Rift Valley fever virus Glycoproteins, Key to Entry and Control

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Chapter 1

General Introduction

A NOVEL DISEASE IN THE RIFT VALLEY

Rift Valley fever (RVF) was first described as enzootic hepatitis among adult sheep and lambs in 1930 (26). In July of 1930, Daubney and co-workers were sent out to investigate a serious outbreak on a farm in the Rift Valley, on the shores of Lake Naivasha in Kenya (26). In less than two months' time, around 3500 lambs and 1200 ewes had succumbed to this disease. Remarkably, animals were found dead within twenty-four hours after the first signs of illness (38). Mortality rates were extremely high in young lambs (>95%) and in ewes mortality was often associated with abortion.

Daubney and colleagues discovered that the causative agent retained its infectivity after filtration, indicating that the pathogen was a virus (10, 86). Further studies led them to suggest that the virus is transmitted by mosquitoes (26, 77). The disease not only affected sheep, goats and cattle but also humans could be infected. Cases were reported in which people that had been in close contact with infected animals developed a dengue-like fever for up to two days, after which they recovered. Inquiries among farmers and shepherds indicated that some had developed a strong fever and had been seriously ill for a couple of days around the peak of the outbreak (26). The symptoms in humans were reproduced by inoculating a volunteer with the filtered agent (38).

The virus was brought to the Wellcome physiological research laboratories in the United Kingdom to investigate the relation between this new zoonotic virus, yellow fever virus and dengue virus, which seemed to share similarities (25). At that time it had been shown that monkeys, previously infected with dengue virus, were subsequently immune to yellow fever virus (29). Although infection could be established in a range of different animals including monkeys, no such cross-protection was found for Rift Valley fever virus (RVFV). In 1932 the RVFV was considered to be a trivial disease with limited geographical distribution. Nobody could foresee the economic and social problems RVF would cause in the years to come.

THE SPREAD OF RIFT VALLEY FEVER VIRUS

In 1944, for the first time, RVFV was isolated from mosquitoes caught in the wild in the Semliki forest of Uganda, supporting the previous suggestion of Daubney and co-workers (122). Mosquitoes could also be experimentally infected, but establishing transmission to susceptible hosts was found to be challenging (44, 122, 123, 139). It is

now well established that transmission of RVFV from animal to animal occurs via mosquito vectors, whereas human cases are generally attributed to contact with aerosols, handling of RVFV infected tissues or by ingesting RVFV contaminated products (7, 18, 41, 68, 126, 148, 151).

RVF outbreaks are generally preceded by heavy rainfall (78), which causes flooding of large grassland areas named dambos. These dambos are the habitat of floodwater mosquitoes of the genus *Aedes*, which are believed to vertically transmit the virus to the eggs (14, 27, 81). These eggs can remain viable during long periods of drought and, since no natural mammalian host was ever identified, are therefore believed to maintain RVFV during inter-epidemic periods (36, 79, 141).

Before the first generation of RVFV vaccines were available, the best way of protecting flocks of sheep against RVF during the lambing season was to move the animals to areas of high-altitude, were limited or no mosquito vectors are present (28). Although this strategy has proven to be effective, occasionally RVF outbreaks are reported in mountainous areas. In 1951, for example, a RVF outbreak in cattle was reported at an altitude of 2286 meter above sea level. Interestingly, this epizootic was accompanied by an enormous increase in the rat population (28). Daubney and co-workers had made a similar observation during the RVFV outbreak of 1930 (34, 25).

Human infections

Human clinical cases are characterized by a sudden onset of high fever, severe headache and myalgia. In a small percentage of cases, the infection results in macular retinitis and can progress to meningoencephalitis or hemorrhagic fever with often fatal outcome (39, 49, 67, 88). Over the years, the human case-fatality rates were estimated at 0.5–1.0%. More recent outbreaks however show considerably higher numbers (84, 146). Vertical transmission of RVFV in humans (1), and transmission via raw milk (5), have also been reported.

Epidemiology

RVFV comprises only one serotype and seven genetic lineages. The low genetic diversity of RVFV suggests a relatively recent ancestor (106). This assumption was reinforced by “the most recent common ancestor calculation” (13). The putative emergence of RVFV around the 1900’s coincided with the establishment of colonial agriculture systems and the importation of large numbers of European livestock. Taken together, it was hypothesized that an ecological niche at the beginning of the

previous century was exploited by the viral ancestor creating the zoonotic pathogen today known as RVFV (106).

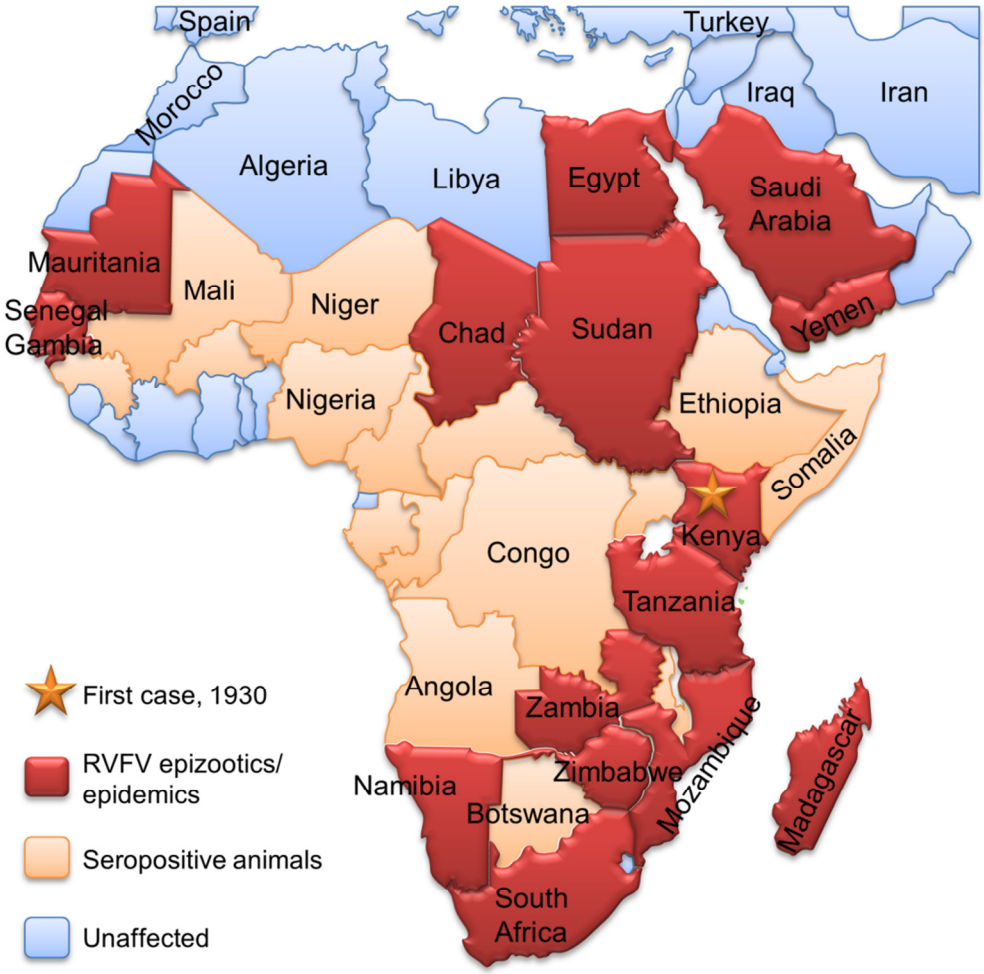

Fig.1. Geographic distribution of RVFV. RVFV was first isolated in 1930. RVFV has since spread throughout sub-Saharan Africa (1950-2008) and Egypt (1977). In 1979 the virus was detected for the first time on the island of Madagascar, demonstrating the ability of the virus to migrate across large distances. Exceptionally high case fatality rates among humans were reported in Egypt (1977), Saudi Arabia and Yemen (2000). Areas with frequent outbreaks are colored in red. Countries with seropositive animals and occasional virus isolation are colored in orange. Areas where RVF is not yet reported are depicted in blue (www.CDC.gov/).

Since its isolation in 1930, RVFV has caused large recurrent outbreaks across the African continent. Interestingly, three large outbreaks of RVF required the

crossing of major physical barriers. The first of these so-called "virgin soil" epidemics, occurred in Egypt between 1977-'78, after the virus had crossed the Sahara desert (43). This epidemic involved ~18 000 human cases, resulting in 30% mortality. The devastating number of fatal cases of this outbreak might be explained by an increase in virus virulence. It was also hypothesized that the affected people were more susceptible to RVFV because of previous liver damage caused by a local endemic disease named schistosomiasis. Due to the hepatotropic nature of RVFV, liver damage might contribute to more acute disease in patients (89). In 1979, RVFV was isolated for the first time outside of mainland Africa, across the Indian Ocean, on the island of Madagascar (94-96). Two decades later, the virus crossed the Red sea into Saudi Arabia and Yemen. During this epidemic, 156 people were hospitalized of which 56 patients (34%) succumbed to the infection (2). These and many other large outbreaks that occurred across the African continent have demonstrated that the virus has a remarkable ability to adapt to new environments (Fig. 1) (39). Therefore, it is important to have control tools available to protect farmers and their livestock both inside and outside endemic areas.

THE BUNYAVIRIDAE FAMILY

RVFV belongs to the *Bunyaviridae* family, which is the largest family of RNA viruses, containing over 350 named members. This family of viruses was established in 1975 after the discovery of a collection of antigenically related viruses (98, 112), including Bunyamwera virus (BUNV) the prototypic member (112, 124). Bunyaviruses are transmitted by insect vectors or rodents and are categorized into four genera comprising animal-infecting viruses (*Orthobunyavirus, Phlebovirus, Hantavirus* and *Nairovirus*) and one genus that contains plant infecting viruses (*Tospovirus*) (Table 1) (112). Several of these viruses are responsible for serious recurrent outbreaks and are considered emerging pathogens.

Rift Valley fever virus is a member of the genus *Phlebovirus* within the family *Bunyaviridae*. Except for RVFV, which is transmitted by mosquitoes, the viruses of this genus are transmitted by Phlebotomine sandflies or ticks (Table 1).

Table.1. Selection of the most common *Bunyaviridae* members (32, 116)

Genus	Virus	Host	Vector	Distribution
Orthobunyavirus	Bunyamwera	Human	Mosquito	Africa,
	La Crosse	Human	Mosquito	North America
	Akabane	Cattle, sheep	Midge	Africa, Asia, Australia
	Oropouche	Human	Midge	South America
Hantavirus	Hantaan	Human	Field mouse	Asia, East Europe
	Puumala	Human	Bank vole	West Europe
	Sin Nombre	Human	Deer mouse	North America
Nairovirus	Nairobi sheep disease	Sheep, Goat	Tick	Asia, Africa
	Crimean-Congo haemorrhagic fever	Human	Tick	Asia, Africa, East Europe
Phlebovirus	Rift Valley fever	Ruminants, Human	Mosquito	Africa, Arabian peninsula
	Sicilian sandfly fever	Human	Sandfly	Africa, Europe
	Toscana	Human	Sandfly	Europe
	Punta toro	Human	Sandfly	North/ South America
	Uukuniemi	Seabirds	Tick	Europe
Tospovirus	Tomato spotted wilt	Plants	Thrips	Worldwide

PROTECTION AGAINST RIFT VALLEY FEVER

Registered and experimental RVFV vaccines

RVFV is recognized as a serious pathogen due to the severity of the outbreaks and proven ability to rapidly adapt to new environments (7, 147). This explains the recognition of RVFV as a Category A agent by the Centers for Disease Control and Prevention and the United States Department of Agriculture and its classification as an emerging notifiable zoonotic viral disease by the World Organization for Animal Health *(*Office International des Épizooties [OIE]) (3). Biosafety level-3 (BSL-3) facilities are required to work safely with the virus. The above mentioned facts highlight the need for protective measures like vaccines and antiviral drugs.

Although no human vaccines are available, two veterinary vaccines for the protection of ruminants are commercialized, both produced by Onderstepoort Biological Products (OBP, Onderstepoort, South Africa). The first is an aluminum hydroxide gel-adjuvanted vaccine that is based on formalin-inactivated virus. This vaccine is expensive to produce and requires a revaccination at 4-6 weeks and yearly

booster vaccination to acquire and sustain the level of protection (8, 9). The second vaccine is based on a live virus that was attenuated by passage in suckling mouse brain (125). This attenuated RVFV strain named "Smithburn" virus was further attenuated in mice and embryonated eggs before it was extensively used as a live-attenuated vaccine to protect livestock. The Smithburn vaccine is highly effective but concerns remain about residual virulence. It can cause abortions and foetal malformations in a small percentage of vaccinated animals, particularly sheep (15, 21). Recently, an additional vaccine has become available for the vaccination of animals older than 2 months of age (OBP, South Africa). This live vaccine is based on a human isolate with a natural 549-nt deletion in the NSs gene rendering the virus highly attenuated. This so-called "Clone 13" vaccine was shown to be safe in pregnant animals and provides good protection (30, 142). A previous study, however, described neurological disorders and paralysis in Clone 13-vaccinated mice (140), suggesting that the Clone 13 virus is not completely avirulent.

RVFV consists of just one serotype and neutralizing antibodies are the only established correlate of protection against RVFV infection, rendering vaccine design fairly straightforward. In recent years, much effort has been made to develop cost-effective vaccines that optimally combine efficacy and safety using various strategies, including subunit (117), DNA (128), virus vector-based (143), live-attenuated virus (12, 20, 92, 93, 97) and inactivated (110) whole virus-based vaccines.

Antivirals

Antiviral drug therapies are most effective when administrated shortly after virus exposure, but can potentially also be effective if administered after onset of the first signs of disease. Different anti-RVFV drugs have already been identified and tested *in vitro* (16, 45, 56) and *in vivo* (107). The results of these preliminary experiments are promising, although the protective efficacy and safety of these drugs still need to be extensively evaluated to exclude untoward effects. The drug ribavirin, for example, was used to treat patients that suffered from RVF during the outbreak in Saudi Arabia in 2000 (2). Treatment with this drug successfully limited the hemorrhagic symptoms in patients with RVF, but did not prevent the neurological symptoms, including hallucinations, lethargy and coma (16). Ribavirin treatment was terminated before the end of the Saudi Arabian outbreak.

Novel drugs that inhibit the function of essential host proteins that support virus replication need to be identified. These proteins are attractive targets for antiviral drug development, since it is difficult for a virus to overcome a drug-induced

inhibition of a critical host factor. Via such a host-oriented approach broad-spectrum inhibitors might be developed, since viruses of the same family share many aspects of the viral life cycle. It is therefore important to study the viral life cycle of viruses to be able to identify these host targets. Once these targets are identified, candidate drugs can be selected, either from approved drug libraries, or by designing the compound at the molecular level (31). This strategy might yield new products that can be used as effective therapies in the battle against pathogenic viruses like RVFV.

VIRUS STRUCTURE, GENOME AND REPLICATION

Architecture of the virion

Ultrastructural studies demonstrate that RVFV particles are ~100nm in size and have a unique icosahedral structure. The outer surface of RVFV virions is made up of oligomers of two glycoproteins named Gn and Gc, which extend ~12nm from the viral envelop. The Gn (47-kDa) and Gc (51-kDa) glycoproteins are the building blocks of 110 hexamers and 12 pentamers, together 122 capsomers, which are organized in a T = 12 lattice (Fig. 2) (42, 77).

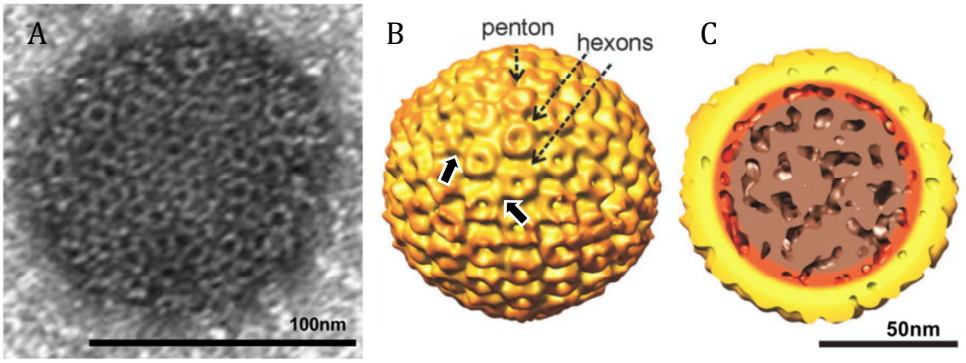

Fig.2. RVFV particle morphology. (A) Negative-stain transmission electron microscopy (TEM) micrograph showing spherical RVFV particles, at neutral pH, revealing a distinct surface structure made up of "doughnut"-shaped structures. (B) Representation of RVFV reconstructed from tomograms at 7.5nm resolution generated by averaging data. The individual doughnut-shaped morphological units have a central cavity in each of the pentameric or hexameric capsomers (indicated by the dotted arrows). Bridging densities between capsomers are also visible (solid arrows). (C) Inside view of the RVFV cryo-EM structure. Reprinted by permission from American Society for Microbiology, Publisher of Journal of Virology (Freiberg, A. N., M. B. Sherman, M. C. Morais, M. R. Holbrook, and S. J. Watowich. 2008. Three-dimensional organization of Rift Valley fever virus revealed by cryoelectron tomography. J Virol 82:10341-10348.), copyright (2008).

Bridging densities between the individual capsomers, observed in cryo-electron tomography analysis, might explain the stable structure of the RVFV particle (42). These bridging densities have also been observed for Uukuniemi virus (UUKV) (101). In contrast to other RNA viruses, bunyaviruses lack a matrix protein (33) that connects the glycoproteins with the viral core, in accordance therewith the cytoplasmic tail of Gn seems to directly interact with the ribonucleoproteins (RNPs) (102, 109).

