infectivity due to DC-SIGN expression was

329 significantly reduced in cells treated with inhibitors (**Figure 4D**). Together, these results clearly

330 indicate that infection by tick cell-derived rUUKV S23 is mediated by DC-SIGN and suggest

331 that the viral glycoproteins have, at least in part, high-mannose carbohydrates recognized by the

332 lectin.

333 **G_N and G_C present distinct glycosylation profiles on tick and mammalian cell-derived**

334 **rUUKV S23 particles.** Like other bunyaviruses, UUKV has several *N*-linked oligosaccharides

335 in its envelope glycoproteins G_N and G_C (four sites each). To analyze the glycosylation pattern of

336 G_N and G_C on tick cell-derived viruses, rUUKV S23 was treated with peptide-N-glycosidase F

337 (PNGase F) under denaturing conditions before SDS-PAGE and western blotting. When rUUKV

338 S23 was produced in IRE/CTVM19 cells, at least one *N*-glycosylation site on G_N appeared

339 sensitive to PNGase F, while all four sites were sensitive when the virus was derived from BHK-

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340 21 cells (**Figure 5A**). In contrast, all four of the *N*-glycans on the glycoprotein G_C were

341 susceptible to the glycosidase regardless of the cells used to produce rUUKV S23 (**Figure 5B**).

342 To further examine the *N*-glycans on rUUKV S23, the virus was subjected to

343 Endoglycosidase H (Endo H). When rUUKV S23 was amplified in BHK-21 cells, the

344 glycoprotein G_N acquired complex glycosylation and was mainly Endo H-resistant (**Figure 5C**),

345 while G_C carried mainly high-mannose *N*-glycans as evidenced by sensitivity to Endo H

346 digestion (**Figure 5D**), i.e. only one site remained resistant. When the virus was produced in the

347 IRE/CTVM19 tick line, both glycoproteins G_N and G_C were sensitive to Endo H, with a

348 digestion pattern identical to that of PNGase F (**Figures 5C and 5D**). These results indicate that

349 all the *N*-glycans on G_N and G_C on tick cell-derived virus particles contain, at least, high-

350 mannose core structures.

351 Of the two viral envelope glycoproteins, G_N shows the most striking differences in

352 glycosylation patterns between tick and BHK-21 cells. To further address these distinctions,

353 rUUKV S23 was exposed to neuraminidase, β-1,4-galactosidase, and β-N-

354 acetylglucosaminidase, which liberate neuraminic acids, β-galactosides, and terminal β-N-

355 acetylglucosamine and N-acetylgalactosamine residues from oligosaccharides, respectively. G_N

356 originating from tick cells, but not from BHK-21 cells, was only sensitive to β-N-

357 acetylglucosaminidase, to a similar extent as PNGase F (**Figures 5E and 5F**). Overall, our data

358 indicate that UUKV particles derived from vector tick cells have glycosylation profiles distinct

359 from those in mammalian cells.

360 **The reduction of di-sulfide bonds impacts the electrophoresis mobility of G_N derived from**

361 **mammalian cells but not from tick cells.** Under reducing and non-reducing conditions, the

362 electrophoretic mobility of G_N on tick cell-derived rUUKV S23 appeared faster than that of the

363 protein on viral particles originating from BHK-21 cells (**Figures 5A and 6A** respectively). In

364 contrast, the electrophoretic mobility of G_C on tick cell-derived virions appeared similar to that

365 of the glycoprotein on viruses produced from BHK-21 cells, under either reducing or non-

366 reducing conditions (**Figures 5B and 6B** respectively).

367 We next assessed whether treatment with β-mercaptoethanol results in a change in the

368 apparent molecular weight of G_N made in tick and mammalian cells. When virions produced in

369 BHK-21 cells were analyzed, the electrophoresis mobility of G_N appeared slower under reducing

370 conditions (**Figure 6C**). In our experimental conditions, no shift was observed when the protein

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371   originated from particles derived from tick cells, suggesting that the number of di-sulfide bonds

372   differs between G_N on tick and mammalian cell-derived viruses. Altogether these results suggest

373   that the viral glycoprotein G_N on viruses produced in tick cells has different maturation and

374   folding properties compared to those of the protein on viral particles derived from mammalian

375   cells.