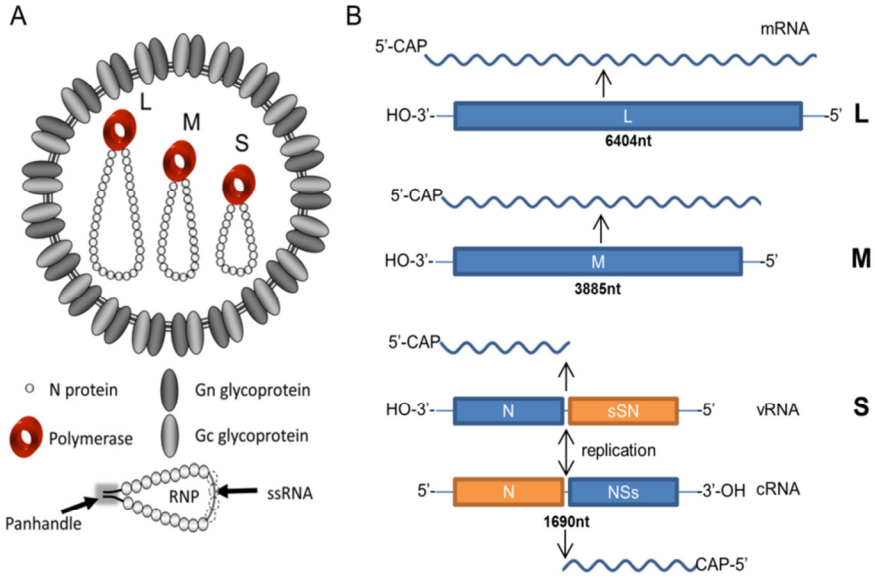

Fig.3. The RVFV virion and transcription strategy (A) Schematic representation of a RVFV particle with the Gn and Gc glycoproteins anchored in the viral envelope. The three genome segments S, M and L are encapsidated by the nucleoprotein (N) into ribonucleoproteins (RNP) and associate with the viral polymerase. The terminal ends of the genomic RNA segments are complementary allowing base-pairing and the formation of a panhandle structures. (B) The RVFV genome consists of three single-stranded RNA segments of which the S segment is of ambisense polarity. The viral polymerase is responsible for primary transcription of the negative-sense RNAs and subsequent replication of all three genomic segments. Blue = genomic sense, orange = antigenomic-sense.

Genome organization

The RVFV genome (~12kb) consists of a tri-segmented single-stranded RNA genome of negative and ambisense polarity. The three virion RNA (vRNA) molecules, designated small (S), medium (M) and large (L) (Fig. 3A), are used as templates to generate complementary RNA (cRNA) and messenger RNA (mRNA). The M segment encodes two glycoproteins, Gn and Gc, and two proteins of 14-kDa and 78-kDa in

mass, both proposed to be non-structural (23, 69). Of note, there are also indications that the 78-kDa protein is a minor constituent of the virion (69, 135). The L segment codes for the RNA-dependent RNA polymerase (RdRp) (Fig. 3B). The S segment uses an ambisense expression strategy, encoding the nucleoprotein (N) in genomic-sense and a non-structural protein (NSs) in the antigenomic-sense orientation (Fig. 3B*).*

The bunyavirus replication cycle

The infection cycle of bunyaviruses can be divided into three events (i) attachment, uptake and fusion, (ii) primary transcription, translation and replication, and (iii) virus assembly and release (Fig. 4). The first step of a productive infection involves the interaction between the virus and the cell surface. This virus-cell interaction is initiated by binding of the virus to specific attachment factors or cell surface receptor(s). Based on available data we assume that after binding, bunyaviruses are taken up by the cell through receptor mediated endocytosis (57, 65, 115, 116). After uptake, the virus is likely to be trafficked along the endocytic pathway towards the perinuclear-localized degradative lysosomes (116). In general, to avoid lysosomal degradation, enveloped viruses escape from this endocytic pathway by fusion of the viral envelope with the endosomal membrane, exposing the particle content to the cytoplasm before it is degraded in lysosomes.

Primary transcription will start after release of the viral genome into the cytosol. In the case of Bunyamwera virus, RNA replication and RNP assembly has been suggested to take place in so-called viral factories. These structures are composed of Golgi membranes, actin and viral proteins and are associated with the rough endoplasmic reticulum and mitochondria that provide essential cellular factors (40, 99). This means that RNA replication, RNP formation and virus assembly all occur adjacent to, or at the Golgi apparatus. These viral factories have not been described for other bunyaviruses. The progeny virus particles egress from the cell through the cellular secretory pathway (Fig. 4).

Replication strategies and viral protein functions

RVFV ribonucleoprotein is composed of viral RNA encapsidated by numerous copies of the N protein (Fig. 3A). The terminal ends of the RVFV RNAs are complementary and due to 5’ and 3’-end base-pairing are shaped like a panhandle (63) (Fig. 3A). This structure is not unique to the bunyavirus family but is also reported in other RNA virus families (62).

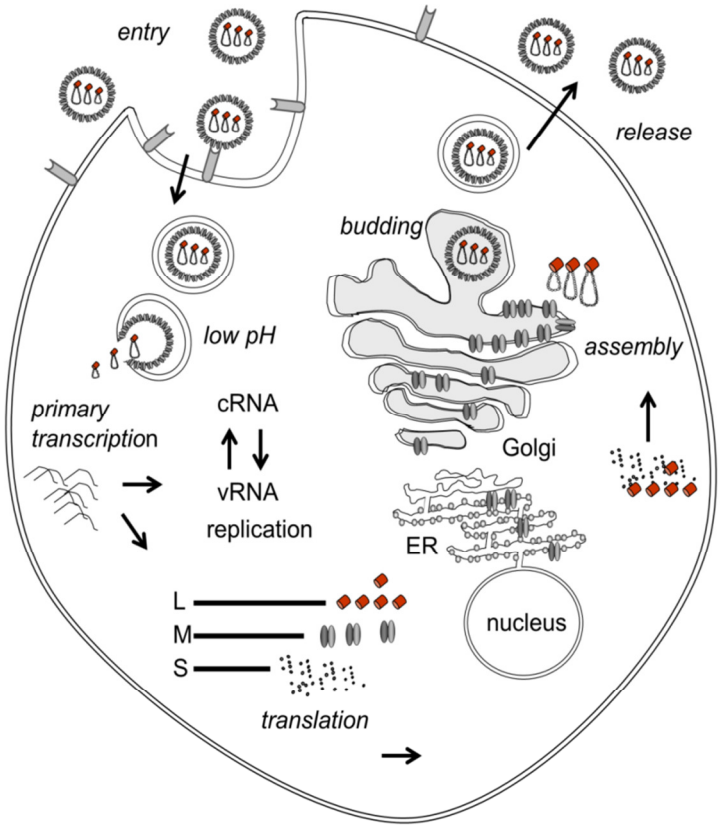

Fig.4. A model of the RVFV replication cycle. RVFV enters the cell by receptor-mediated endocytosis. The low pH in endosomal compartments triggers fusion of the viral and endosomal membrane. The viral genome is released into the cytosol, where primary transcription of the genomic-sense RNA (vRNA) into mRNA is initiated by the ribonucleoprotein (RNP)-associated RNA polymerases. Messenger RNAs transcribed from the L, M and S segments are translated and viral proteins accumulate. Complementary RNA (cRNA) serves as template for vRNA replication and *vice versa*. In Golgi-associated structures newly formed vRNAs are encapsidated by the nucleocapsid protein and associate with the polymerase protein, resulting in the formation of RNPs. Upon synthesis the structural glycoproteins Gn and Gc exit the ER and accumulate in the membranes of the *trans*-Golgi network where RVFV particles are assembled through budding. Newly formed particles subsequently travel from the Golgi to the plasma membrane where they are released.

The single stranded RNA genome of RVFV is complementary to mRNA and therefore non-infectious. In order to commence infection, translation-competent RNA strands are generated by virion associated viral RNA-dependent RNA polymerases (RdRp) (Fig. 3B). The RVFV RdRp is responsible for transcription and replication and thereby plays a central role in viral RNA synthesis. Viral mRNA synthesis is initiated

by a "cap-snatching" mechanism (105, 116). In this process, the viral polymerase cleaves off short capped-primers of cellular mRNAs by its endonucleolytic activity. These host-derived capped-primers serve as transcription initiation sites through limited base-pairing with the vRNAs (66, 73, 104). Guanine- or purine-rich secondary RNA structures that reside within the UTR or intergenic region (S segment) serve as 3'-termination signals (22, 35, 55, 63, 120).

At some point during infection, the viral RdRp changes from cap-primed mRNA synthesis to unprimed vRNA replication. The mechanism that initiates this switch is unknown, though it has been suggested that the concentration of nucleoprotein plays a role in this process. RVFV N protein is involved in RNA packaging and essential for genome transcription and replication. For these reasons it is assumed that RNA encapsidation by N stimulates genome replication (116).

The vRNA's that result from replication of cRNA intermediates are directly encapsidated by the N protein protecting the RNA from degradation and preventing innate immune responses. Surprisingly, besides genomic vRNA, it has been reported that antigenomic cRNAs are also packaged in RVFV particles (64). The antigenomic S segment seems to serve as template for the synthesis of the NSs mRNA, independent of viral mRNA synthesis (64). By this strategy, the NSs gene is expressed early in infection suggesting that this protein plays an important role, already in the primary stages of infection. The NSs protein is a multifunctional suppressor of the host innate immune response, and is the only RVFV protein found in the nucleus, were it forms filamentous structures (137). NSs strongly antagonizes type-I interferon production (11, 17), inhibits cellular RNA synthesis (76) and blocks the antiviral response of host cells through interaction with nuclear localized Sin3A-associated protein 30. A deletion of the NSs gene renders the virus avirulent (97). Downregulation of the host defense by NSs promotes efficient replication of RVFV in the host cell.

The M segment codes for an mRNA that contains five in-frame AUG codons, of which four are used for synthesis of proteins (136) (Fig. 5). Interestingly, only the fourth start codon is in the context of an optimal translational initiation Kozak sequence (74) and translation seems not to commence from the third methionine. The 78-kDa protein is translated from the first AUG codon, combining the complete pre-Gn and Gn coding sequences. This protein has a so far unknown function (Fig. 5). Translation from the second AUG codon results in a 14-kDa non-structural protein that seems to suppress virus-induced apoptosis (150) (Fig. 5). Both proteins are non-essential for replication of RVFV in cell culture, suggestion that these proteins have a function *in vivo* either in the mammalian or insect host (50, 149). Translation

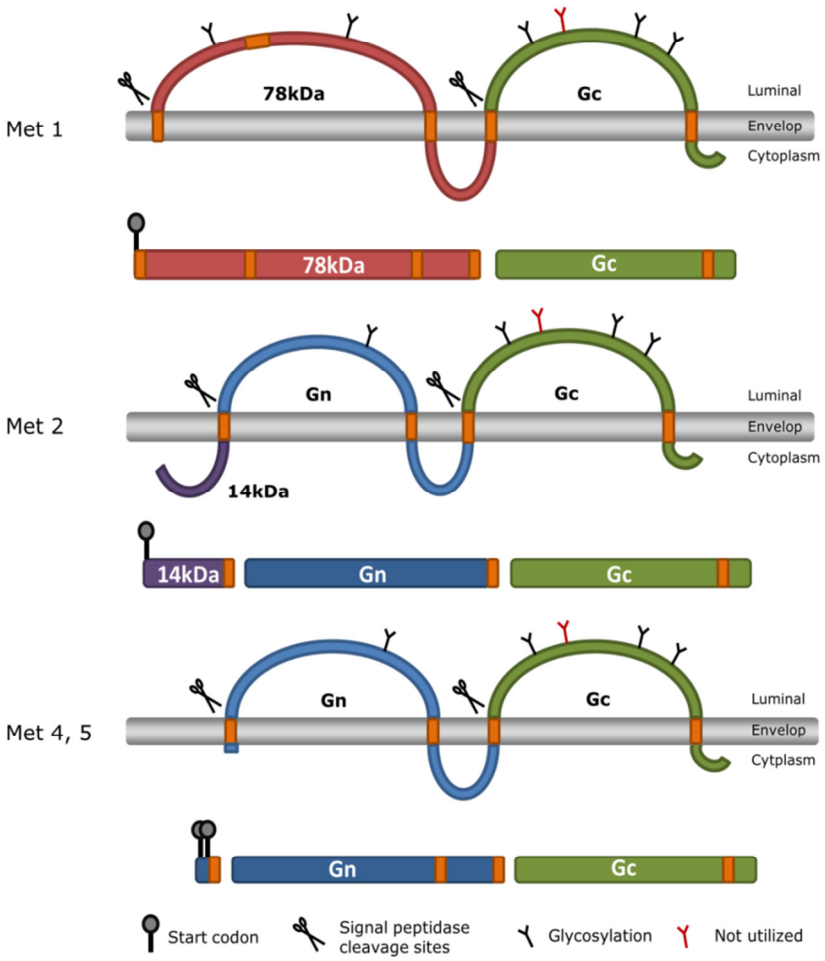

Fig.5. Membrane topology and proteolytic processing of RVFV polyproteins. Membrane topology of the RVFV M-segment-encoded polyproteins. Shown are the translation products starting from the in-frame AUG codons 1,2 and 4/5 of the open reading frame. The ´Y´ symbols indicate the predicted N-linked glycosylation sites. The red ´Y´ symbol is known not to be utilized. The 14-kDa, 78-kDa, Gn and Gc proteins are colored in purple, red, blue and green, respectively. The orange blocks represent the predicted transmembrane spanning regions.

from the fourth and fifth start codons results in a polyprotein precursor that is co-translationally cleaved into Gn and Gc (Fig. 5) (52). Both newly synthesized proteins interact in order to be transported from the ER to the Golgi, by virtue of a Golgi-localization signal in Gn (51, 136). Eventually the Gn-Gc heterodimers accumulate in the membranes of the *trans*-Golgi network, awaiting the final step in particle assembly (Fig. 4). In all probability, interactions between the Gn cytoplasmic tails and viral RNPs drive the inward budding, the start of particle formation (102, 103).

RVFV ENTRY IN MORE DETAIL

Virus binding

Before binding to its cognate receptor, many viruses first interact with cellular attachment factors that are abundantly expressed on the cell surface. The function of these often low-affinity interactions is to concentrate virus particles on the cell surface thereby promoting higher affinity interactions with the dedicated receptor and co-receptors. These high-affinity interactions actively promote entry by inducing conformational changes in the viral proteins and/or through signal transduction pathway activation. Two cellular receptors of bunyaviruses have so far been identified, the β3 integrin for hantavirus (48) and DC-SIGN for the phleboviruses UUKV and RVFV (82). DC-SIGN is primarily found on the surface of dendritic cells. The broad host range of most bunyaviruses suggests that besides DC-SIGN other surface molecules mediate entry into the cell.

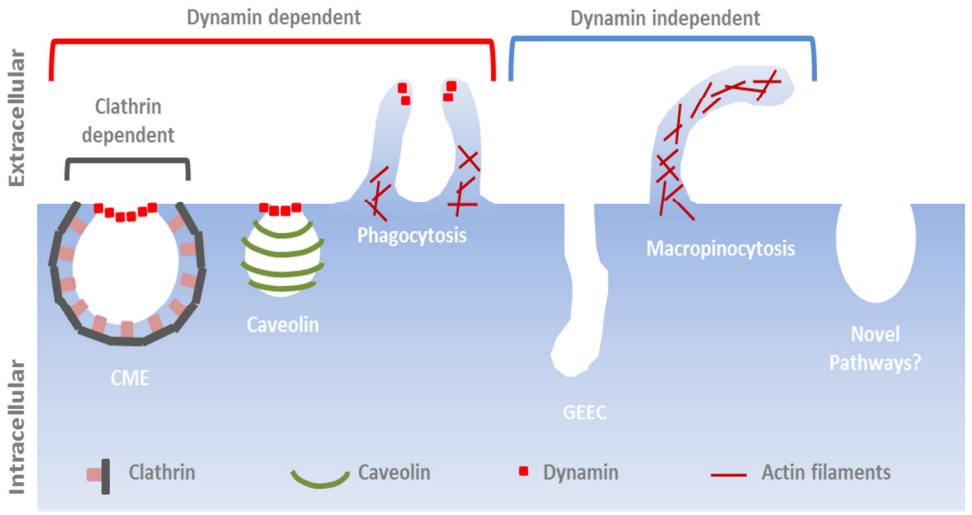

Fig.6. Cellular endocytic mechanisms. Endocytosis in animal cells can be divided into dynamin-dependent and dynamin-independent pathways. Dynamin assists in the process of endocytosis by pinching off vesicles. Clathrin-mediated endocytosis (CME) is the most studied endocytic pathway which involves the formation of clathrin-coated pits. Entry via the caveolar pathway is cholesterol-dependent. Macropinocytosis is an actin-dependent form of endocytosis normally involved in fluid uptake. Large particles can be taken up by phagocytosis, which is a property of a few specialized cell types. GPI-anchored proteins and the fluid phase can also enter the cell via tubular invaginations named GEECs (GPI-anchored protein-enriched early endosomal compartments) (85, 87, 118).

Virus endocytosis

Enveloped viruses can penetrate the cell at the plasma membrane, but most viruses first undergo endocytosis. They are either internalized by ongoing cellular endocytosis, or induce their uptake by activating or stimulating cellular signal transduction pathways through virus-receptor interactions (90, 118). By trafficking along these endocytic routes viral proteins are not left behind on the plasma membrane, reducing immune recognition of infected cells. Concomitantly, viruses hitch a ride through the cell cortex and crowded cytoplasm (127) and are able to traffic to specific locations in the cell. Endocytosis in animal cells can occur via several different mechanisms among which clathrin- or caveolin-mediated endocytosis, or macropinocytosis (Fig. 6) (for reviews on this subject 24, 85, 87, 105, 118). The entry pathway of bunyaviruses has not been extensively studied. In case of Hantaan virus, Oropouche virus and Crimean-Congo hemorrhagic fever virus entry seems to occur through clathrin mediated endocytosis (65, 115, 119).

When moving into the degradative branch of the endocytic pathway, viruses get exposed to a variety of endosomal factors (*e.g.* low pH, endosome resident lipids and proteolytic enzymes) that may trigger penetration.