376   **The structural proteins G_N, G_C, and N in infectious viral progeny produced in tick and**

377   **mammalian cells.** We then further examine potential differences in the structure of rUUKV S23

378   particles derived from tick and mammalian cells. For this purpose, viruses were purified through

379   a 25% sucrose cushion, to retain only viral particles with an intact envelope. Identical numbers

380   of infectious particles, based on titration on BHK-21 cells, were analyzed by SDS-PAGE and

381   western blot using the polyclonal antibody U2 against the whole virus, i.e. enabling the detection

382   of G_N, G_C, and N. The amount of nucleoprotein N and glycoproteins G_N and G_C appeared

383   markedly different in virions produced in tick and BHK-21 cells, with substantially less protein

384   N and more glycoproteins G_N and G_C in tick cell-derived viruses (**Figure 7A**).

385   The amount of proteins G_N, G_C, and N incorporated into rUUKV S23 particles was

386   determined by quantitative western blot using the anti-UUKV U2 and an anti-rabbit infrared

387   fluorescence secondary antibody prior analysis with an Odyssey Imaging Systems. The ratio

388   between protein N and ffu was significantly lower in tick cell-derived viruses (**Figure 7B**) while

389   that between the viral glycoproteins and ffu was greater (**Figure 7C**). It was also apparent that

390   the numbers of G_N and G_C molecules per nucleoprotein N (**Figure 7D**) and the number of ffu per

391   N (**Figure 7E**) were higher in virions originating from tick cells. Similar results were obtained

392   with rUUKV (data not shown). From these results, it is most likely that the global structural

393   organization of viral particles exhibit differences between viruses produced in tick and

394   mammalian cells.

395   **Wild type UUKV originating from Swedish ticks in the 2010s.** All RNA viruses are known to

396   have high polymerase error rates. To determine whether the wild type UUKV in vector tick

397   populations has genetically evolved since the 1960s, and thus, whether our tick-borne virus

398   model is still representative of the circulating virus, we analyzed 16 pools of 25 nymphs of the

399   tick *I. ricinus* recently collected in the region of Ramsvik and Hindens Rev (Sweden, 2013) for

400   the presence of the UUKV RNA segment M. The cDNA corresponding to the full-length M

401   segment of UUKV was first synthesized by RT-PCR from vRNA extracted with magnetic beads

402   from nymphal homogenates and then amplified by a single PCR. Out of the 16 homogenates,

403   four were positive, from which it was possible to obtain the full-length nucleotide M sequence

404   for two samples (GenBank accession numbers KX219593 and KX219594). Due to limited

405   amounts of material, a partial sequence only was obtained from one of the two other samples.

406   Nucleotide-sequence analysis showed identity greater than 93% between the UUKV S23 and the

407   virus circulating currently. At the amino-acid level, the sequence identity reached a minimum of

408   98.4%, with 7-11 amino-acid variations located in G_N and 2-4 in G_C (**Table 2**). Together, our

409   data indicates a modest genetic evolution of UUKV glycoproteins in ticks.

410   **Dependence on low pH of rUUKV S23 infectious entry.** We have recently showed in human,

411   rodent, and monkey cells that infectious entry of UUKV is pH-dependent (23). We examined

412   whether the envelope proteins G_N and G_C remain dependent on endosomal acidification to trigger

413   the infectious entry of tick cell-derived viruses into mammalian cells. To this end, DC-SIGN-

414   expressing Raji and HeLa cells were exposed to IRE/CTVM19 cell-derived rUUKV S23 in the

415   presence of agents that neutralize vacuolar pH. The lysomotropic weak base ammonium chloride

416   (NH_4Cl) induced a dose-dependent inhibition of infection in both cell lines (**Figures 8A and**

417   **8B**). Infection of both IRE/CTVM19 and IRE/CTVM20 cells was also sensitive to NH_4Cl

418   (**Figures 8C and 8D**). Similar results were obtained when chloroquine, another lysomotropic

419   agent, was employed (data not shown). These data show that rUUKV S23 infection relies on

420   vacuolar acidification regardless of the host cell origin of the virus and the targeted cell type.

Discussion

421 **Discussion**

422 Ticks belong to the *Arachnida*, a class distinct from that of insects (*Insecta*) (49, 50). They are

423 arthropods of huge economic significance worldwide, both as harmful ectoparasites and as

424 vectors of many agents of disease, including protozoan and helminth parasites, bacteria, and

425 viruses (10, 50). As obligatory parasites, tick-borne viruses share dependence on biochemical

426 and biophysical features gained in ticks for production in arthropod vectors and then

427 transmission to mammalian hosts and other vertebrates. However, very little is known about the

428 molecular and cell biology of ticks. In the *Bunyaviridae* family, a number of novel pathogenic

429 tick-borne phleboviruses, all closely related to UUKV, have recently emerged in different parts

430 of the world (24, 25). In this context, there is a renewed interest in using UUKV as a model

431 system to examine the initial infection of human hosts by these emerging pathogenic viruses.