THE MECHANISM OF MEMBRANE FUSION

Triggers for fusion

Host cell penetration by enveloped viruses occurs via membrane fusion, executed by specialized viral fusion proteins anchored in the viral envelope (58). These fusion proteins are generally folded in a metastable energy state. The metastable energy barrier can be overcome by fusion protein-specific triggers. These triggers can activate the fusogenic functions by initiating conformational changes in the fusion proteins' metastable prefusion state leading to the formation of a stable postfusion structure (59). The signal that acts as the trigger ensures that the critical fusion event occurs in the right place at the right time. The two common cues that trigger fusion proteins are receptor binding and proton binding (the latter in the acidic environment of endosomes), or a combination thereof (144).

Previous work on bunyaviruses has suggested that exposure to low pH plays an important role during the course of infection (4, 100, 111). In accordance to this, low pH exposure of cells that overexpress the RVFV glycoproteins result in extensive cell-cell fusion (37). The optimal pH to trigger RVFV fusion is estimated to be ~pH 5.4, as

determined in a cell-cell fusion assay (37). This low pH corresponds to the acidic milieu of late endosomal compartments (91).

Classes of viral fusion proteins

Viral fusion proteins are subdivided into three classes (class I, II and III) based on their structural features. In the prefusion state, these fusion proteins can assemble into dimers or trimers, but in the post fusion state are characterized by a typical homotrimeric arrangement. Structurally, the post fusion form of Class I fusion proteins consists of trimeric hairpins that contain a central helical coiled-coil structure, whereas class II fusion proteins are characterized by trimers of hairpins composed of beta structures (59, 75, 121, 133). A third class of fusion proteins, that has recently been added, combines the structures of both class I and class II fusion proteins (6, 60, 114, 131). The trimeric hairpins of class III fusion proteins consist of a central α-helical trimeric core and two fusion loops. These fusion loops are located at the tip of an elongated β-sheet resembling class II fusion proteins (59, 113, 144). Remarkably, in the case of the vesicular stomatitis G and baculovirus gp64 class III fusion proteins the low-pH induced-conformational change is reversible, indicating that the native state of these fusion proteins is not metastable (6, 47, 113, 154).

RVFV Gc: A PUTATIVE CLASS II FUSION PROTEIN

Proteomic computational analyses, structural modeling and mutational analyses suggest that the bunyavirus Gc glycoprotein fits the class II fusion protein characteristics (46, 111, 138). From these studies it became clear that RVFV Gc has structural similarities with alpha- and flavivirus fusion proteins and that similar motifs, like a hydrophobic segment predicted to correlate with a fusion peptide, are located in equivalent positions on the bunyavirus Gc, and alphavirus E1 fusion protein (46). Similar to class II fusion proteins of alphaviruses and flaviviruses (70), RVFV glycoproteins form dimers in the ER and seem to utilize low pH to activate the fusion process as suggested by preliminary data (37).

Based on structural, biochemical and functional data of the alpha- and flavivirus fusion proteins, a membrane fusion model for class II fusion proteins has been proposed (Fig.6) (59, 71, 145). In this model, membrane fusion by class II fusion proteins is initiated by proton binding in the acidic endosomal environment, triggering the alphavirus E2-E1 heterodimers or flavivirus E-E homodimers to dissociate (Fig. 7A) and to expose the membrane-interacting fusion loop. The

conformational change is followed by the insertion of the fusion peptide in the target membrane which, depending on the virus, may require the presence of cholesterol in the target membrane (Fig. 7B/C) (70, 72, 83, 108, 132, 134). After insertion, the fusion protein trimerizes and folds back like a jackknife. During this fold-back process domain III and the stem region of the fusion protein move toward the target membrane-associated viral fusion loop (Fig. 7D/E). The stem and domain III segments fold back against domain II and, concomitantly, drag the transmembrane domain and fusion loops into close proximity. Due to these pulling forces, both the viral and host lipid bilayers get distorted and move closer, ultimately leading to lipid mixing and hemifusion stalk formation (Fig. 7F). The final step, the creation of a fusion pore, is achieved through the concerted action of multiple homotrimers that form a ring and ultimately the pore (Fig. 7G) (53, 54).

Besides similar fusion characteristics, the structures of the bunyavirus pre- and postfusion state need first to be resolved before the above described membrane fusion cascade can be verified to apply also for bunyaviruses.

Premature (in)activation of fusion proteins

Low pH-dependent viruses somehow have to solve the problem of premature activation of their acid-sensitive fusion proteins in the acidic compartments of the biosynthetic transport pathway (19, 80, 152). The acidity in the exocytic organelles has been reported to be as low as pH 5.5 (19). Premature activation would trigger irreversible conformational changes in the fusion proteins leading to inactivation (130). To overcome this barrier, low-pH triggered enveloped viruses, like flavi- and alphaviruses, have established sophisticated ways to prevent premature fusion activation during exocytosis (58, 61, 153).

In the case of flaviviruses, premature activation is blocked by a scaffolding protein that shields the immature precursor membrane protein envelope complex (prM-E) during Golgi-to-plasma membrane transport. Digestion of the prM by furin in the slightly acidic Golgi compartments, cleaves the “pr” domain from the M domain, leaving both parts non-covalently attached (129). In the final step of maturation the “pr” domain is released upon exposure to the neutral pH of the extracellular space, rendering the virus glycoproteins fusion competent (153). Viruses produced from furin-deficient LoVo cells yield immature particles that are non-infectious, confirming the importance of the proteolytic processing for infectivity. The polyproteins of RVFV do not seem to undergo such a proteolytic priming event. It is unknown how premature activation of the RVFV fusion protein is prevented.

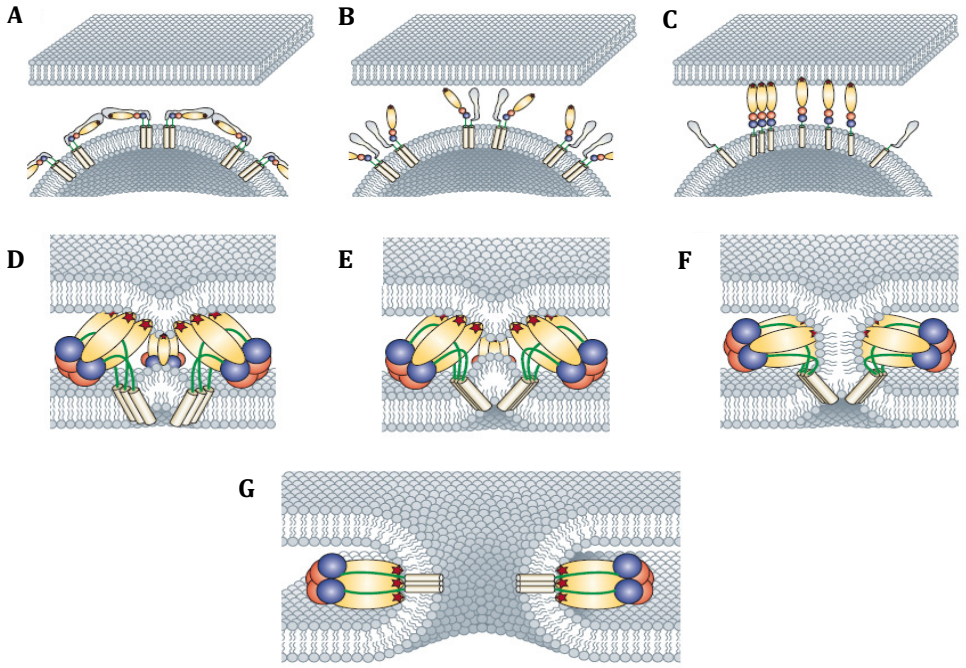

Fig.7. Class II membrane fusion model (Alphavirus). (A) Alphavirus virion, showing E1 in colors and E2 in light grey interacting with E1 covering the fusion loop. (B) The low pH triggers E1–E2 dissociation and exposure of the fusion loop. (C) Low-pH- and cholesterol-dependent insertion of the fusion loop, leading to the alignment of E1 subunits parallel to each other, favouring trimerization. (D) Fold-back process: domain III and the stem region move towards the fusion loop. Cooperative interactions between trimers through their fusion loops distort the target membrane. (E) Folding of the domain III and stem segments against the body of the trimer pulls the membrane interacting segments (TM and fusion loops) towards each other, distorting the viral membrane. (F) Opposing dome-like deformations in the two membranes lead to mixing of the outer leaflets (hemifusion). (G) To reach the final stable post- fusion conformation, the TM segments get closely juxtaposed to the fusion loop. This is only possible by opening an initial fusion pore. Reprinted by permission from Macmillan Publishers Ltd:Nature Reviews Microbiology, (Kielian, M., and F. A. Rey. 2006. Virus membrane-fusion proteins: more than one way to make a hairpin. Nature reviews. Microbiology 4:67-76.), copyright (2006).

THESIS AIM AND OUTLINE

RVFV is a serious pathogen that has a demonstrated ability to spread across large distances. Introduction of this virus into Europe, America or Asia could have a significant impact on veterinary and human health with serious socio-economic consequences. Farmers and veterinarians in and around endemic areas are in need of affordable and safe vaccines that induce long term protective immunity in livestock, preferably after a single vaccination. Vaccine stockpiles should be established in countries at risk.

The aim of the work described in this thesis was to develop control measures to protect livestock against RVFV infection. We have studied the RVFV glycoproteins, which are exposed on the surface of the virus and are the first targets of the host immune defence system upon infection. Antibodies directed against the Gn and Gc glycoproteins are able to neutralize the virus, which is the only established correlate of protection against RVFV infection. We have developed and characterized different veterinary vaccines that are considered safe for animals of all ages and evaluated their efficacy in mice and sheep animal models. We have also studied the RVFV entry mechanism and paid specific attention to the fusion process, in order to identify virus and host targets that can be explored for the development of antivirals. The results of our studies are described in the following chapters:

Chapters 2 and 3

In chapters 2 and 3 we describe the development of three experimental vaccines. In chapter 2 we evaluate two subunit vaccines, the first is based on the purified ectodomain of the RVFV Gn protein (Gn-e) and the second comprises both the RVFV Gn and Gc glycoproteins in the form of virus-like particles (VLPs). Both candidates were developed using the *Drosophila* expression system. In chapter 3 we assess the protective efficacy of a recombinant Newcastle disease virus (NDV) vaccine virus that produces both RVFV structural glycoproteins. We have studied the efficacy of these experimental vaccines in a lethal mouse model and demonstrate that a single vaccination with the NDV vector vaccine induces a neutralizing antibody response in lambs.

Chapter 4

In chapter 4 we evaluate the efficacy of three of our candidate vaccines after a single vaccination of lambs. This study included the Gn-e subunit vaccine, the NDV-based vector vaccine as well as a newly developed vaccine, based on RVFV replicon particles. These replicon particles resemble the virus structurally, are capable of genome replication, but incapable of autonomous spread. We demonstrate that a single vaccination with each of the vaccines induces a neutralizing antibody response and protects lambs from pyrexia, viremia and mortality.

Chapter 5

In this chapter we discuss the role of heparan sulfate in RVFV infection. We demonstrate that this glycosaminoglycan is required for efficient RVFV infection of different mammalian cells. Heparan sulfate is abundantly present on the surface of most animal cells and the involvement of this attachment factor might explain the broad tropism of RVFV.

Chapter 6

In the work described in chapter 6 we have studied RVFV entry using replicon particles. These studies confirm the hypothesis that RVFV membrane fusion is triggered by low pH. The activating pH and the kinetics of RVFV entry suggest that fusion occurs in late endosomal compartments. We also demonstrate biochemically that exposure of particles to low pH results in the formation of highly stable Gc multimers. Mutagenesis studies of Gc in combination with biochemical and functional analyses suggest that proton binding by histidines in Gc plays a central role in the conformational rearrangement of the Gc fusion protein and virus entry.

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Chapter 2

Rift Valley fever virus subunit vaccines confer complete protection against a lethal virus challenge

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ABSTRACT

Rift Valley fever virus (RVFV) is an emerging mosquito-borne virus causing significant morbidity and mortality in livestock and humans. Rift Valley fever is endemic in Africa, but also outside this continent outbreaks have been reported. Here we report the evaluation of two vaccine candidates based on the viral Gn and Gc envelope glycoproteins, both produced in a *Drosophila* insect cell expression system. Virus-like particles (VLPs) were generated by merely expressing the Gn and Gc glycoproteins. In addition, a soluble form of the Gn ectodomain was expressed and affinity-purified from the insect cell culture supernatant. Both vaccine candidates fully protected mice from a lethal challenge with RVFV. Importantly, absence of the nucleocapsid protein in either vaccine candidate facilitates the differentiation between infected and vaccinated animals using a commercial recombinant nucleocapsid protein-based indirect ELISA.

INTRODUCTION

Rift Valley fever virus (RVFV) is a mosquito-borne zoonotic virus that causes recurrent and massive outbreaks affecting humans and ruminants. The virus is endemic in Africa and emerged in Saudi Arabia and Yemen in 2000 (14). Sheep, goat and cattle are the main species affected during a RVFV outbreak. Abortion storms and high newborn fatality rates, which can approach 100%, are typical features of such outbreaks (39, 56). The virus can be transmitted to humans via direct contact with infected animal tissues and by the bite of infected mosquitoes. Disease in humans is generally mild consisting of fever, myalgia, headache and photophobia (39). A small percentage of infected individuals, however, develop more severe symptoms like retinitis, retinal lesions, hepatitis or hemorrhagic fever (37, 39). Although the overall case-fatality rate is estimated at 0.5-1.0% (15), recent outbreaks show considerably higher numbers (57-59). The high case-fatality rates combined with the potential of rapid spread via its vector explains the recognition of RVFV as a potential bioterrorism agent by the United States government (10, 46). Given the impact of RVF outbreaks on livestock, the human population, and the economy, there is an urgent need for a safe and effective vaccine.

RVFV is a member of the *Phlebovirus* genus within the *Bunyaviridae* family. The virus is membrane-enveloped and contains a segmented negative-sense genome. The three genome segments are called L, M and S referring to the large, medium and small segment. The L segment encodes the viral polymerase. The S segment is of ambisense polarity and codes for the non-structural protein NSs in sense polarity and the nucleocapsid protein (N) in antisense polarity (48). The M segment encodes the two surface glycoproteins Gn and Gc, and the two non-structural proteins NSm1 and NSm2. Gn (~54 kDa) and Gc (~59 kDa) (22) form a heterodimer after processing of the polyprotein by host proteases in the endoplasmic reticulum (ER) (27). The Gc glycoprotein harbours a C-terminal lysine-based ER retention signal whereas the Gn protein contains a C-terminal Golgi localization signal. Heterodimerization of Gn and Gc is thus required for the transport of Gc to the Golgi compartment, where virus assembly takes place (21). According to a T = 12 icosahedral symmetry (19), RVFV incorporates 720 Gn-Gc heterodimers into its envelope, which function in virus binding to and entry into cells (27).

Over the years, much effort has been put forth developing RVFV vaccines using various strategies, including subunit (36, 40, 49), DNA (31, 53), virus vector-based (26, 55), live-attenuated virus (8, 13, 38, 44, 51) and inactivated whole virus

vaccines (45) (for a recent review see: 12). This work provided considerable evidence that humoral immunity is sufficient for protection against RVFV. The Gn and Gc glycoproteins are supposed to be crucial for generating a protective humoral immune response.

The production of a RVFV vaccine outside endemic areas must be safe, preferably without the need for a high biosafety containment facility. In addition, animal transport regulations require that the vaccine enables the differentiation between infected and vaccinated animals (DIVA) using a field validated diagnostic test. Viral subunit vaccines are safe for the environment, since neither production nor vaccination involves a replicating virus. Virus-like particles (VLPs) can be used as a special type of viral subunit vaccine, mimicking the overall virus structure, thus preserving the antigenicity of the authentic virus (2, 47).

VLPs have been successfully produced for different members of the *Bunyaviridae* family including Uukuniemi virus (UUKV), Hantaan (HTN) virus and RVFV (25, 35, 36). Whereas expression of HTN virus VLPs was described to depend on the presence of the N protein (7), UUKV and RVFV VLPs can be produced by expressing only the Gn and Gc glycoproteins (36, 42). Vaccination of mice with RVFV VLPs containing the N protein provided 90% (40) or 56% (36) protection upon RVFV challenge after three immunizations. In the latter study, vaccination with VLPs without the N protein, however, provided only 19% protection (36).

Here, we have generated RVFV VLPs comprising only the Gn and Gc protein using a robust *Drosophila* insect protein expression system. Since studies have indicated that antibodies to Gn alone are sufficient for virus neutralization (49, 55) we compared the vaccine efficacy of VLPs - with or without adjuvant - with that of adjuvanted, purified Gn ectodomain using a mouse infection model. All vaccine candidates formulated with adjuvant provided full protection against a lethal RVFV challenge. Contrary to a recent report (36), vaccination with VLPs lacking the N protein also provided 100% protection, even without adjuvant. The absence of the N protein in all vaccine preparations facilitates the development of a safe and highly effective RVFV DIVA vaccine for use in livestock.

MATERIALS AND METHODS

Virus and cells

The RVFV strain 35/74 was kindly provided by Prof. dr. Janusz Paweska of the National Institute for Communicable Diseases (Johannesburg, South Africa) and Dr.

Christiaan Potgieter of the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI, Onderstepoort, South Africa). The virus was handled under BSL-3 laboratory conditions in biosafety class III glove boxes. Virus stocks were produced by infection of BHK-21 cells grown on CO_2-independent medium (GIBCO™, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO), 2 mM L-glutamine (GIBCO) and 5% fetal calf serum (FCS) at 37°C. Virus stocks were titrated on BHK-21 cells using 6-fold dilutions. Titres are depicted as 50% tissue culture infectious doses ($TCID_{50}$).

Plasmid construction

The GnGc-coding sequence of the M segment of RVFV strain 35/74, codon optimized for optimal expression in human and insect cells, was synthesized by the GenScript cooperation (Piscataway, NJ, USA). For expression of the GnGc gene and the gene encoding the Gn ectodomain (Gn-e), the insect expression vector pMT/BiP/V5-HisA (Invitrogen™, Carlsbad, CA, USA) was used, which contains an inducible metallothionein (MT) promoter and the *Drosophila* BiP secretion signal. The sequences to be expressed were cloned into the pMT/BiP/V5-HisA vector, yielding plasmids pMT-GnGc and pMT-Gn-e. In both, the sequence encoding the Gn signal peptide was replaced by that coding for the BiP signal peptide, specifying the junction sequence "GLSLG-**RSL**-*AEDPH*" (BiP, **linker**, *Gn ectodomain*). In the pMT-Gn-e plasmid, the Gn ectodomain sequence was extended 3' terminally to add a C-terminal 6xHis-tag to the protein as follows: "YQCHT-**DPTG**-*HHHHHH*" junction (Gn-e-**linker**-*His tag*). To allow expression in mammalian cells, the codon-optimized GnGc gene was cloned into the pCAGGS expression vector (41), yielding pCAGGS-GnGc.