432 The orthobunyavirus Bunyamwera virus was the first bunyavirus for which a reverse genetics

433 system was developed to allow the recovery of infectious viral particles from plasmid DNAs

434 (51). Since then, similar or derivative methods have been adapted to some other bunyaviruses,

435 including the tick-borne phlebovirus SFTSV (1, 52-55). In this study, we have added UUKV to

436 the list with a reverse genetics system that relies on the use of the cellular RNA Pol I promoter,

437 enabling modification of the viral genome. The UUKV particles recovered from plasmids were

438 infectious and had replication characteristics similar to those of the authentic virus. An

439 alternative approach to rescuing UUKV from cDNAs that utilizes the T7 promoter was recently

440 described (56). In contrast to this latter method, our Pol I-driven system requires the co-

441 transfection of plasmids coding for the proteins N and L to trigger viral replication and

442 production. This may be explained by the fact that the RNA transcripts under the control of the

443 T7 promoter are exclusively processed in the cytosol, whilst those regulated by the Pol I

444 promoter follow the classical cellular messenger RNA maturation cycle through the nucleus. The

445 main critical advantage of our system concerns the stability of the rescued virus that we showed

446 stable after long-term passage.

447 The reference UUKV strain 23 was isolated from the tick *I. ricinus* in the 1960s and then

448 adapted to tissue culture, being passaged and amplified over many years, first in chicken embryo

449 cells, and subsequently in BHK-21 cells (23, 39, 57). However the nucleotide sequence of the

450 three RNA segments of our UUKV lab strain did not exhibit major evolution compared to that

451 published for the initial virus. As a consequence of this conservation, remarkably high for a RNA

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452 virus, it was possible to obtain all the original UUKV RNA transcripts with only one site-
453 directed mutagenesis in the sequence coding for the glycoprotein G_C. In addition, this single
454 mutation does not seem to confer any significant advantages to the virus in terms of infectivity
455 and replication capacity, either in tick or mammalian cells.

456 When UUKV S23 was compared with viruses currently circulating in tick populations, we
457 found a rather modest number of point mutations in the nucleotide sequence of the M segment,
458 which codes for the glycoproteins G_N and G_C. Furthermore, most of these mutations were silent
459 at the amino acid level, with sequence identities reaching 98% and higher. It is not possible to
460 conclude whether the few differences result from (1) the genetic evolution of the virus since the
461 1960s, (2) different sites for the collection of ticks, i.e. Finland for the strain 23 and Sweden for
462 the new isolates, or (3) adaptation of the strain 23 to tissue culture. However, while cloning the
463 circulating virus in our reverse genetics system for future investigations into the new point
464 mutations remains paramount, it is apparent that our tick cells-virus model already allows for
465 addressing many aspects of both the cell biology of ticks and the early steps of initial infection in
466 mammalian hosts, from virus transmission to entry.

467 Several bunyaviruses are vectored by ticks: a few orthobunyaviruses, all the nairoviruses, and
468 all of the phleboviruses related to UUKV (1, 49, 58). While some tick cell lines have been shown
469 to be sensitive to bunyaviruses, including the nairovirus Crimean-Congo hemorrhagic fever
470 virus, we have extended these observations to tick-borne phleboviruses (49). We found that both
471 the IRE/CTVM19 and IRE/CTVM20 cell lines originating from the tick *I. ricinus* are sensitive to
472 UUKV. Both lines supported a complete virus life cycle, from infectious entry to release of new
473 infectious progeny. Although the interaction of additional phleboviruses with tick cell lines must
474 be assessed experimentally, it is likely that many lines can be infected by tick-borne
475 phleboviruses.

476 Arthropod-borne viruses infecting humans and other animals are generally maintained in
477 arthropod vectors and amplified in non-human vertebrates. This is also the case for bunyaviruses
478 (1). In contrast to vertebrate hosts, there is no clear evidence of pathology or lethal outcomes
479 following bunyavirus infection in arthropod hosts. In line with these observations, IRE/CTVM19
480 and IRE/CTVM20 cells infected by UUKV grew normally without any sign of cytopathic effects
481 for many weeks, as reported previously for other arboviruses propagated in tick cell lines (49).
482 Cells exposed to UUKV remained infected for months, demonstrating the asymptomatic

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483   persistence of UUKV infection in tick cells. This contrasts with the inability of mammalian cells
484   to survive infection after a couple of days. In UUKV, we have found a suitable surrogate to study
485   important emerging tick-borne pathogens such as SFTSV and HRTV in both tick vectors and
486   mammalian host cells, as well as to reproduce host alternation *in vitro*.

487   Previous studies have mainly involved virus stocks produced in mammalian cells. As a
488   consequence, tick-arbovirus interactions are poorly understood at the cellular level, and the
489   characteristics of viral particles produced from ticks are largely unknown. Using our model
490   system to produce UUKV particles with a genome and biophysical properties very close to those
491   of the virus isolated from ticks, we applied immunoblot-based approaches to analyze the
492   envelope glycoproteins G_N and G_C on tick cell-derived progeny virions. To different extents,
493   both UUKV glycoproteins were sensitive to PNGase F treatment. This highlights the presence of
494   classical *N*-glycan structures on tick cell-derived viral glycoproteins, at least in part.

495   After synthesis in rodent cells, all of the eight *N*-glycosylation sites in the UUKV envelope
496   proteins G_N and G_C have previously been found to carry oligosaccharides, mainly high-mannose
497   glycans (17, 23, 29). In the present study, DC-SIGN enhanced infection of human cells by
498   viruses isolated from tick cells, indicating the existence of high-mannose *N*-glycans on tick cell-
499   derived glycoproteins. The sensitivity of G_N and G_C to Endo H confirmed this presence. It is
500   reasonable to assume that tick cell-derived viruses have the capacity to target cells expressing
501   such lectinic virus receptors in the skin dermis, following introduction into humans and other
502   vertebrates by infected ticks, and before subsequent spread throughout the host.

503   The glycoprotein G_N was much less sensitive to PNGase F and Endo H when the virus was
504   isolated from IRE/CTVM19 cells than from BHK-21 cells. In addition, the β-N-
505   acetylglucosaminidase recognized G_N glycosylations only on tick cell-derived virions. Though
506   we cannot completely exclude differential glycosylation processes in tick cells, our data rather
507   support the view that tick cell-derived G_N carries atypical glycans, not recognized by the
508   classical glycosidases PNGase F and Endo H. Very little is known about the nature of
509   oligosaccharides on tick-derived glycoproteins (14, 59). Specific tick glycans may impact the
510   initial infection of human hosts in the skin dermis by broaden the spectrum of potential receptors
511   and target cells or by influencing subsequent entry pathways, especially those mediated by lectin
512   receptors. For instance, DC-SIGN is able to trigger selective signal transduction pathways, which
513   depend on the nature and glycosylation pattern of the captured antigens (60, 61). UUKV can use

514   distinct lectin receptors for entering cells through different mechanisms (22). With regards to
515   this, elucidating the type of glycans synthesized in tick cells and on derivative viruses remains of
516   paramount importance.

517   Both glycoproteins G_N and G_C on virions originating from BHK-21 cells showed molecular
518   features distinct from those on viruses produced in tick cells. G_C differed by one *N*-glycan site,
519   which remained insensitive to Endo H in BHK-21 cells. The glycoprotein G_N had the highest
520   degree of dissimilarity, with striking differences in glycans but also in electrophoresis mobility
521   under reducing and non-reducing conditions. The apparent molecular weight of G_N was lower for
522   viruses produced in IRE/CTVM19 cells than in BHK-21 cells. No deletion in the virus genome
523   segment M, which could explain the translation of a smaller sized protein, was found after
524   several weeks of virus propagation in tick cells (data not shown). Smaller size of glycan units or
525   divergent transcription and maturation processes in tick cells may be responsible for the variation
526   in molecular weight. In addition, that only BHK-21 cell-derived G_N molecules were sensitive to
527   β-mercaptoethanol indicate a difference in the number of di-sulfide bonds between the protein
528   made in tick and mammalian cells. It is likely that the folding of G_N on virions differs depending
529   on whether they are produced in tick or mammalian cells.

530   It is clear from our data that viral particles derived from IRE/CTVM19 and BHK-21 cells
531   exhibit substantial differences in the amount of viral structural proteins, with less nucleoprotein
532   N and more glycoproteins G_N and G_C on virions produced in tick cells. It is tempting to postulate
533   that the level of N protein correlates with the quantity of RNPs and the number of particles, and
534   therefore, that the ratio between ffu and N reflects the ratio between infectious and non-
535   infectious particles. This ratio is known to be low for phleboviruses produced in mammalian
536   cells (23). Though additional experiments are needed to confirm this model, production in tick
537   cells arguably confers an advantage in terms of infectivity to the virus, i.e. with a higher ratio
538   between infectious and non-infectious particles.