For the quantification of Gn and Gc glycoproteins by quantitative Western blot analysis, a DNA fragment was synthesized (GenScript) that encodes two previously described linear epitopes of Gn (residues 374-CFEHKGQYKGTMDSGQTKRE-393) and Gc (residues 975-VFERGSLPQTRNDKTFAASK-994) (18). The synthesized DNA fragment, containing an upstream *Bam*HI site and a downstream *Eco*RI site, was ligated into the *Bam*HI/*Eco*RI site of the pGEX-2T bacterial expression vector (GE Healthcare, Diegem, Belgium) in frame with the glutathione *S*-transferase (GST) gene, yielding the pGEX-GnGc plasmid. All constructs were verified by DNA sequencing (Baseclear, Leiden, The Netherlands).

Expression and purification of RVFV VLPs and the Gn ectodomain

The pMT-GnGc and pMT-Gn-e constructs were each co-transfected into *Drosophila* Schneider (S2) cells (29, 50) at a ratio of 19:1 with the Blasticidin resistance vector pCoBlast (Invitrogen™) according to manufacturers' recommendations. Stably transformed cells were selected by growth at 27°C in serum-free InsectXpress medium (Lonza, Westburg, Leusden, The Netherlands) containing 25 µg/ml Blasticidin-S-HCl (Invitrogen™) and maintained in this culture medium in the presence of 10 µg/ml Blasticidin-S-HCl. For protein expression, these stable cell lines were seeded at a density of 1.5 x 10^6 cells/ml in 175m² cell culture flasks (Greiner Bio-One B.V.) and induced by addition of copper sulphate (500 µM) to the culture medium. Ten days later RVFV VLPs were purified from the pre-cleared (10,000 x *g*, 20 min, 4°C) culture supernatant by sedimentation through a sucrose cushion. Typically, 32 ml of pre-cleared supernatant was placed on top of 4 ml 20% (wt/wt) sucrose in TN buffer (10 mM Tris pH 7.4, 100 mM NaCl) and centrifuged for 2 h at 100,000 x *g* at 4°C in a Beckman SW32Ti rotor. The pellet was dissolved in 100 µl TN buffer on ice. For flotation experiments, concentrated VLPs were mixed with a TN-buffered 60% sucrose solution (total volume 1.4 ml) and used as the bottom layer of a discontinuous sucrose gradient. Layers of 50, 40, 30 and 20% (wt/wt) sucrose, of each 0.9 ml, were placed on top, respectively. After a 72 h spin at 160.000 x *g* at 4°C in a Beckman SW55Ti rotor, 0.5 ml fractions were collected. The sucrose densities were determined and all fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting for the presence of RVFV glycoproteins.

The secreted Gn ectodomain was purified from the cell culture supernatant by virtue of its C-terminal 6xHis-tag using ProBond™ nickel-chelating resin according to the manufacturer's recommendations (Invitrogen™). The Gn-e protein was eluted from the washed beads with 250 mM imidazole (Sigma) and concentrated using an Amicon® Ultra-4 concentrator with a molecular mass cut-off of 30 kDa (Milipore™, Billerica, MA, USA).

Expression and purification of GST-GnGc

The GST-GnGc fusion protein was expressed and purified essentially as described before (11). Briefly, BL21 cells (Novagen, Gibbstown, NJ, USA), transformed with the pGEX-GnGc plasmid, were grown in 2 x yeast-tryptone medium to log phase and subsequently induced by adding isopropyl-ß-D-thiogalactopyranoside to a final concentration of 0.5 mM. Three hours later, the cells were pelleted, resuspended in

1/25 volume of 10 mM Tris (pH 8.0), 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and sonicated on ice. The cell homogenate was centrifuged at 1,600 x *g* (JA-10 rotor) for 30 min at 4°C. To each 50 ml of supernatant, 2 ml of glutathione-Sepharose 4B (GE Healthcare, 50% [vol/vol] in phosphate-buffered saline [PBS]) was added, and the mixture was incubated overnight at 4°C under rotation. The beads were washed three times with 50 ml of PBS and resuspended in a final volume of 1 ml PBS. Purified GST-GnGc was eluted from the beads with 10 mM glutathione in PBS. The purity and concentration of the purified GST-GnGc protein was determined by gel electrophoresis followed by Coomassie staining and by Nanodrop1000 (Wilmington, DE, USA) measurements, respectively (data not shown).

Western blotting

Proteins were separated in 10% SDS-PAGE gels (Biorad system) and subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Biorad). After blocking, the blots were incubated with rabbit polyclonal antisera raised against peptides derived from the Gn or the Gc protein (GenScript, Piscataway, NJ, USA). The Gn antiserum (α-Gn) was raised against a synthetic peptide comprising residues 374-CFEHKGQYKGTMDSGQTKRE-393. For obtaining the Gc antiserum (α-Gc) a peptide comprising residues 975-VFERGSLPQTRNDKTFAASK-994 was used (18). Goat anti-rabbit horseradish peroxidase-conjugate (Biorad) was used as the secondary antibody and protein detection was performed using the Amersham ECL™ Western blotting detection reagents (GE Healthcare). Protein quantification of Gn and Gc was performed by quantitative Western blotting using a two-fold serial dilution of the purified GST-GnGc fusion protein as a calibration marker (data not shown).

Vaccination and challenge of mice

Female BALB/c mice (Charles River laboratories, Maastricht, The Netherlands) were housed in groups of five animals in type III filter-top cages and kept under BSL-3 conditions. The light regime was set at 14 h light/10h dark, the temperature at 22°C and the relative humidity at 55%. Food and water was provided *ad libitum*. Groups of ten 7-week-old mice were vaccinated via the intraperitoneal route on day 0 with saline, Gn-e protein (10 µg/mice) or VLPs (10 µg/mice) formulated in Stimune, a water-in-oil adjuvant (Prionics, Lelystad, The Netherlands) (32), in a total volume of 500 µl. Two additional groups of ten mice were either vaccinated intraperitoneally with non-adjuvanted VLPs (10 µg/mice) or were left untreated (non-vaccinated). We daily monitored for clinical signs and measured body weights of the mice every week.

Blood samples, to be used for serological tests, were obtained from the tail vein at different time points. All groups were boosted on day 21, performed in the same way as the initial immunization. On day 42, all mice were challenged via the intraperitoneal route with 10^2.7 $TCID_{50}$ of RVFV strain 35/74 in 0.5 ml culture medium. This optimal challenge dose, for intraperitoneal inoculation, was determined by two independent dose titration studies, performed with 7 and 11-week-old female BALB/c mice (Antonis A.F. et al., manuscript in preparation). Challenged mice were monitored daily for visual signs of illness and mortality. At day 62 post initial immunization, all animals that survived the RVFV challenge were bled via orbital puncture under general anaesthesia using xylazine (7 mg/kg) and ketamine (70 mg/kg) and euthanized by cervical dislocation. This experiment was approved by the Ethics Committee for Animal Experiments of the Central Veterinary Institute of Wageningen UR.

Analysis of the antisera by ELISA

The commercial recN-ELISA was originally developed for analysis of sera from livestock (43). For analysis of the mouse sera, the ELISA was performed essentially according to the manufacturer's instructions (BDSL, Ayrshire Scotland, UK), but with the following modifications. Plates were coated with stock antigen, diluted 1:3000 and all mouse sera were analyzed in duplicate. As the secondary antibody, a peroxidase-conjugated rabbit anti-mouse antibody (DAKO, Glostrup, Denmark) was used. Optical densities were measured at 405 nm and the cut-off was arbitrarily set at 0.80.

For the GnGc peptide ELISA, 96 well plates were coated overnight at 4°C with GST-GnGc protein (0.27 µg/well) comprising the previously described selected linear epitopes of Gn and Gc fused to the GST protein. Plates were subsequently blocked with 10% skimmed milk in PBS for 1 h at 37°C. Mouse serum was diluted (1:16) in dilution buffer (PBS with 2% skimmed milk) and 50 µl was added to the wells. After 1 h incubation at 37°C and three washes with wash buffer (PBS with 0.05% Tween-20) incubation with a peroxidase-conjugated rabbit anti-mouse antibody (DAKO) was performed for 1 h at 37°C. After three washing steps, 100 µl of substrate buffer (100 mM NaAc pH 6.0) containing 0.1 mg/ml 3,3',5,5'-Tetramethyl-benzidine (TMB, SIGMA-ALDRICH™, St. Louis, MO, USA) and 0.005% H_2O_2 was added. After 30 min incubation at room temperature (RT), 50 µl 0.5 M sulphuric acid was added to each well. Optical densities were measured at 450 nm.

Virus neutralization tests

Virus neutralization tests were performed under BSL-3 conditions using RVFV strain 35/74. Sera collected one day before the second immunization or challenge were pooled and analyzed in quadruplet. The serum pools were diluted (1:32) in 100 µl CO_2-independent medium (GIBCO), supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO), L-glutamine 2 mM (GIBCO) and 5% FCS. Two-fold serial dilutions of the sera (50 µl) were mixed in 96-well plates with 50 µl of culture medium containing ~150-250 $TCID_{50}$ of RVFV. After 2.5 h incubation at RT, 50 µl culture medium containing 4 x 10^4 BHK-21 cells was added to each well. After a 3-4 day incubation period at 37°C, the cultures were scored for cytopathic effect. Fifty percent end point titres were calculated using the Spearman-Kärber method (30, 52).

Immunoperoxidase monolayer assay (IPMA)

Monolayers of BHK-21 cells in 96 well plates were transfected with 100 ng of pCAGGS-GnGc using jetPEI™ according to manufacturers' instructions (Polyplus transfection, Illkirch, France). At 24 h after transfection, plates were washed with PBS and dried for 1 h at RT. Cells were disrupted via a freeze-thaw step at -20°C and fixed with 4% paraformaldehyde for 10 min at RT. After three washes with PBS, mouse serum was added to the cells in a four-fold serial dilution in 100 µl block buffer (4% horse-serum, 0.5 M NaCl, 1% Tween-80 and 0.1% NaN_3). The cells were washed three times with wash buffer (PBS with 0.15 M NaCl and 0.05% Tween-80), and subsequently incubated with peroxidase-conjugated rabbit anti-mouse antibodies (DAKO) diluted in conjugate buffer (4% horse serum, 0.5 M NaCl and 1% Tween-80) for 1 h at 37°C. Cells were washed three times with wash buffer after which activity of peroxidase was detected using 3-amino-9-ethyl-carbazole (Sigma) as the substrate. Titres were calculated using the Spearman-Kärber method (30, 52).

Quantitative real-time reverse-transcriptase PCR (qRRT-PCR)

Livers and brains of deceased or euthanized mice were collected during the experiment. Autoclaved Zikonia beads ~1.2 gr (Zirconia 1.0 mm beads, Biospec) were mixed with ~75mg of tissue and 600 µl lysis buffer (RNeasy mini kit, Qiagen). The tissue was disrupted by vortexing for 15 minutes before pelletation for 10 minutes at 7200 x *g*. An eluate volume of 350 µl was used to isolate total RNA using the Qiagen mini RNeasy kit according to the manufacturers' recommendations. The total RNA was eluted with 30 µl elution buffer of which 5 µl was used for analysis by qRRT-PCR as described (16).

Transmission electron microscopy (TEM)

RVFV VLPs present in the clarified (10,000 x *g*, 20 min, 4°C) S2 cell culture supernatant were fixed (10 minutes, 4°C, 0.5% glutaraldehyde [Polysciences, Warrington, PA, USA] in 0.5 mM phosphate buffer pH 7) and purified by sedimentation (1.5 h, 4°C, 100.000 x *g*) through a 28% (w/v) sucrose cushion onto a 45% (w/v) sucrose cushion. The collected interphase was diluted using distilled water and spun (1.5h, 4°C, 100.000 x *g*) onto a 45% (w/v) sucrose cushion. The VLP fraction which settled on top of the 45% (w/v) sucrose was applied to copper Formvar-carbon coated grids (Stork Veco BV, Eerbeek, The Netherlands). The VLP-coated grids were stained with 1% or 2% sodium phosphotungstate pH 7 (Merck, Darmstadt, Germany). Images were recorded at a calibrated magnification of 60,000 x using a Jeol 1010 electron microscope.

Statistical analyses

Statistical analyses were performed with the Kruskal-Wallis one-way analysis of variance (ANOVA) or two-way ANOVA Bonferroni post-test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). Statistical differences with p values <0.05 were considered significant.

RESULTS

Expression of RVFV glycoproteins in *Drosophila* cells

The *Drosophila* expression system (Invitrogen™) is an attractive system for expression of secretable proteins. It allows inducible expression and secretion of soluble glycoproteins under non-lytic conditions and in serum-free medium. In order to produce RVFV VLPs we generated a stable Schneider 2 (S2) cell line allowing the inducible expression of the GnGc polyprotein (Fig. 1A). Upon induction both glycoproteins could be detected in the cell culture supernatant by Western blotting (Fig. 1B). Consistent with their being secreted in the form of VLPs, Gn and Gc co-sedimented through a 20% (w/w) sucrose cushion (Fig. 1B) and subsequently co-floated in a discontinuous sucrose gradient (Fig. 1C). The Gn and Gc proteins accumulated at a buoyant density of ~1.14 g/cm^3 which is close to the buoyant density of RVFV of 1.16-1.18 g/cm^3 (23, 28). The observations indicate that expression of the RVFV GnGc polyprotein in insect cells results in the formation and secretion of VLPs. The morphology of the generated VLPs analysed by transmission electron microscopy indicated a circular or donut shaped morphology with spiky surface

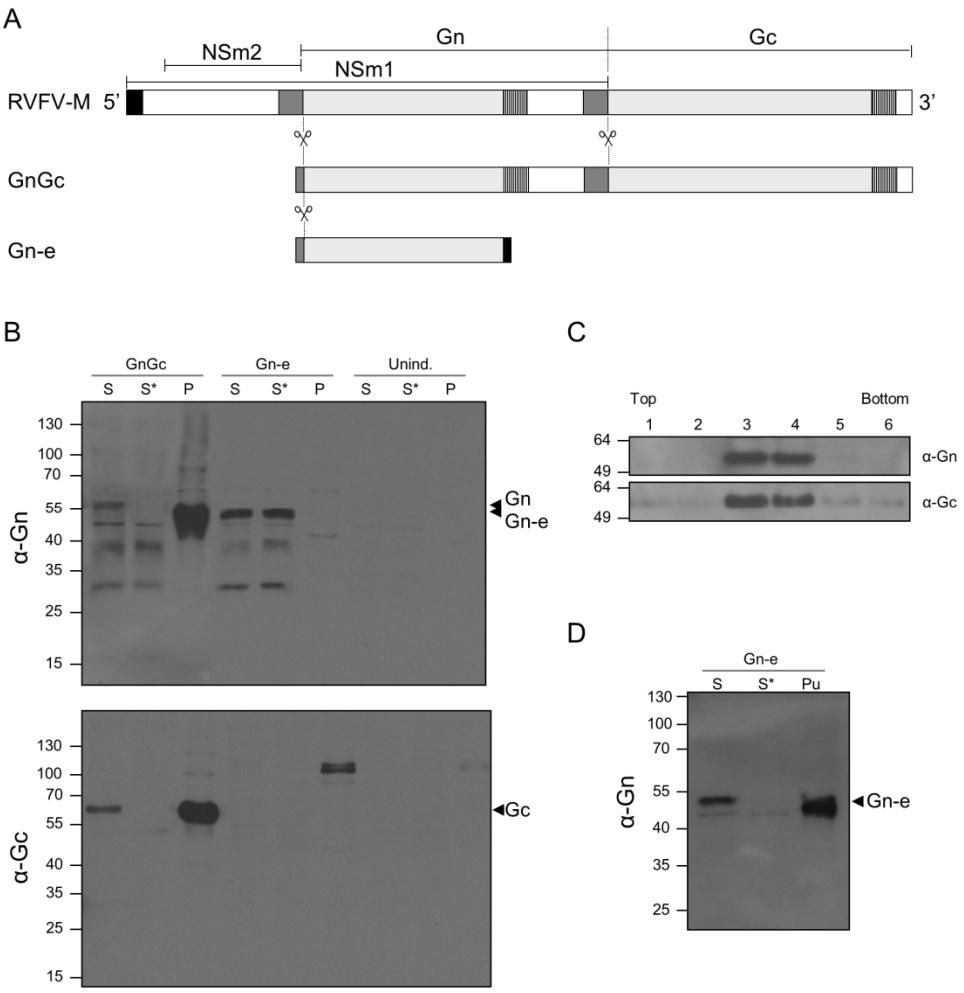

Figure.1. Expression and analyses of the RVFV Gn and Gc glycoproteins. (A) Schematic representation of the RVFV M segment, encoding the Gn and Gc glycoproteins as well as two non-structural proteins NSm1 and NSm2 (12). The constructs, encoding parts of the M segment used for expression in *Drosophila* Schneider 2 (S2) cells, named GnGc and Gn-e are also depicted. The positions of a hydrophobic domain (black box), signal peptides (dark grey box), transmembrane domains (vertical-hatched box) and the signal peptide cleavage sites (scissors) are indicated. The expression constructs encode the GnGc or Gn ectodomain (Gn-e) with an N-terminal *Drosophila* BiP signal sequence (dark grey bar) and - in case of Gn - a C-terminal-fused His–tag (black bar). (B) Western blot analysis of culture supernatant of S2 cells expressing the GnGc polyprotein or Gn-e before and after ultracentrifugation. Cell culture supernatant before (S) and after (S*) pelletation and the pellet (P) were analyzed for the presence of Gn or Gc by Western blotting using an α-Gn (upper panel) or α-Gc (lower panel) peptide serum. Culture supernatant of uninduced S2 cells (unind.) was taken as a negative control. Position of Gn, Gn-e or Gc are indicated on the right (arrowheads); sizes of molecular weight markers are indicated on the left in kilodaltons (B-D).

protrusions (Fig. 2). To also produce the Gn protein we separately generated a stable cell line expressing the Gn ectodomain (Gn-e, Fig. 1A). Gn-e could readily be detected in the culture supernatant but, as expected for a soluble protein and unlike the VLPs, could not be concentrated by ultracentrifugation (Fig. 1B). Gn-e was successfully purified from the cell culture supernatant using nickel chelate beads via its C-terminal-fused 6xHis-tag (Fig. 1D).