539   The ratio between the nucleoprotein N and the glycoproteins G_N and G_C for tick cell-derived
540   viruses significantly differs from that for viral particles produced in mammalian cells. It is
541   reasonable to believe that virions originating from tick cells present an overall structure different
542   from that of the particles derived from mammalian cells. Electron microscopy pictures and
543   tomography-based approaches have shown pleomorphic virions heterogeneous in size for UUKV
544   produced in BHK-21 cells, with spike-like projections of 5-10 nm composed of the two

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545   glycoproteins G_N and G_C (27, 32). Structural analyses of UUKV and other tick-borne viruses

546   obtained from arthropod vector cells are still lacking.

547   As with other bunyaviruses, tick-borne phleboviruses rely on vacuolar acidification for

548   infectious entry into mammalian cells (1, 9, 16, 23). The fusion of the virus envelope with the

549   cell membranes is triggered following the acidic activation of the viral glycoproteins. We found

550   that the presence of NH_4Cl resulted in blocking UUKV infection of DC-SIGN-expressing human

551   cells by tick cell-derived viral particles, and of both tick cell lines by viruses produced from

552   BHK-21 cells. In other words, regardless of the host cells that are targeted and those from which

553   the virus originates, UUKV depends on low pH for penetration and infection. Structural data

554   support the view that the phlebovirus glycoprotein G_C is the fusion protein (class II) (62). The

555   possible roles of G_N in the entry and fusion processes of UUKV and other tick-borne

556   phleboviruses, and also in host alternation, remain to be uncovered.

557   The lipid composition of the viral envelope, adaptive mutations in the virus genome, and the

558   nature of oligosaccharides in the virus glycoproteins on tick cell-derived viruses arguably

559   influence the initial infection in humans and other vertebrates, and subsequent spread throughout

560   the host. Tick cell lines and UUKV provide an interesting, functional model to investigate not

561   only tick-borne phleboviruses, but many other intracellular pathogens transmitted by ticks. The

562   results gained here will open avenues in research on tick vectors, the detailed cell biology of

563   which remains a challenge for future work.

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746 **Figure legends**

747 **Fig 1. Recovery of UUKV from Pol I-driven plasmid DNAs. (A)** The tri-segmented, negative-

748 sense RNA genome of UUKV. The black arrowhead shows the cleavage site in the polyprotein

749 precursor of the glycoproteins G_N and G_C. The black bars indicate the nucleotides found to be

750 mutated in the UUKV laboratory strain relative to UUKV strain 23. The penetrance of each

751 mutation is indicated underneath. Four to five clones were analyzed for each viral genome

752 segment. **(B)** Schematic representation of the Pol I-driven UUKV rescue system. **(C)** Focus-

753 forming assay used for the titration of UUKV strains. Examples are shown for the UUKV lab

754 strain (UUKV) and the viruses rescued from plasmid DNAs after five passages in BHK-21 cells

755 (rUUKV and rUUKV S23). After three days of incubation at 37°C, foci were immuno-stained

756 with the rabbit polyclonal antibody U2 against the viral proteins N, G_N, and G_C. **(D)** rUUKV and

757 rUUKV S23 production 5 days after transfection of plasmids expressing L, M, and S segments

758 under control of the Pol I promotor together with the UUKV L and N expression plasmids

759 pUUK-L and pUUK-N in BHK-21 cells. **(E)** Titer of rUUKV and rUUKV S23 after rescue (P0)

760 and up to five passages (P1-P5) in BHK-21 cells. ffu: focus-forming units. **(F)** Sequence analysis

761 of the rUUKV S23 M segment compared to that of rUUKV carried out from vRNA purified

762 extracts after five passages in BHK-21 cells.

763 **Fig 2. Characterization of UUKV rescued from plasmids. (A)** The UUKV lab strain and

764 rUUKV were analyzed by SDS-PAGE and western blotting under reducing conditions using the

765 rabbit polyclonal antibody U2 against the three structural viral proteins N, G_N, and G_C or **(B)**

766 under non-reducing conditions with the mouse monoclonal antibodies 8B11A3, 6G9E5, and

767 3D8B3 that recognize each of the structural proteins N, G_N, and G_C, respectively. **(C)** BHK-21

768 cells were exposed to the UUKV lab strain or rUUKV at a MOI of 0.1 for 24 hours. After

769 fixation and permeabilization, infected cells were immuno-stained for N, G_N, and G_C with the

770 mouse monoclonal antibodies 8B11A3, 6G9E5, and 3D8B3, respectively, and analyzed by flow

771 cytometry. **(D)** Infection of BHK-21 cells by UUKV and rUUKV was monitored over 64 hours

772 using the flow cytometry-based assay used in C. Infection is given as the percentage of N

773 protein-positive cells. **(E)** Supernatants collected from cells infected at a MOI of 0.1 and at

774 indicated times were assessed for the production of infectious viral progeny by focus-forming

775 assay.

776 **Fig 3. Infection of tick cells by rUUKV and rUUKV S23 is persistent. (A)** Tick cell lines

777 IRE/CTVM19 and IRE/CTVM20 were exposed to BHK-21 cell-derived rUUKV S23 at the

778 indicated MOIs for 48 hours. Infection was analyzed by flow cytometry after immuno-staining

779 against the nucleoprotein N. **(B)** IRE/CTVM19 and IRE/CTVM20 cells were exposed to various

780 MOIs of rUUKV S23 derived from BHK-21 cells. The next day, infected cells were immuno-

781 stained for the intracellular UUKV nucleoprotein N using the anti-N primary mouse monoclonal

782 antibody 8B11A3 and an AF488-coupled anti-mouse secondary monoclonal antibody (green).

783 Nuclei were stained with Hoescht (blue) and samples analyzed by wide-field microscopy. **(C)**

784 and **(D)** IRE/CTVM19 and IRE/CTVM20 cells were exposed to rUUKV S23 (C) and rUUKV

785 (D). 200 µL of supernatant of infected cells was harvested daily during the first ten days, and

786 every ten days thereafter. The production of infectious viral particles in the supernatant was

787 determined by focus-forming assays. The cells were subcultured in fresh complete medium (1:1)

788 after sampling of the parent cells on days 34, 54, and 74 (black arrows).

789 **Fig 4. The C type lectin DC-SIGN enhances infection of human cells by tick cell-derived**

790 **rUUKV S23. (A)** BHK-21 cells were infected (at a MOI of 0.1) with rUUKV S23 derived from

791 IRE/CTVM19 cells for 18 hours and immuno-stained for N, G_N, and G_C proteins prior to flow

792 cytometry analysis. **(B)** Parental and DC-SIGN-expressing Raji cells (Raji and Raji DC-SIGN+)

793 were infected with IRE/CTVM19 cell-derived rUUKV S23 and analyzed by flow cytometry 16

794 hours later after immuno-staining against the viral nucleoprotein. **(C)** Parental and DC-SIGN-

795 expressing HeLa cells (HeLa and HeLa DC-SIGN+) were exposed to various MOIs of

796 IRE/CTVM19 cell-derived rUUKV S23. The next day, infected cells were immuno-stained for

797 the intracellular virus nucleoprotein N using the anti-N primary mouse monoclonal antibody

798 8B11A3 and an AF488-coupled anti-mouse secondary monoclonal antibody (green). Nuclei

799 were stained with Hoescht (blue) and samples analyzed by wide-field microscopy. **(D)** Raji DC-

800 SIGN+ cells were exposed to IRE/CTVM19 cell-derived rUUKV S23 (MOI ~1) in the presence

801 of inhibitors blocking DC-SIGN, namely EDTA (5 mM) or the neutralizing mouse monoclonal

802 antibody mAb1621 (25 µg.mL^{-1}). Intracellular viral antigens were detected by immuno-staining

803 with an anti-UUKV rabbit polyclonal antibody followed by incubation with AF647-conjugated

804 secondary antibodies. Infection was analyzed by flow-cytometry 18 hours later and normalized

805 to infection of DC-SIGN-expressing Raji cells in the absence of inhibitor (% of control).

806 **Fig 5. Glycosylation of the rUUKV S23 envelope glycoproteins G_N and G_C on virions**
807 **produced from tick and mammalian cells. (A) to (F)** IRE/CTVM19 and BHK-21 cell-derived
808 rUUKV S23 purified through a 25%-sucrose cushion was reduced and denatured before
809 digestion with PNGase F (A, B, E, and F), Endo H (C and D), or neuraminidase, β-1,4-
810 galactosidase, and β-N-acetylglucosaminidase (E and F). Proteins were analyzed by SDS-PAGE
811 and western blotting using the rabbit polyclonal antibodies K1224 and K5 against linear epitopes
812 in the viral glycoprotein G_N (A, C, E, and F) and G_C (B and D), respectively.