Immunogenicity of RVFV VLPs and the Gn ectodomain

To investigate and compare the immunogenic properties of the RVFV GnGc VLPs and of the Gn-e protein, groups of 10 mice were immunized via the intraperitoneal route and boosted three weeks later. Sucrose-cushion concentrated VLPs and affinity-purified Gn-e were each formulated in Stimune adjuvant. Stimune, previously known as specol (9), is a water-in-mineral oil emulsion which strongly promotes the induction of antibodies with a low incidence of site reactivity (4). Another 10 animals served as an adjuvant control group and were administered Stimune only. Since VLPs are regarded morphological representatives of the authentic viral particles and should therefore be able to stimulate a strong immune response in the absence of adjuvant (40, 47, 60) we additionally included a group of mice that were vaccinated with non-adjuvanted VLPs. All formulations were applied at a dose of 10 µg in 500 µl per mouse. Unfortunately, of the Stimune control group 4 mice were lost due to an experimental handling error. A fifth group of 10 non-vaccinated mice was therefore added as an additional challenge control group. In none of the groups clinical symptoms were observed before challenge.

Sera of mice were collected weekly until the day of challenge. After challenge, deceased mice were bled when possible and surviving mice were bled before euthanasia via orbital puncture. Sera were analyzed for the presence of antibodies against Gn and/or Gc by enzyme-linked immunosorbent assay (ELISA, Fig. 3A), immunoperoxidase monolayer assay (IPMA, Fig. 3B) and virus neutralization tests (VNT, Fig. 3C). The GST-GnGc peptide ELISA was developed to specifically detect antibodies against a linear epitope in Gn and one in Gc. Mice vaccinated with VLPs

(C) Resuspended GnGc pellet was analyzed by flotation through a discontinuous sucrose gradient. Gradient fractions (1-6) were analyzed for the presence of Gn or Gc by Western blotting using an α-Gn (upper panel) and α-Gc (lower panel) peptide serum, respectively. (D) Purification of Gn-e from culture supernatant of S2 cells expressing Gn-e. Cell culture supernatant before (S) and after (S*) His-tag affinity purification and purified Gn-e (Pu) were analyzed by Western blotting using the α-Gn peptide serum.

(with or without adjuvant) seroconverted already after the first vaccination, whereas seroconversion induced by Gn-e required two vaccinations (Fig. 3A). This result demonstrates that the GST-GnGc peptide ELISA is suitable to detect differences in antibody responses induced by the different vaccines. In view of the inherent limitations of this peptide ELISA, we also used IPMAs to compare GnGc antibody levels induced by the different vaccine candidates. The Stimune formulated VLP ($p < 0.05$) and Gn-e ($p < 0.001$) vaccines elicited significantly higher antibody titres after the second immunization when compared with the non-adjuvanted VLPs (Fig. 3B). Since the GST-GnGc ELISA results were confirmed by IPMA, we consider the GnGc peptide ELISA a reliable tool to compare antibody responses induced by glycoprotein-based RVFV vaccine candidates.

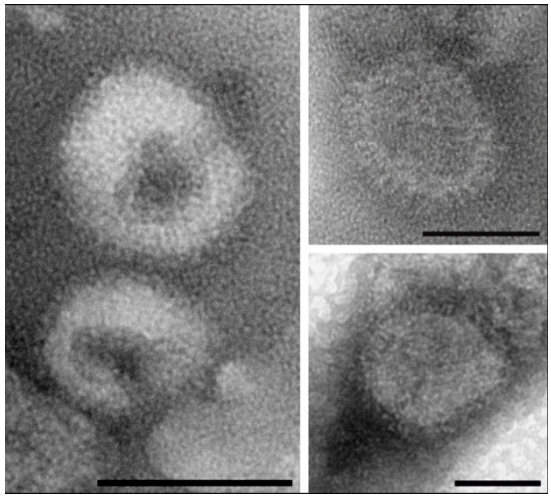

Figure.2. Morphological analysis of GnGc VLPs using transmission electron microscopy (TEM). Glutaraldehyde-fixated VLPs were concentrated by sucrose-cushion sedimentation, stained with 1-2% sodium phosphotungstate (PTA) - particles left and upper right 1% PTA, bottom right 2% PTA - and analyzed by TEM. Four representative virus-like particles are depicted showing spiky structures on their surface. Scale bar on the left represents 100nm, both scale bars on the right represent 50nm.

To determine whether the sera obtained after the first and second vaccination were able to neutralize the virus in vitro, virus neutralization tests were performed. Neither a negative control serum (data not shown) nor a pooled serum from Stimune vaccinated mice was able to neutralize the virus (Fig. 3C). Sera obtained from mice vaccinated with VLPs, with or without adjuvant, obtained after the primary vaccination were able to neutralize the virus, and the level of these antibodies increased after the secondary vaccination. In contrast, the induction of neutralizing

antibodies by Gn-e required two vaccinations (Fig. 3C). The neutralizing antibody levels obtained after vaccination with Stimune adjuvanted VLPs were significantly higher than those obtained after vaccination with non-adjuvanted VLPs ($p < 0.001$) or Gn-e ($p < 0.001$) (Fig. 3C). In conclusion, Gn-e induces the highest overall antibody titres (Figs. 3A and B), though the antibodies induced by VLPs are more potent in neutralizing RVFV. Secondly, Stimune adjuvanted VLPs induce significantly higher levels of neutralizing antibodies in mice than adjuvanted Gn-e or non-adjuvanted VLPs (Fig. 3C).

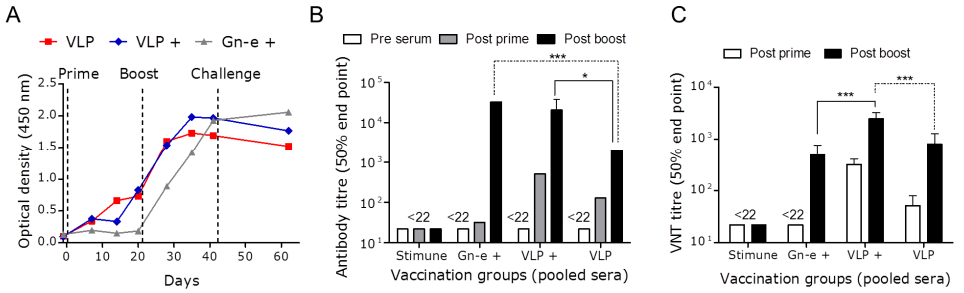

Figure.3. Analysis of antibody responses in mice, induced by different vaccine candidates. (A) Comparison of the reactivity of antibodies from antisera raised against different vaccine candidates (VLP with [VLP +] and without [VLP-] Stimune adjuvant or Gn-e with Stimune adjuvant) with the GST-GnGc fusion protein by ELISA. First and second vaccination and challenge moments are indicated. Antibody titres are depicted as average (n = 10) optical density. (B) BHK-21 cells were transfected with a plasmid encoding the Gn and Gc protein. The monolayers were fixed and incubated with pooled antisera (n = 10) as the primary antibody and peroxidase-conjugated rabbit anti-mouse antibodies as the secondary antibody. Antibody titres are depicted as 50% end-points (n = 2, ± S.D.). (C) Virus neutralization test of pooled mice (n = 10) sera collected after the first (1st) and second (2nd) immunization, from mice that received Stimune only, VLPs, VLP formulated in Stimune adjuvant, or Gn-e formulated in Stimune adjuvant. The results are depicted as 50% end-point titres (n = 4, ± S.D.). Titres were calculated using the Spearman-Kärber method. *p <0.05; ***p <0.001.

Protection against a lethal challenge

Three weeks after the second immunization mice were challenged via the intraperitoneal route with a lethal dose (10^2.7 $TCID_{50}$) of the RVFV strain 35/74. The lethal dose was previously determined by two independent dose-titration experiments (Antonis AF., et al. manuscript in preparation). All mice were monitored weekly for weight-loss and daily for clinical signs.

The adjuvant-only and non-vaccinated groups showed 83% and 100% mortality, respectively (Fig. 4C). In contrast, all mice immunized with RVFV antigens survived, regardless of the antigen composition and whether or not adjuvant was used. Moreover, the body weights of these mice were stable and the mice did not

develop any clinical signs throughout the experiment (Figs. 4A and 4B). In contrast, the non-vaccinated mice developed severe clinical signs (Fig. 4A), which resulted in 100% mortality within 4 days. Interestingly, the Stimune control group showed delayed symptoms. Twelve days after challenge, five out of the total of six mice from this group had succumbed to the infection, whereas the sixth mouse was negative in all our serological assays. However, in this mouse, viral RNA was detected in both the liver and the brain (Figs. 5B/C). The delayed disease in mice inoculated with Stimune might be explained by unspecific immune modulatory effects (3, 17).

Since our RVFV vaccines are only comprised of the viral envelope glycoproteins, the detection of antibodies against the nucleocapsid protein in sera after challenge would be a good indicator of viral replication. To further verify if vaccination suppressed virus replication, we screened sera for the presence of anti-N antibodies using a commercially available recombinant N-ELISA (43) (Fig. 5A) and performed quantitative real-time reverse-transcriptase PCR on liver and brain samples to detect viral RNA (Figs. 5B and C).

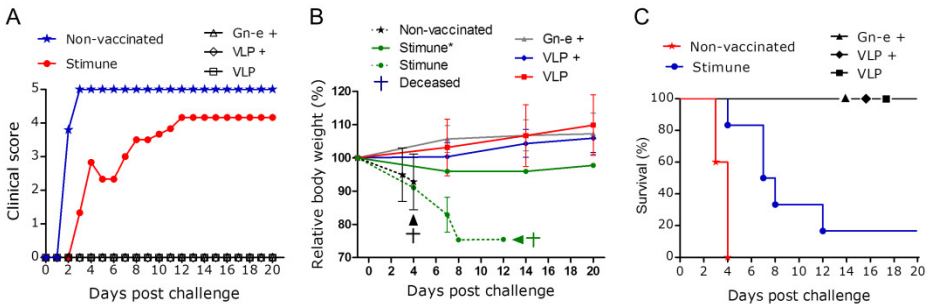

Figure.4. Vaccination with RVFV VLPs or the Gn ectodomain protects mice against lethal RVFV infection. Groups of mice (n = 10) were either non-vaccinated or vaccinated twice with adjuvant only (Stimune; n = 6), 10 µg VLPs with (VLP +; n = 10) or without (VLP; n = 10) Stimune adjuvant, or 10 µg adjuvanted Gn-e (Gn-e +; n = 10). Mice were challenged with $10^{2.7}$ $TCID_{50}$ of RVFV strain 35/74 via the intraperitoneal route. Mice were monitored daily, until 20 days post challenge, for mortality (A) and clinical scores (C, n = 6 or n = 10) and weekly for weight (B, n = 6 or n = 10 ± S.D.). The body weights of the only survivor of the Stimune-vaccinated control group are depicted separately (Stimune*). Number coding related to the clinical scores (C) are 0 = Normal healthy; 1 = Active, hairs raised; 2 = Less active, hairs raised; 3 = Less active, hairs raised, accelerated respiration, coiled; 4 = Less active, hairs raised, accelerated respiration, hunchback like posture. Mouse fails to roll from posterior to anterior, when place on its back; 5 = Mouse deceased.

The post-challenge sera from four out of ten Gn-e vaccinated mice did show some antibodies against N (Fig. 5A) and in two liver samples and one brain sample viral RNA was detected (Figs. 5B and C). This indicates that despite these mice survived RVFV infection, some viral replication had occurred. In the group vaccinated

with adjuvanted VLPs, only one animal was positive in the recN-ELISA, whereas none of the mice of the non-adjuvanted VLP group had detectable levels of N-antibodies. In five of the ten mice that were vaccinated with non-adjuvanted VLPs, viral RNA was detected in liver samples (Fig 5B). Intriguingly, - with one exception (mouse #3.5) - vaccinated mice which contained viral RNA in the liver did not seroconvert for antibodies against the N protein (Figs. 5B and C) and - vice-versa - mice positive in the recN-ELISA did not have detectable levels of viral RNA in neither the liver nor the brain. Experiments are in progress to further elucidate these findings.

In conclusion, our findings suggest that all vaccine candidates provide full protection against a lethal RVFV challenge, and that vaccination with adjuvanted VLPs even results in sterile immunity in 90% of the mice.

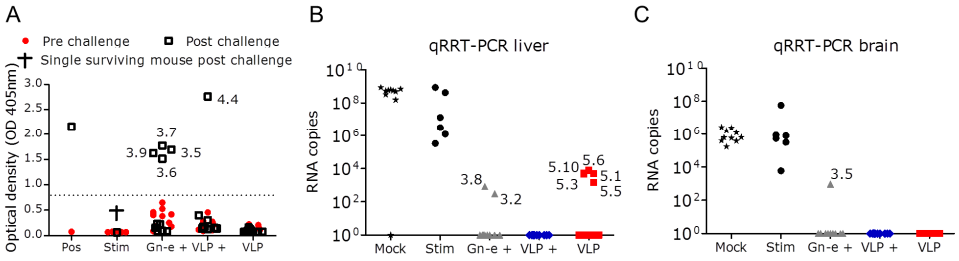

Figure.5. Detection of N-antibodies in mice and viral RNA in liver and brain samples. (A) The pre- and post-challenge sera of mice vaccinated with Stimune only (Stimune), adjuvanted Gn-e or VLP (Gn-e + and VLP +) or non-adjuvanted VLP (VLP), were screened for the presence of antibodies against the nucleoprotein using an indirect recombinant N-ELISA (n = 2) (43). Serum from a RVFV infected mouse was used as a reference control (pos = positive). The dotted line represents the cut-off value, which was arbitrarily set at 0.80. Numbers of some individual mice are depicted in the graph. (B/C) The non-vaccinated mice, mice inoculated with Stimune adjuvant (Stim), mice vaccinated with adjuvanted Gn ectodomain or VLPs with or without adjuvant were challenged with RVFV. The livers (B) and brains (C) of the deceased or euthanized mice were screened for the presence of viral RNA using qRRT-PCR. Numbers of some individual mice are depicted in the graph.

DISCUSSION

RVFV is a mosquito-borne virus causing disease in livestock and humans. Although the virus has so far been confined to the African continent and the Arabian Peninsula global warming and globalization increase the risk of virus migration towards new virgin soils (1). Hence, there is an urgent need for a safe and effective vaccine that is applicable to both humans and livestock. Here we report the successful production of two vaccine candidates against RVF based on the RVFV surface glycoproteins. Both vaccine candidates fully protected mice from a lethal challenge with RVFV.

Until recently (36), RVFV VLPs were successfully produced, using alternative expression systems, only when the nucleocapsid protein was included. Specifically, VLPs were obtained by coexpression of GnGc with the nucleocapsid protein using the baculovirus insect expression system (35) or in combination with a mini-replicon system consisting of the nucleocapsid protein, the polymerase protein and a mini-genome RNA using a mammalian expression system (25). Our nucleocapsid protein-independent assembly of VLPs is consistent with recent work of Mandell et al. (36), in which expression of the Gn and Gc proteins was shown to be sufficient for the formation and secretion of VLPs (36). In the work of Mandell et al., three vaccinations of mice with VLPs containing only Gn and Gc provided protection in only 19% of the animals, whereas in the current work, two vaccinations with VLPs lacking the N protein resulted in 100% protection. Moreover, when these VLPs were formulated in Stimune adjuvant, sterile immunity was even obtained in nine out of ten mice.

Due to the increased international trade of animals and concomitant surveillance, the development of DIVA vaccines is of great importance. The absence of the nucleocapsid protein in our vaccine candidates will facilitate the development of a DIVA vaccine for livestock that can be accompanied by the recN ELISA (43). The N protein of RVFV is highly immunogenic, inducing antibodies already within the first days after infection (54) and is therefore an ideal target in diagnostic ELISAs (43). Surprisingly, Näslund et al. (40) observed that mice vaccinated with VLPs containing the nucleocapsid protein could be serologically distinguished from infected animals using an N-based ELISA. Apparently, the antibody response against the N protein induced by these VLPs was sufficiently low to enable this differentiation. Yet, use of VLPs that lack the nucleocapsid protein, such as those developed in the current work, will minimize the chances of obtaining false-positive results in field diagnostics.

Based on its limited but significant similarity with the alphavirus E2 glycoprotein, it has been suggested that the Gn protein of Bunyaviruses might function as the receptor binding protein (20) and would therefore be a good target antigen for inducing virus neutralizing antibodies. Indeed, Gn is known to contain neutralizing epitopes (5, 6). Accordingly, protection against RVFV challenge based on Gn could be achieved by inoculation of animals with bacterial lysates or baculovirus-infected eukaryotic cells containing only the Gn protein (49, 55), or by alphavirus vectors expressing the Gn ectodomain (24). We demonstrate that immunization with soluble adjuvanted Gn ectodomain provides full protection against RVFV. Of note, the antibody titres induced by the Gn ectodomain (i.e. Gn-e) were equal to those induced by VLPs formulated in Stimune adjuvant and significantly higher than those induced

by VLPs only ($p < 0.001$). It is important to stress, however, that neutralizing antibodies were readily obtained after a single vaccination with VLPs, whereas induction of these antibodies by Gn-e required two vaccinations. This observation might be explained by the repetitive arrangement of the Gn and Gc antigens on the VLP surface allowing efficient cross-linking of the immunoglobulin receptors on B cells which can greatly facilitate B-cell activation (2).