813 **Fig 6. Electrophoretic mobility of the glycoproteins G_N and G_C on rUUKV S23 produced in**
814 **tick and mammalian cells. (A) and (B)** BHK-21 and IRE/CTVM19 cell-derived rUUKV S23
815 purified through a 25%-sucrose cushion were analyzed by non-reducing SDS-PAGE and western
816 blotting, using the mouse monoclonal antibodies 6G9E5 and 3D8B3 against conformational
817 epitopes in G_N (A) and G_C (B) respectively. **(C)** rUUKV S23 viruses produced in BHK-21 and
818 IRE/CTVM19 cells were purified through a 25%-sucrose cushion and analyzed by non-reducing
819 (-) or reducing (+) SDS-PAGE and western blot with the rabbit polyclonal anti-G_N antibody
820 K1224.

821 **Fig 7. The structural rUUKV S23 proteins G_N, G_C, and N in infectious particles derived**
822 **from tick and mammalian cells. (A)** The amount of viral glycoproteins and N protein for 10^5
823 and 5.10^5 foci forming units (ffu) of purified rUUKV S23, produced in either tick cells or BHK-
824 21 cells, were analyzed by SDS-PAGE and western blot using the rabbit polyclonal anti-UUKV
825 antibody U2, which recognized G_N, G_C, and N. **(B) to (E)** The amount of viral glycoproteins and
826 protein N for identical amounts of purified infectious rUUKV S23 was determined by
827 quantitative western blot (Odyssey Imaging Systems) with the anti-UUKV U2 and an anti-rabbit
828 infrared fluorescence secondary antibody. The ratios of N protein (B) or viral glycoproteins (C)
829 per ffu and the ratios of viral glycoproteins (D) or ffu (E) per relative unit of N protein are
830 shown.

831 **Fig 8. Low pH dependence of rUUKV S23 for infection. (A) and (B)** Raji (A) and HeLa (B)
832 cells that stably express DC-SIGN were pre-treated with NH_4Cl, a weak base that neutralizes the
833 endosomal pH, and then exposed to IRE/CTVM19 cell-derived rUUKV S23 (MOI ~5) in the
834 continuous presence of the inhibitor. Infected cells were harvested 16 hours later and immuno-
835 stained for the UUKV nucleoprotein N. Infection was analyzed by (A) flow cytometry or (B)

31

836   wide-field microscopy counting at least 200 cells in more than three independent fields. Data

837   were normalized to DC-SIGN-expressing cells infected in the absence of inhibitor (% of

838   control). **(C) and (D)** IRE/CTVM19 (C) and IRE/CTVM20 (D) cells were infected with BHK-21

839   cell-derived rUUKV S23 at a MOI of 5 for 36 hours in the continuous presence of NH_4Cl.

840   Infection was analyzed by flow cytometry and normalized against data obtained in the absence of

841   inhibitor (% of control).

842   **Table 1. Names and sequences of the primers used for cloning and mutagenesis.** Lower case

843   nucleotide letters indicate the virus RNA sequence that is targeted while those in capital letters

844   show the sequences introduced for cloning. Underlined nucleotide sequences indicate a BsmBI

845   restriction site. Bold nucleotides are the point mutation introduced in the M segment sequence of

846   the UUKV lab strain. Forw: forward, Rev: reverse, and RT: reverse transcription.

847   **Table 2. Full-length sequence of the viral glycoproteins G_N and G_C obtained from UUKV-**

848   **infected field *Ixodes ricinus* nymphal ticks.** Total RNAs were extracted from 16 pools of 25

849   nymphs collected in the region of Ramsvik and Hindens Rev Sweden in 2013. The cDNA

850   corresponding to the M segment of UUKV was synthesized by RT-PCR and amplified by a

851   single PCR. Out of the 16 nymphal pools, four were positive for the virus, and from these four

852   samples, one partial and two full-length nucleotide sequences could be obtained and. The list is

853   shown of the amino acids found to be mutated in the full-length amino acid sequences of isolates

854   RVS and HRS (GenBank accession numbers KX219593 and KX219594 respectively) relative to

855   the polypeptide precursor of the two glycoproteins G_N and G_C of UUKV S23 (GenBank

856   accession number NC_005220.1). The point mutations in light gray indicate positive

857   substitutions.

Table 1

Primer	Sense	Sequence (5' -> 3')	Purpose
RT-S	Forw.	acacaaagacctccaacttagctatcg	RT S segment
RT-M	Forw.	acacaaagacggctaacatggtaagg	RT M segment
RT-L	Forw.	acacaaagacgccaagatgcttttagcg	RT L segment
UUKV-S-5NC	Forw.	AATCGTCTCTAGGTacacaaagacctccaacttagctatcg	Cloning S segment into pRF108 (pRF108-S)
UUKV-S-3NC	Rev.	AATCGTCTCTGGGacacaaagaccctcc	
UUKV-M-5NC	Forw.	AATCGTCTCTAGGTacacaaagacggctaacatggtaagg	Cloning M segment into pRF108 (pRF108-M)
UUKV-M-3NC	Rev.	AATCGTCTCGGGGacacaaagacacggctacatgg	
UUKV-L-5NC	Forw.	AATCGTCTCTAGGTacacaaagacgccaagatgcttttagcg	Cloning L segment into pRF108 (pRF108-L)
UUKV-L-3NC	Rev.	AATCGTCTCGGGGacacaaagtccgccaagatggaagtaaagg	
Mut-M-S	Forw.	caaggattcagtggattgtc **a** atcatcaatcatagatccca	Mutagenesis M segment (G2386A)
Mut-M-AS	Rev.	tgggatctatgattgatgat **t** gacaatccactgaatccttg	

Table 2

Region (start .. end)	Isolate (name)	Identity (%)	Positives (%)	Substitutions relative to the UUKV S23 polypeptide precursor of G_N and G_C (1,008 amino acids)						
G_N (18 .. 496)	RVS	98.5	99.6	L8I	L29I		S124T	T167S	L207V	A219T
	HRS	97.6	98.7		L29I	T44A	S124T	T167S	L207V	A219T
				T237A						
					S282T	T287A	N476K	N477Q	A479C	
Intra region (497 .. 513)	RVS	100.0	100.0							
	HRS	97.1	97.1	A508T						
G_C (514 .. 1008)	RVS	99.6	100.0		S695T	Q790R				
	HRS	99.2	99.8	K577R		Q790R	T841S	L1003F		

Fig. 1

Fig. 2

A rUUKV S23

B

C rUUKV S23

D rUUKV

Fig. 3

A

BHK-21 cells (SSC-H)

N G_N G_C

No virus

Tick cell-derived rUUKV S23

B

Infected cells (% of total)

- - - - Raji
———— Raji DC-SIGN+

Tick cell-derived rUUKV S23 (MOI)

C

HeLa

HeLa DC-SIGN+

0   1.25   5

50 μm

Tick cell-derived rUUKV S23 (MOI)

D

Raji DC-SIGN+

Infected cells (% of control)

No virus | No inhibitor | EDTA | mAb1621

Tick cell-derived rUUKV S23

Fig. 4

A

Anti-G$_N$

rUUKV S23 produced in

BHK-21   IRE/CTVM19

kDa

62 —

49 —

− + − +

PNGase F

B

Anti-G$_C$

rUUKV S23 produced in

BHK-21   IRE/CTVM19

kDa

62 —

49 —

− + − +

PNGase F

C

Anti-G$_N$

rUUKV S23 produced in

BHK-21   IRE/CTVM19

kDa

62 —

49 —

− + − +

Endo H

D

Anti-G$_C$

rUUKV S23 produced in

BHK-21   IRE/CTVM19

kDa

62 —

49 —

− + − +

Endo H

E

rUUKV S23
from IRE/CTVM19 cells

Mock PNGase F Neuraminidase β-1,4-galactosidase β−N-acetyl-glucosaminidase All enzymes

kDa

62 —

49 —

Anti-G$_N$

F

rUUKV S23
from BHK-21 cells

Mock PNGase F Neuraminidase β-1,4-galactosidase β−N-acetyl-glucosaminidase All enzymes

kDa

62 —

49 —

Anti-G$_N$

Fig. 5

A Anti-G$_N$

rUUKV S23 produced in

B Anti-G$_C$

rUUKV S23 produced in

C Anti-G$_N$

β-mercapto-ethanol

IRE/CTVM19 cell-derived rUUKV S23

BHK-21 cell-derived rUUKV S23

Fig. 6

A

rUUKV S23 (ffu)

IRE/CTVM19 cell-derived   BHK-21 cell-derived

Fig. 7

A IRE/CTVM19 cell-derived rUUKV S23

B IRE/CTVM19 cell-derived rUUKV S23

C BHK-21 cell-derived rUUKV S23

D BHK-21 cell-derived rUUKV S23

Fig. 8