The absence of clinical signs, seroconversion and our inability to detect viral genome in livers and brains in 90% of the mice vaccinated with adjuvanted VLPs suggest that sterile immunity was obtained in these animals. Although no clinical signs were observed in mice vaccinated with VLPs without adjuvant, low levels of viral RNA were detected in 50% of the livers. Apparently, some viral replication occurred in these animals.

Although RVFV has the potential to cause severe disease in humans, no vaccine or antiviral agent for human application is available. Efforts are being made to produce live attenuated vaccines for use in humans (12). The safety of such vaccines has to be thoroughly tested, however. Such safety concerns do not apply to subunit vaccines as those reported here. The *Drosophila* S2 expression system, used to produce the VLPs and the Gn ectodomain in the current work, offers the additional advantage of serum-free production and has been used previously to produce secreted forms of native-like glycoproteins in large amounts for vaccine applications (33, 34). In addition, the recombinant subunit approach allows for convenient antigen affinity purification by appending a purification tag, which is beneficial especially for human vaccine development.

In summary, we report here the efficient production of RVFV VLPs and the Gn ectodomain using a robust insect expression system. The vaccine candidates provide full protection against a lethal RVFV challenge. Additional studies should be performed to establish the DIVA property and minimal protective dose of these vaccine candidates in sheep. The necessity of repeated vaccination should also be evaluated. It is furthermore important to study contribution of humoral and cellular immunity and the durability of protection. Our study demonstrates the potential of a RVFV subunit vaccine, either in the form of VLPs or soluble protein.

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Chapter 3

Rift Valley fever virus immunity provided by a paramyxovirus vaccine vector

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ABSTRACT

Rift Valley fever virus (RVFV) causes recurrent large outbreaks among humans and livestock. Although the virus is currently confined to the African continent and the Arabian Peninsula, there is a growing concern for RVFV incursions into countries with immunologically naïve populations. The RVFV structural glycoproteins Gn and Gc are preferred targets in the development of subunit vaccines that can be used to control future outbreaks. We here report the production of Gn and Gc by a recombinant vaccine strain of the avian paramyxovirus Newcastle disease virus (NDV) and demonstrate that intramuscular vaccination with this experimental NDV-based vector vaccine provides complete protection in mice. We also demonstrate that a single intramuscular vaccination of lambs, the main target species of RVFV, is sufficient to elicit a neutralizing antibody response.

INTRODUCTION

Rift Valley fever virus (RVFV) is a negative-strand RNA virus that belongs to the phlebovirus genus of the *Bunyaviridae* family. RVFV is maintained in the environment in an enzootic vertebrate-mosquito-vertebrate cycle. Typically, RVFV outbreaks are preceded by the mass hatching of RVFV-infected eggs of floodwater *Aedes* mosquitoes during periods of heavy rainfall. The RVFV-carrying *Aedes* mosquitoes transmit the virus to susceptible mammalian species and several other mosquito species are subsequently involved in further spread (26, 56).

RVFV causes significant morbidity and mortality among sheep, goat, cattle (18, 19, 25) and humans (1, 43). Mortality in adult sheep and cattle is estimated at 20% and 10%, respectively, whereas in unborn and infant sheep, mortality is considerably higher, approaching 100% (25). Humans can be infected with the virus either by mosquito bite or aerosols released from contaminated animal products. Most infections in humans run a subclinical course or manifest as a self-limiting febrile illness. RVFV infection can, however, result in severe complications, such as retinitis with visual impairment, encephalitis, or fulminant hepatitis with hemorrhagic fever (1, 43). Although mortality in humans is historically reported to be below 1%, mortality rates in recent outbreaks were considerably higher (59-61). Because of its impact on both animal and human health, RVFV is listed as a so-called overlap agent by the Department of Health and Human Services and the United States Department of Agriculture. Furthermore, because of its large impact on animal health and its potential for rapid spread, RVFV was included on the former list-A of the World Organization for Animal Health (the Office International des Epizooties [OIE]).

Outbreaks of RVFV have so far remained confined to the African continent and the Arabian Peninsula. However, because the virus is transmitted by a wide variety of mosquito species that are not confined to these areas, there is a growing concern that RVFV will expand its current habitat. Several governments of industrialized countries are supporting the development of RVFV control strategies in order to be properly prepared for a possible future RVF incursion.

In African countries, RVF is controlled in livestock using vaccines based on the live-attenuated Smithburn strain or inactivated whole-virus. Vaccines based on the Smithburn strain can provide protection after a single vaccination, but these vaccines are not safe for young animals or gestating adults (11, 36). Inactivated vaccines can be applied in animals of all ages, but these vaccines are less effective than live-attenuated

vaccines and repeated vaccination is necessary for optimal immunogenicity (3, 4, 31). No vaccine for human use is currently available.

In the past decade, significant progress has been made in the development of novel experimental RVF vaccines (7, 8, 12, 20, 30, 32, 33, 38, 44, 47). To optimally combine efficacy and safety, several candidate vaccines are based on virus subunits (20, 30, 32, 38, 44, 47). We are exploring the potential of using Newcastle disease virus (NDV) as a vaccine vector for the *in vivo* expression of these subunits. NDV is a paramyxovirus that causes disease in birds. Vaccination of poultry with avirulent strains of NDV is common practice. The availability of an NDV reverse-genetics system provides opportunities to use NDV as a vaccine vector for the control of other viral diseases of birds (27, 35, 50). In addition, NDV is considered a particularly interesting vaccine vector for applications in mammals. Although NDV is well capable of infecting mammals, spread in these non-natural hosts is very limited, rendering application in these species inherently safe (14, 15, 21).

We previously reported the production of a recombinant NDV vaccine virus that produces the RVFV Gn glycoprotein and demonstrated that a homologous prime-boost vaccination of calves elicited RVFV-specific neutralizing antibodies (38). Although these first results were promising, the induction of neutralizing antibodies required two vaccinations, and the titres obtained were modest. To improve vaccine efficacy, we proceeded with the construction of an NDV vaccine virus that produces both RVFV glycoproteins. We and others previously demonstrated that expression of both structural glycoproteins Gn and Gc results in the production and secretion of virus-like particles (VLPs) (20, 38, 44, 47). The particulate structure and repetitive arrangement of the antigenic building blocks of VLPs explains their generally high immunogenicity (2). In accordance with this, we have previously reported that a homologous prime-boost vaccination of mice with VLPs provides solid protection against a lethal dose of RVFV (20). We now report the successful production of a recombinant NDV vaccine virus that produces both RVFV structural glycoproteins. A homologous prime-boost vaccination via the intramuscular route protected mice from a lethal virus challenge. Moreover, a single vaccination of lambs was sufficient for the induction of RVFV-neutralizing antibodies.

MATERIALS AND METHODS

Cells, plasmids and viruses

The cDNA clone of NDV strain LaSota, named pNDFL and all other tools required for the production of recombinant NDV were described previously (38, 53). RVFV strain 35/74 was kindly provided by Prof. Dr. Janusz Paweska (National Institute for Communicable Diseases, Johannesburg, South Africa) and Dr. Christiaan Potgieter (Agricultural Research Council-Onderstepoort Veterinary Institute, Onderstepoort, South-Africa).

Production of NDFL-GnGc

The nucleotide sequence of NDFL (53) differs by a few nucleotides from the sequence of the LaSota strain (GenBank accession numbers AF077761.1). The nucleotide differences result in four amino-acid changes: F protein, R189(Q); HN protein D393(N); L protein, Q97(E), K191(R) and N369(I) (consensus in parentheses). Before introducing the GnGc gene into NDFL, we decided to change the corresponding codons to match those of the LaSota sequence. The resulting plasmid, named pNDFL2, was used in the current work for the production of recombinant virus.

Plasmid pUC57-GnGcOpt encodes the open reading frame of the M genome segment (nucleotides 411 to 3614) of RVFV strain 35/74 starting from the fourth methionine codon. The sequence was previously codon-optimized and synthesized by the GenScript corporation (Piscataway, NJ, USA) (20). The pUC57-GnGcOpt plasmid contains two *Lgu*I sites, which were used to transfer the GnGc gene to a plasmid named pGEM-PM-cassette (Fig. 1A) (38). The pGEM-PM-cassette plasmid contains the sequence that is located between unique *Apa*I and *Not*I sites in the pNDFL2 plasmid, as well as newly introduced NDV transcription start and stop boxes and two *Lgu*I sites to facilitate insertion of foreign genes (Fig. 1A). The sequence between the *Apa*I and *Not*I sites of pNDFL2 was exchanged for the corresponding fragment of plasmid pGEM-PM-cassette-GnGc. The resulting plasmid, pNDFL2-GnGc (Fig. 1A), was designed in such a way, that the DNA copy of the NDV genome complies to the rule of six (17, 54). Recombinant virus was generated from plasmid pNDFL2-GnGc using previously described methods (38). Virus titres were determined as tissue culture 50% infective dose ($TCID_{50}$) on quail fibrosarcoma (QM-5) cells.

Characterization of NDFL-GnGc

Immunoperoxidase monolayer assays (IPMAs) and immunofluorescence assays (IFAs) were performed as described (38). For Western blot analysis of Gn and Gc, rabbit polyclonal antibodies were used that were previously raised against a Gn-derived peptide (residues 374-CFEHKGQYKGTMDSGQTKRE-393) or a Gc-derived peptide (residues 975-VFERGSLPQTRNDKTFAASK-994) (20). Proteins were separated in Bis-Tris gradient gels (NuPAGE, Invitrogen, Carlsbad, CA, USA) and analyzed by Western blotting as described (38). Goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (DAKO, Heverlee, Belgium) were used as the secondary antibodies. Peroxidase activity was detected using the Amersham ECL™ Western blotting detection reagents (GE Healthcare, Diegem, Belgium).

Flotation analysis (*i.e.* sucrose density centrifugation analysis) was performed as described (38). Briefly, allantoic fluids containing NDFL or NDFL-GnGc were placed on top of 20% (w/w) sucrose and centrifuged at 100 000 x *g* for 1.5 hours at 4°C. Pellets were mixed with a TN (10 mM Tris pH 7.4, 100 mM NaCl)-buffered 60% sucrose solution (total volume 1 ml) and layers of 50, 40, 30 and 20% (w/w) sucrose, of each 1 ml, were placed on top. After centrifugation at 160 000 x g for 72 hours, the sucrose densities of 250 µl fractions were determined, and samples were analyzed by Western blotting. For detection of NDV proteins, the blots were incubated with a sheep anti-NDV antiserum and subsequently with peroxidase-conjugated rabbit anti-sheep IgG antibodies (Abcam, Cambridge, MA, USA).

Vaccination and challenge of mice

Female BALB/c mice (Charles River laboratories, Maastricht, The Netherlands) were housed in groups of five animals in type-III filter-top cages and kept under biosafety level-3 conditions. Ten 7-week-old mice were vaccinated via the intramuscular route on days 0 and 21 with either 10^5.3 $TCID_{50}$ NDFL or NDFL-GnGc, present in 50 µl culture medium. One group of ten mice was left untreated (non-vaccinated). The body weights of the mice were monitored weekly and blood samples, to be used for serological tests, were obtained from the tail vein at different time points. On day 42, all mice were challenged via the intraperitoneal route with 10^2.7 $TCID_{50}$ of RVFV strain 35/74 in 0.5 ml culture medium. This optimal challenge dose was established by two previously performed dose-titration experiments (Kortekaas J. et al., manuscript in preparation). Challenged mice were monitored daily for visual signs of illness and mortality. On day 62, mice that survived the RVFV challenge were bled via orbital puncture under general anaesthesia using xylazine (7 mg/kg) and ketamine

(70 mg/kg) and euthanized by cervical dislocation. Livers were tested for the presence of viral RNA by Taqman quantitative reverse-transcriptase real-time PCR (qRRT-PCR). The LightCycler RNA Amplification Kit HybProbe (Roche, Almere, The Netherlands) was used. Primers and probes, targeting the Gn gene, and cycling conditions were used as previously described (24). Sera were analyzed for the presence of antibodies against the nucleoprotein using a commercially available N protein-based indirect ELISA (BDSL, Ayrshire Scotland, UK). This so-called recN ELISA was originally developed for analysis of sera from livestock (52) and was previously modified for analysis of mouse sera (20). Sera obtained prior RVFV challenge were analyzed for Gn and Gc-specific antibodies by IPMAs as described (20). Briefly, BHK-21 cells were transfected with plasmid pCAGGS-GnGc, which encodes the GnGc protein. After fixation, the cells were incubated with the mouse sera and subsequently incubated with peroxidase-conjugated anti-mouse IgG antibodies. Activity of peroxidase was detected using 3-amino-9-ethyl-carbazole (Sigma, St. Louis, MO, USA) as the substrate. The presence of neutralizing antibodies was determined by virus-neutralization tests (VNTs) as described (20).

Vaccination of lambs

Conventional mixed-breed lambs were randomly allotted in two groups of 6 animals. Group 1 received 10^7.3 $TCID_{50}$ of NDFL-GnGc in a total volume of 1 ml allantoic fluid via the intramuscular route on days 0 and 21. Group 2 was used as a control group and received an equivalent amount of control NDFL virus. None of the lambs displayed any clinical sign throughout the experiment. Serum samples were collected weekly until day 42. Serum samples were used for quantification of NDV antibodies by ELISA using the FlockChek NDV ELISA (IDEXX, Hoofddorp, The Netherlands), which was modified for analysis of sheep antibodies. Plates coated with NDV were blocked using ELISA-buffer (10% skimmed milk; 10% bovine serum albumin; 1% Tergitol NP-9; 0.05% Tween-80; 0.64 M NaCl; 2.7 mM KCl; 2.8 mM KH_2PO_4; 8.1 mM Na_2HPO_4; pH 7.4) and then incubated with 100-fold diluted sheep sera. Bound antibody was then detected with peroxidase-conjugated rabbit anti-sheep IgG antibody (Abcam, Cambridge, UK) diluted in conjugate buffer (PBS supplemented with 5% FCS; 2% NaCl; and 0.05% Tween-80) and staining with 3,3',5,5' tetramethylbenzidine. The optical density at 450 nm was determined.

RVFV-specific neutralizing antibody titres were determined by VNT as described (38).

RESULTS

Construction and characterization of NDFL-GnGc

We have previously reported the construction of an NDV recombinant virus that produces the RVFV Gn glycoprotein (*i.e.* NDFL-Gn) (38). This virus was readily recovered after expression of the viral RNA and helper proteins in QM-5 cells and subsequent inoculation of the collected supernatant into 9-11 day-old embryonated hens' eggs. The production level of the NDFL-Gn virus was about a hundred-fold lower than that of the NDFL virus, which can be attributed to the insertion of the foreign gene (40, 46). It was anticipated that insertion of the GnGc gene (3204 bps) which is considerably larger than the Gn gene alone (1635 bps) would result in a further decrease in virus yield. Before proceeding with this construction, we therefore aimed to optimize the fitness of the NDFL backbone. The NDFL genome encodes five amino acids in three different proteins, (one in HN, one in F and three in L, for specifications see materials and methods) that differ from the LaSota sequence. Since these differences could influence the fitness of the virus, we changed the codons of the five amino acids listed above, to match those of the LaSota sequence. The potential beneficial effects of these changes were not studied.

NDFL-GnGc was readily recovered from embryonated eggs. The maximum production levels were in the range of 10^9 $TCID_{50}$/ml. As expected, QM-5 or BHK-21 cells infected with NDFL-GnGc could be stained with antibodies directed against NDV and antibodies directed against RVFV in IPMAs and IFAs (data not shown).

RVFV produces the glycoproteins Gn and Gc from a single protein precursor [40]. The two glycoproteins form a heterodimer after processing of the polyprotein by host proteases in the endoplasmic reticulum (29). We have previously demonstrated that the Gn glycoprotein is present in the NDFL-Gn virion (38). The amount of Gn that was detected in allantoic fluid containing NDFL-Gn virus was, however, somewhat disappointing. Production of Gn from the GnGc precursor protein by NDFL-GnGc could result in higher production levels of Gn. To compare the expression levels of Gn from NDFL-Gn and NDFL-GnGc, allantoic fluids containing equivalent amounts of these viruses were placed on top of a sucrose cushion and centrifuged at 80 000 x g for 2h. As a reference, previously prepared culture medium of Schneider-2 (S2) cells containing RVFV virus-like particles (VLPs) (20) was used as a control. The proteins present in the resulting pellets were analyzed by Western blotting using Gn and Gc-specific polyclonal antibodies. As previously described (38), the Gn protein could be detected in pellets containing NDFL-Gn (Fig. 1B, left panel). The Gn protein was also

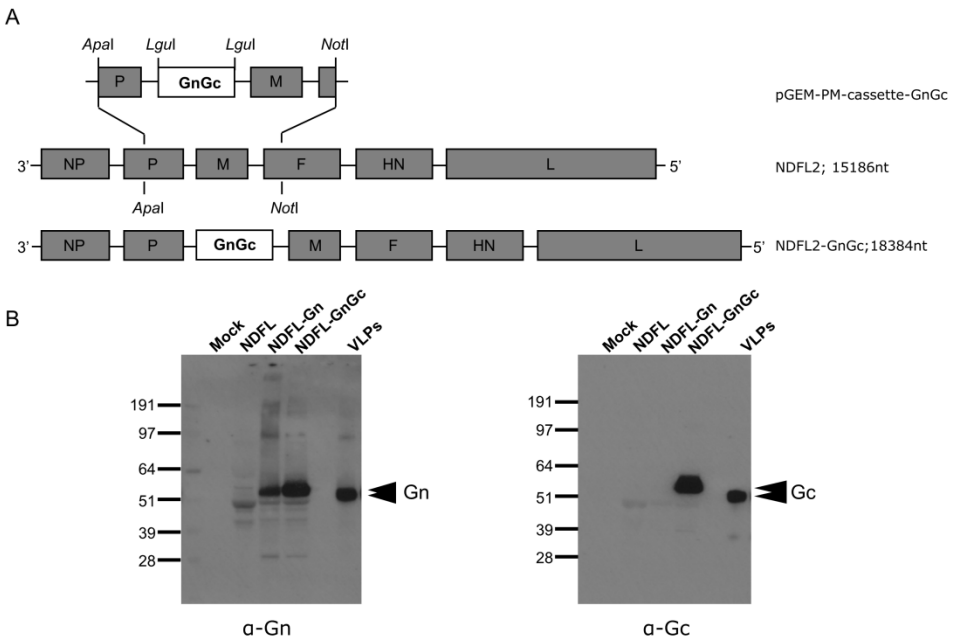

Figure.1. Construction of NDFL-GnGc and expression of the Gn and Gc glycoproteins. (A) Construction of NDFL-GnGc. The GnGc gene was introduced between the *Lgu*I sites of plasmid pGEM-PM-cassette (38). The *Apa*I/*Not*I fragment of pNDFL2 was exchanged for the corresponding fragment of pGEM-PM-cassette-GnGc, resulting in pNDFL2-GnGc. (B) Western blot analysis of Gn and Gc. Allantoic fluids containing no virus (mock) or containing NDFL, NDFL-Gn (38) or NDFL-GnGc were placed on top of 20% sucrose and centrifuged at 80 000 x g for 2 h. The proteins present in the collected pellets were separated in NuPAGE gradient gels and analyzed by Western blotting using Gn (left panel) and Gc (right panel)-specific polyclonal peptide antisera (20). The positions of the Gn and Gc monomers are indicated by arrowheads. The positions of molecular weight standard proteins are indicated to the left.

detected in allantoic fluid containing NDFL-GnGc (Fig. 1B, left panel). Shorter exposures of the blot depicted in Fig. 1B demonstrated that the amount of Gn was substantially higher in allantoic fluids containing NDFL-GnGc than in similar samples produced from NDFL-Gn (data not shown). As expected, the Gc protein was only detected in allantoic fluid containing NDFL-GnGc (Fig. 1B, right panel). The molecular weights of Gn and Gc produced in embryonated eggs were somewhat larger than those of the same proteins produced by S2 cells (Fig. 1B). This is most likely explained by the known difference in size of sugars on glycoproteins when produced in vertebrate cells or insect cells (39), Gn having one and Gc having four potential N-linked glycosylation sites (29).

The detection of both the Gn and Gc protein in pellets containing NDFL-GnGc suggested that either Gn and Gc are incorporated in the NDFL particle, and/or that

RVFV VLPs are produced by NDFL-GnGc. To investigate this further, we aimed to separate NDV particles and possibly produced RVFV VLPs by sucrose gradient centrifugation (Fig. 2).

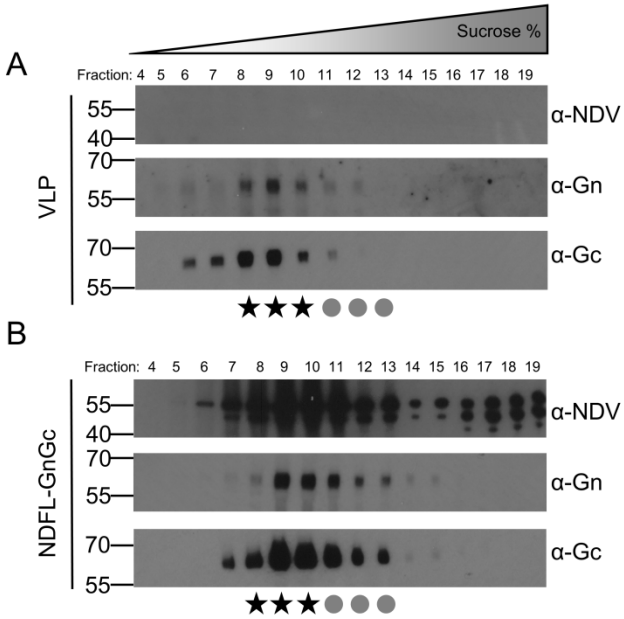

Figure.2. Western blot analyses of sucrose fractions obtained from flotation experiments. Culture medium containing RVFV VLPs produced by drosophila cells (A) (20) or allantoic fluids containing NDFL-GnGc (B) were placed on top of a 20% sucrose cushion and centrifuged at 100 000 x g for 1.5 h. The resuspended pellets were mixed with 60% (w/w) sucrose. This mixture was used as the bottom fraction of a 60 to 20% discontinuous sucrose gradient. Fractions of 250 µl were collected after 72 h centrifugation at 160 000 x g. The proteins in sucrose fractions 4 (top) - 19 (bottom) were separated in 4-12% NuPAGE Bis-Tris gels and subsequently transferred to nitrocellulose blots. Specific proteins were detected by a polyclonal anti-NDV sheep serum (α-NDV, top panels), or rabbit polyclonal antibodies raised against a Gn (middle panels) or Gc (bottom panels) peptide. The positions of molecular weight standard proteins are indicated to the left in kilodaltons. Stars: buoyant density of RVFV VLPs (20); Circles: buoyant density of paramyxoviruses (28).

We previously reported sucrose gradient centrifugation analysis (*i.e.* flotation analysis) of the NDFL virus (38). These experiments demonstrated that NDFL was consistently detected throughout most of the sucrose gradient, which is explained by the known pleiomorphic nature of paramyxoviruses (28, 51). In our experiments, the peak fractions of NDFL proteins generally reveal a buoyant density of ~1.16 - 1.19 g/cm^3, which closely corresponds to the known buoyant density of paramyxoviruses (1.18 - 1.20 g/cm^3) (28). Similar as in previous work, the buoyant density of RVFV VLPs that consist of only Gn and Gc was between 1.14 and 1.15 g/cm^3 (Fig. 2A; peak

fractions 8 – 9) (20). Thus, based on their buoyant densities, it should be possible to separate RVFV VLPs from NDV particles containing Gn and Gc.

The peak fractions of NDV proteins from NDFL-GnGc revealed a slightly lower buoyant density when compared to NDFL, of about 1.15-1.18 g/cm^3 (Fig. 2B; peak fractions 9 - 11). The peak fractions of Gn and Gc were found to overlap with those of NDV proteins (Fig. 2B) and those of the drosophila-produced VLPs (compare Fig. 2A with 2B). Thus, although the flotation experiments demonstrate that Gn and Gc are present in membrane-enveloped particles, it remains unclear whether Gn and Gc are present in the form of VLPs, and/or if these proteins are incorporated in the NDV particle.

Vaccination and challenge of mice

Two groups of 10 mice were immunized via the intramuscular route with either NDFL or NDFL-GnGc and boosted three weeks later. A third group of 10 non-vaccinated mice was added as an additional challenge control group. Sera of mice collected weekly until the challenge were analyzed for the presence of antibodies against Gn and/or Gc by IPMAs (Fig. 3A) and for the presence of virus-neutralizing antibodies by VNTs (Fig. 3B) as described (20).

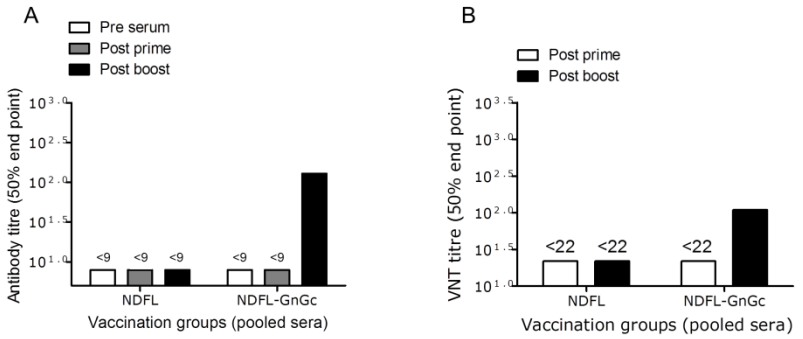

Figure.3. Analyses of antibody responses induced by NDFL-GnGc. Groups of mice (n = 10) were either non-vaccinated or vaccinated twice, via the intramuscular route, with 10^5.3 $TCID_{50}$ of NDFL or NDFL-GnGc. (A) For analysis of the antibody responses against Gn and Gc, BHK-21 cells were transfected with plasmid pCAGGS-GnGc (20), which encodes the Gn and Gc proteins. The monolayers were fixed and incubated with pooled antisera as the primary antibody and peroxidase-conjugated rabbit anti-mouse IgG antibodies as the secondary antibody. Antibody titres are depicted as the average reciprocal value of the highest serum dilution (n = 2, ± S.D.). (B) Virus neutralization test of pooled sera obtained from mice (n = 10) before vaccination or after the first (1st) and second (2nd) immunization. The results depicted are the average reciprocal values of the highest serum dilutions that neutralized the virus, determined in quadruplicate. Titres were calculated using the Spearman-Kärber method (37, 57).

Both experiments suggested that two vaccinations were required to obtain GnGc-specific antibodies. Importantly, the VNTs demonstrated the ability of these antibodies to neutralize the virus *in vitro* (Fig. 3B). Three weeks after the second vaccination, all mice were challenged with a known lethal dose of RVFV strain 35/74.

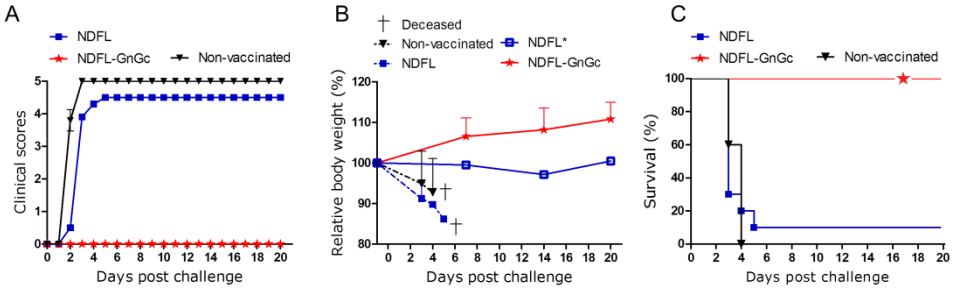

Figure.4. Vaccination with NDFL-GnGc protects mice from RVFV. Groups of mice (n = 10) were either non-vaccinated or vaccinated twice, via the intramuscular route, with 10^5.3 $TCID_{50}$ of NDFL or NDFL-GnGc. Mice were challenged with 10^2.7 $TCID_{50}$ of RVFV strain 35/74 via the intraperitoneal route. Mice were monitored daily, until 20 days post challenge, for clinical signs (A), weekly for weight (B, n = 10 ± S.D.) and daily for mortality (C). The relative body weight of the only survivor of the NDFL control group is depicted separately (NDFL*). Numbers represent clinical scores as described (20).

All unvaccinated mice displayed overt clinical signs (Fig. 4A) and weight loss (Fig. 4B) and succumbed to the infection within 4 days after challenge (Fig. 4C). Nine of the ten mice inoculated with NDFL succumbed to the infection within 5 days after challenge (Fig. 4C). At the end of the experiment, all these mice were positive for RVFV RNA in both the liver (Fig. 5A) and the brain (Fig. 5B). One mouse (mouse no. 2.8) of the group inoculated with NDFL survived the RVFV challenge. Productive infection in this mouse was confirmed by the detection of viral RNA in both the liver and the brain at the end of the experiment (Fig. 5) and the detection of N protein-specific antibodies by ELISA (Fig. 6). However, all mice vaccinated with NDFL-GnGc survived the challenge showing no clinical signs or weight loss (Fig. 4). Remarkably, the sera of these mice, obtained at the end of the experiment, were negative in the recN ELISA (Fig. 6). This suggested that sterile immunity was obtained by vaccination. PCR on liver and brain, however, demonstrated the presence of viral RNA in the livers of five mice (Fig. 5A). These results demonstrate that NDFL-GnGc, administered via the intramuscular route, protects mice from a lethal RVFV challenge.

Immune response in lambs

Sheep are the most susceptible target species for RVFV infections and lambs generally display the most severe disease symptoms. Therefore, after obtaining

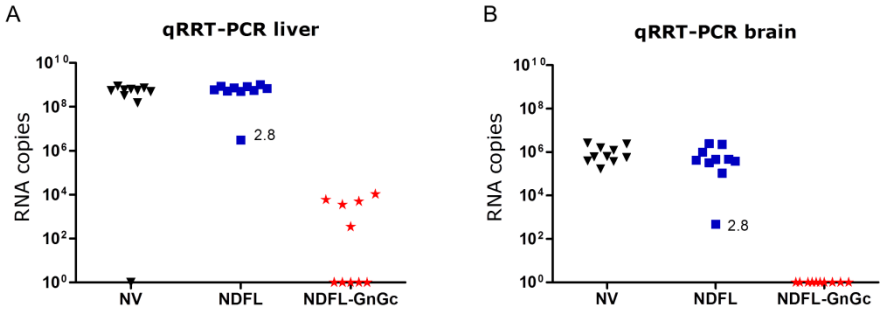

Figure.5. Detection of viral RNA in liver and brain samples by qRRT-PCR. Mice were non-vaccinated (NV) or inoculated with NDFL or NDFL-GnGc and subsequently challenged with RVFV. Liver samples (A) and brain samples (B) were collected from deceased or euthanized mice at the end of the experiment and were screened for the presence of viral RNA by Taqman qRRT-PCR.

promising results from the vaccination-challenge experiment in mice, we proceeded with determining the immunogenicity of NDFL-GnGc in lambs. Groups of six lambs were inoculated with NDFL-GnGc or NDFL control virus, via the intramuscular route, on days 0 and 21. The NDV-specific antibody responses were analyzed using an NDV ELISA. This ELISA demonstrated that NDV antibodies were induced in lambs vaccinated with NDFL-GnGc or control NDFL virus by the primary vaccination and that this response was boosted by the second vaccination (Fig. 7). Analysis of sera obtained three weeks after the first and second vaccination demonstrated that a single vaccination was sufficient to induce RVFV-neutralizing antibodies in all lambs and that the second vaccination clearly boosted this response (Fig. 8). Sera obtained prior to vaccination and sera obtained from the control group were incapable of neutralizing the virus.

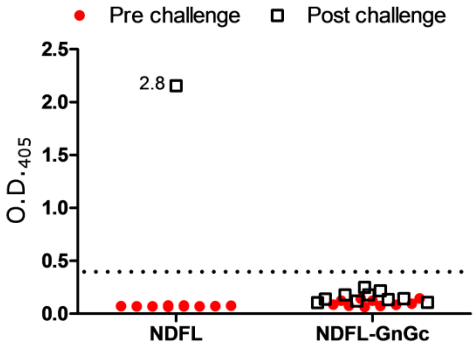

Figure.6. Detection of antibodies against the N protein pre- and post-RVFV challenge. The pre- and post-challenge sera of mice vaccinated with NDFL or NDFL-GnGc were screened for the presence of antibodies against the nucleoprotein using an indirect recombinant N-ELISA (n = 2). Cut-off value was set at 0.40 (mean of negative population [n =50], plus 2 times the corresponding standard deviation).

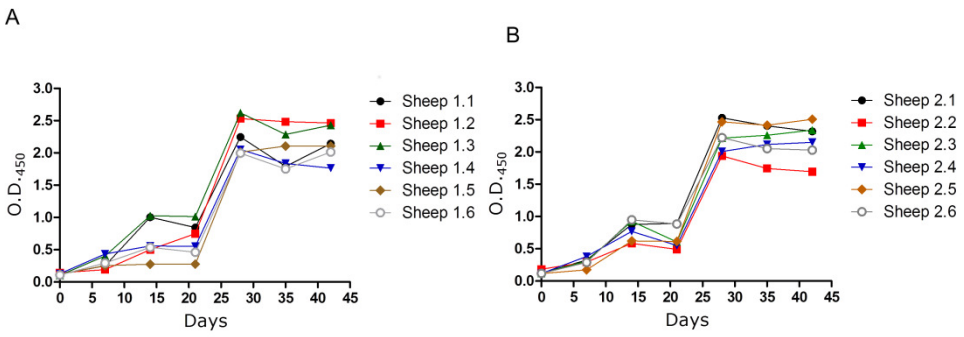

Figure.7. Detection of NDV-specific antibodies in lambs inoculated with NDFL-GnGc or NDFL control virus. Lambs were inoculated with 10^7.3 $TCID_{50}$ of control NDFL virus (A) or NDFL-GnGc. (B) via the intramuscular route, on days 0 and 21. Sera were analyzed by the FlockChek NDV ELISA, which was modified for the analysis of sheep antibodies.

DISCUSSION

We previously reported the production of a recombinant NDV that produces the RVFV Gn glycoprotein, named NDFL-Gn, and demonstrated that a homologous prime-boost vaccination of calves via the intramuscular route induces antibodies that are capable of neutralizing the RVFV *in vitro* (38). Although these first results were promising, the titres of the neutralizing antibodies were modest, varying from 8-to-32. We therefore aimed to improve the efficacy of our vaccine by expressing not merely the Gn but also the Gc protein. Adding the Gc protein provides an additional target for neutralizing antibodies (5, 6). Furthermore, simultaneous production of Gn and Gc by both mammalian (44) and insect (20, 42) cells was shown to result in the assembly of these proteins into VLPs and subsequent release from the producing cells. RVFV VLPs were shown to be highly immunogenic, being capable of providing solid protection without the need of an adjuvant (20, 47).

We have previously demonstrated that the production of Gn by NDV results in the insertion of the Gn protein in the NDV particle (38). Based on this result, it can be expected that the Gn and Gc proteins produced by NDFL-GnGc-infected cells, besides possibly assembling into VLPs, could also be incorporated into NDV virions. Although flotation experiments demonstrated that Gn and Gc are present in membrane-enveloped particles, it remained unclear whether the RVFV glycoproteins are present in NDV particles and/or VLPs. Experiments are ongoing to further address this issue.

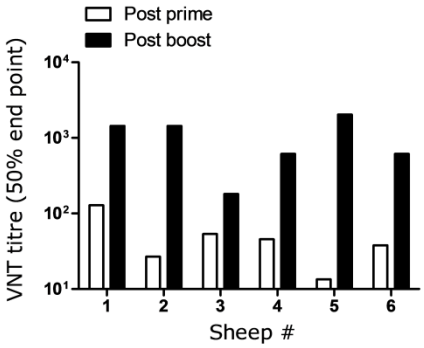

Figure.8. Virus neutralization test with sera from lambs inoculated with NDFL-GnGc. Individual sera obtained three weeks after the first or second inoculation were analyzed in quadruplet. Titres were calculated using the Spearman-Kärber method (37, 57).

The presence of the RVFV glycoproteins in the NDV virion could, at least in theory, influence the tropism and/or the infectivity of the virus. It is important to note that NDV is already very well capable of infecting mammalian species. Subsequent spread of the virus in mammals is, however, highly restricted (14-16, 22, 23, 58). In birds, propagation of NDV is promoted by the V protein, which counteracts the type-I interferon response and prevents apoptosis (48, 49). The fact that V-protein function is species specific (48) explains the highly restricted spread of NDV in mammals (14, 15, 21). Thus, even if the RVFV glycoproteins could provide NDV with new tools to expand its tropism or increase its infectivity for mammalian cells, it is considered unlikely that this will increase the virulence of the virus. Nevertheless, animal experiments are planned in which the spread and tropism of NDFL-GnGc will be studied in detail.

A homologous prime-boost vaccination of mice with NDFL-GnGc provided solid protection against a lethal challenge. Although no clinical signs were observed, in 50% of the mice, low levels of viral RNA were detected in the livers at the end of the experiment (Fig. 5A). Interestingly, in previous experiments where we inoculated mice with purified VLPs, virtually identical results were obtained (20). This suggests that the protective efficacy of NDFL-GnGc is very similar to that of VLPs when administered without adjuvant. It is interesting to note, however, that neutralizing antibodies were previously obtained after a single vaccination with VLPs (20), whereas two vaccinations with NDFL-GnGc were required to obtain such antibodies in the current work. That both vaccines were of comparable protective efficacy, despite the fact that VLPs were more potent in inducing neutralizing antibodies, suggests that vaccination with NDFL-GnGc also induces cell-mediated immunity. This is a plausible assumption, given that NDV is a potent inducer of type-I interferons (10, 13), which are known to promote antigen presentation via the MHC class-I pathway (9, 34, 41,

55) and that NDV has already been shown to induce strong CD8+ T-cell responses against a foreign antigen (45).

Another resemblance between the current work and our previous study, is the finding that none of the surviving mice that contained virus in the liver, seroconverted for antibodies against the N protein (Figs. 5 and 6). The amount of viral RNA copies detected in the livers of these mice were in the order of 10^3 RNA copies per ~5 mg of liver sample. In the mouse inoculated with NDFL that survived the challenge (mouse 2.8) ~10^7 viral RNA copies per liver sample were detected at the end of the experiment, and this mouse did seroconvert for antibodies against N (Figs. 5 and 6). Both our previous (20) and current findings suggest that RVFV infections can remain unnoticed by serology if the spread of the virus is sufficiently restricted by vaccination. The significance of these findings remains to be elucidated.

Although all mice vaccinated with NDFL-GnGc were completely protected from a lethal RVFV challenge, the induction of neutralizing antibodies required two vaccinations with a vaccine dose of 10^5.3 $TCID_{50}$. In our pilot vaccination experiment with lambs, a higher dose, namely 10^7.3 $TCID_{50}$, was used. In this experiment, the induction of neutralizing antibodies required only a single vaccination and this response was significantly boosted by a second vaccination. Experiments are planned to determine the protective efficacy of NDFL-GnGc after a single intramuscular vaccination in lambs.

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Chapter 4

Efficacy of three candidate Rift Valley fever vaccines in sheep

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ABSTRACT

Rift Valley fever virus (RVFV) is a mosquito-transmitted Bunyavirus that causes high morbidity and mortality among ruminants and humans. The virus is endemic to the African continent and the Arabian Peninsula and continues to spread into new areas. The explosive nature of RVF outbreaks requires that vaccines provide swift protection after a single vaccination. We recently developed several candidate vaccines and here report their efficacy in lambs within three weeks after a single vaccination. The first vaccine comprises the purified ectodomain of the Gn structural glycoprotein formulated in a water-in-oil adjuvant. The second vaccine is based on a Newcastle disease virus-based vector that produces both RVFV structural glycoproteins Gn and Gc. The third vaccine comprises a recently developed nonspreading RVFV. The latter two vaccines were administered without adjuvant. The inactivated whole virus-based vaccine produced by Onderstepoort Biological Products was used as a positive control. Five out of six mock-vaccinated lambs developed high viremia and fever and one lamb succumbed to the challenge infection. A single vaccination with each vaccine resulted in a neutralizing antibody response within three weeks after vaccination and protected lambs from viremia, pyrexia and mortality.

INTRODUCTION

RVFV is a zoonotic mosquito-borne Bunyavirus of the *Phlebovirus* genus. The RVFV genome comprises three single-stranded RNA segments (15). The large (L) segment encodes the viral polymerase. The medium (M) segment encodes two surface glycoproteins Gn and Gc (18). This segment also encodes a non-structural protein named NSm, which was shown to have an anti-apoptotic function, and a 78-kDa protein of which the function is not known (40). The small (S) segment encodes the nucleocapsid protein and a non-structural protein named NSs. The latter is recognized as the major virulence factor of the virus by counteracting host innate immune responses (3, 8, 20, 33).

The first recorded outbreak of RVF occurred on the shores of Lake Naivasha in Kenya in 1930, where 3500 lambs and 1200 ewes died of acute liver necrosis within a period of seven weeks. This outbreak and follow up studies revealed that the virus affects cattle, goats as well as humans (10, 16). RVFV has since been responsible for large outbreaks across the African continent and the Arabian Peninsula (1, 17, 21, 27-29), resulting in many casualties among both domesticated livestock and humans. RVF epidemics are generally preceded by explosions of mosquito vector populations after periods of exceptionally heavy rainfall (26). In semi-arid areas where the virus is endemic, outbreaks tend to occur every 25-35 years, whereas in savannah grasslands outbreaks occur more often, on average every 5 to 15 years. This cyclical nature of RVFV epidemics is still poorly understood. The largely unpredictable and explosive nature of RVFV outbreaks requires that vaccines are available that induce swift immunity after a single vaccination. These vaccines should not only be available to countries where RVFV is currently endemic but also to countries outside these areas since potential mosquito vectors are globally prevalent (32, 37, 38).

The first vaccine that was developed to control RVFV in livestock was produced by attenuation of a field isolate by serial passage in mouse brain (35). This so-called Smithburn vaccine is still commercially available and can be used to protect adult animals from disease. Due to residual virulence, use of the Smithburn vaccine in gestating and young animals, which are the most susceptible to disease, is however not recommended. The commercial vaccine based on inactivated whole virus, which was included in the current study, is expensive to produce and requires a booster and annual revaccination for optimal protection. Considering these shortcomings, efforts were made to develop safer live-attenuated vaccines. This work resulted in the development of the MP-12 vaccine (9), the Clone 13 vaccine (34) and, more recently,

in a recombinant virus that lacks both the NSs and NSm coding regions (4). Several efficacy and safety trials in natural target species were recently reported (5, 13, 30, 31, 39) and the Clone 13 vaccine was recently registered and commercialized in South Africa.

The vaccines based on live-attenuated RVFV that are now available hold great promise for the future control of RVFV in endemic areas. Mass deployment of these vaccines outside these areas could however suffer from safety concerns. Subunit vaccines, DNA-based vaccines or vector vaccines can provide alternatives of optimal safety. The efforts made in the past decades to develop novel RVFV vaccines were recently reported in several comprehensive reviews (6, 7, 19).

Four different candidate vaccines were recently developed in our laboratory. The first is a recombinant avian paramyxovirus that expresses the RVFV structural glycoprotein Gn (24) or both Gn and Gc (23). The LaSota strain of the Newcastle disease virus (NDV) that was selected for this application is used across the world as a vaccine for the control of NDV and is proven highly safe, even in the natural target species. Mammals are not natural reservoirs of NDV, which further adds to the safety of this approach and minimizes the chance of vaccination failure due to pre-existing immunity in the field. Another advantage of this vaccine is its efficient and low-tech production in embryonated eggs. We previously demonstrated that the NDV-based RVFV vaccine (here referred to as NDV-GnGc) provides protection in mice and that a single vaccination in sheep results in a neutralizing antibody response (23).

In another approach, more focused on application in humans, we developed two subunit vaccines. The first is based on the ectodomain of the Gn structural glycoprotein, the second on virus-like particles (VLPs) resulting from the co-expression of the Gn and Gc proteins in *Drosophila* cells. Both vaccines were shown to provide complete protection against a lethal RVFV challenge dose in mice (11). With the aim to further improve the efficacy of VLPs, we recently developed RVFV replicon particles, here referred to as nonspreading RVFV (NSR). NSR particles are capable of genome replication but incapable of autonomous spread. A single intramuscular vaccination with the NSR vaccine without added adjuvant provided solid protection in the mouse model (25).

We now report the efficacy of our candidate vaccines in sheep. Lambs were vaccinated once and challenged with a highly virulent RVFV isolate within three weeks after vaccination. Antibody responses were analysed by virus-neutralization tests and a commercial ELISA and viremia was monitored by quantitative real-time PCR and virus isolation. We show that a single vaccination with these novel vaccines

induces a neutralizing antibody response and protects against viremia, pyrexia and mortality.

MATERIALS AND METHODS

Preparation of the challenge virus

The RVFV virus that was used for challenge was previously rescued from cDNA (25). The recombinant 35/74 (rec35/74) virus was derived from the sequence of strain 35/74 (2) and was titrated on baby hamster kidney (BHK) cells as tissue culture infective dose 50 ($TCID_{50}$) using the Spearman-Kärber algorithm (22, 36) as previously described (25). The virus was handled under biosafety level-3 laboratory conditions in class-III biosafety cabinets.

Preparation of the vaccines

The ectodomain of the Gn protein was produced using the *Drosophila* expression system (Invitrogen, Carlsbad, CA, USA) essentially as previously described (11), although an improved purification procedure was used. Briefly, the sequence encoding the ectodomain of the Gn protein was fused to a sequence encoding a combined FLAG-tag/enterokinase (EK) cleavage site and three Strep-tags separated by glycine linkers to allow easy detection and purification of the monomeric protein. The gene was introduced in pMT/BiP/V5-HisA (Invitrogen), which was used to express the protein in *Drosophila* Schneider 2 (S2) cells that were cultured in 175m^2 cell culture flasks (Greiner Bio-One B.V.). The protein was purified from the culture medium using Strep-Tactin Sepharose according to the manufacturer's recommendations (IBA, Göttingen, Germany) and concentrated using an Amicon® Ultra-4 concentrator with a molecular mass cut-off of 30 kDa (Millipore, Billerica, MA, USA). The protein, named GneS3, was formulated in Stimune water-in-oil adjuvant (Prionics, Lelystad, The Netherlands) to a final concentration of 20 µg/ml.

Production of the NDFL-GnGc, here referred to as NDV-GnGc, (23) and the NSR vaccine (25) were previously reported. The administered doses and routes of vaccination are depicted in Table 1.

Vaccination and challenge

Thirty conventional European breed lambs were purchased from a commercial sheep farm in The Netherlands and divided over five groups. Lambs were vaccinated once at the age of six weeks (day 0), as depicted in Table 1. Mock-vaccinated lambs were

inoculated with PBS. On day 19 (days post challenge [DPC] 0), all lambs were challenged via the intraperitoneal route with 10^5 $TCID_{50}$ of RVFV rec35/74. EDTA blood samples were collected daily starting from day 19 (DPC 0) until day 26 (DPC 7) and again on days 28, 30, 33, 35, 37 and 40 (DPC 9, 11, 14, 15, 17 and 20). Serum samples were collected on days -7, 0, 7, 14, and daily from day 19 (day of challenge) to 26 (DPC 7) and finally on days 33 (DPC 14) and 40 (DPC 20). Body weights were determined weekly, on DPI -7, -1, 6, 13, 18, 25, 32, 39. Rectal body temperatures were determined on days -2 to 4 and, starting from day 17 (DPC -2), daily until the end of the experiment.

This experiment was conducted in accordance with the Act on Experimental Animals of The Netherlands and approved by the Ethical Review Committee of the CVI-WUR.

Table.1. Route and dose of vaccines

Vaccine	Route	Dose[a]	Adjuvant
OBP vaccine	Subcutaneous	Prescribed	Aluminum hydroxide gel
NDV-GnGc	Intramuscular	2 x 10^7 $TCID_{50}$	None
NSR	Intramuscular	10^7 $TCID_{50}$	None
GneS3	Subcutaneous	20 µg	Stimune water-in-oil

[a] All vaccines were administered in a volume of 1 ml

Quantitative real-time PCR

Viral RNA was isolated from plasma samples using the QuickGene DNA tissue kit S (DT-S, Fuji Photo Film Europe GmbH, Dusseldorf, Germany) with the following modifications. Proteinase K solution (EDT, DT-S kit, 30 µl) and 3 µl polyadenylic acid A (polyA 5 µg/µl, Sigma, St. Louis, MO, USA) were added to 250 µl lysis buffer (LDT, DT-S kit). Of this mixture, 250 µl was added to 300 µl plasma. The mixture was heated at 72°C for 10 min in a heating block and stored at -20°C until further analysis. RNA isolation was subsequently performed using the QG-Mini80 Workflow (Fuji Film). The lysate was mixed with 350 µl 99% ethanol before loaded on the column. After three wash steps with 750 µl wash buffer (WDT, DT-S kit) the RNA was eluted with 50 µl elution buffer (CDT, DT-S kit). The material was stored at -70°C until further analysis.

RNA samples (5 µl) were used for quantitative Taqman reverse-transcriptase real-time PCR (qPCR). The LightCycler RNA Amplification Kit HybProbe (Roche,

Almere, The Netherlands) was used and primers, probes and cycling conditions were used as previously described (12).

Virus isolation

Virus isolation from blood was performed on plasma samples. The plasma was mixed with an equal volume of culture medium (CO_2-independent medium [GIBCO™, Carlsbad, CA, USA] supplemented with 100 U/ml penicillin [GIBCO] and 100 µg/ml streptomycin [GIBCO], 2 mM L-Glutamine [GIBCO] and 5% fetal calf serum [FCS]). To prevent clotting of the plasma following contact with divalent cations present in the culture medium, 10 ml medium was put in a Vacutainer® heparin tube (BD, Franklin Lakes, NJ, USA) and after mixing combined with 30 ml of culture medium. The amount of heparin present in the resulting 40 ml of medium was sufficient to prevent clotting during virus isolation assays. The plasma samples were diluted by 2-fold dilution steps and added to 96-wells plates containing 40 000 BHK cells/well. The plates were incubated for 1h at RT, after which the culture medium was replaced. After five days, the plates were scored for cytopathic effect.

Serology

The ID Screen competition ELISA was used for the detection of RVFV-specific antibodies, according to the instructions of the manufacturer (ID-VET Montpellier, France). This ELISA makes use of plates coated with recombinant RVFV nucleocapsid (N) protein. Specifically, the ELISA detects competition of N protein-specific antibodies present in sera with a peroxidase-conjugated diagnostic antibody. Peroxidase activity is detected by conversion of 3,3′,5,5′-Tetramethylbenzidine.

Neutralizing antibodies were detected by virus neutralization test (VNT). Briefly, approximately 200 $TCID_{50}$ of virus was incubated with two-fold serial dilutions of sera for 2.5h at room temperature, after which BHK cells were added. After a 4-5 days incubation at 37 °C, the cultures were scored for cytopathic effect. Fifty percent end point titres were calculated using the Spearman–Kärber method as described previously [33].

Clinical chemistry

Clinical chemistry was performed with serum collected on the day of challenge (study day 19, DPC 0) and subsequently on days 20-25 (DPC 1-6), and on days 32 (DPC 14) and 38 (DPC 21). Enzyme analysis was performed using the Spotchem EZ SP-4430 analyser (Menarini Diagnostics, Valkenswaard, The Netherlands) using strips capable

of detecting alkaline phosphatase (ALP), alanine transaminase (ALT), creatinine, total protein (TP) and blood urea nitrogen (BUN).

Statistical analysis

Statistical analyses were performed with the one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Statistical differences with p-values <0.05 were considered significant.

RESULTS

Vaccination and challenge

After acclimatization for one week, all lambs were vaccinated as depicted in Table 1. On different time points after vaccination, the injection sites were inspected for possible adverse reactions. These inspections revealed mild to moderate swelling in four and five out of six lambs vaccinated with the OBP and GneS3 vaccine, respectively. No adverse reactions at injection sites were observed in lambs vaccinated with NDV-GnGc or NSR (data not shown).

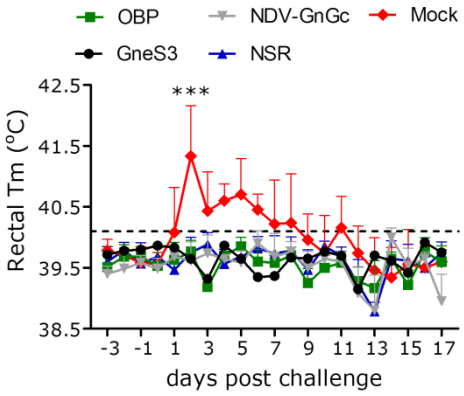

Figure.1. Rectal temperatures of vaccinated and mock-vaccinated (Mock) lambs before and after challenge with RVFV. Rectal body temperatures (°C) were determined daily. Fever was defined as a body temperature above 40.1°C (interrupted line). Rectal body temperatures of vaccinated lambs are depicted as averages (n=6) with SD. Rectal body temperatures of mock-vaccinated lambs determined after DPC 8 are depicted as averages of five measurements since one lamb from this group died on this day. A one-way ANOVA with Bonferroni's posttest was used to determine the statistical significance of peak rectal temperatures between groups, which is denoted by asterisks (***; p<0.001).

After challenge the rectal temperatures in the control group peaked at 2 DPC (Fig. 1). Peak rectal temperatures and the total days of fever (rectal body temperature ≥ 40.1°C) were both significantly lower (one-way ANOVA, $p<0.0001$) in all vaccinated groups compared to the mock-vaccinated group.

Viral RNA in plasma samples was detected by qPCR. High levels of viral RNA were detected in all but one of the mock-vaccinated animals, peaking on DPC 3 (Fig. 2A). Mean viral RNA levels, determined between DPC 0-21, were significantly lower in all vaccinated groups (repeated measures one-way ANOVA, $p<0.0001$) compared to the mock-vaccinated group.

Virus was isolated from plasma samples obtained from five of six mock-vaccinated lambs. In accordance to PCR results, the viral load peaked on day DPC 3 (Fig. 2B).

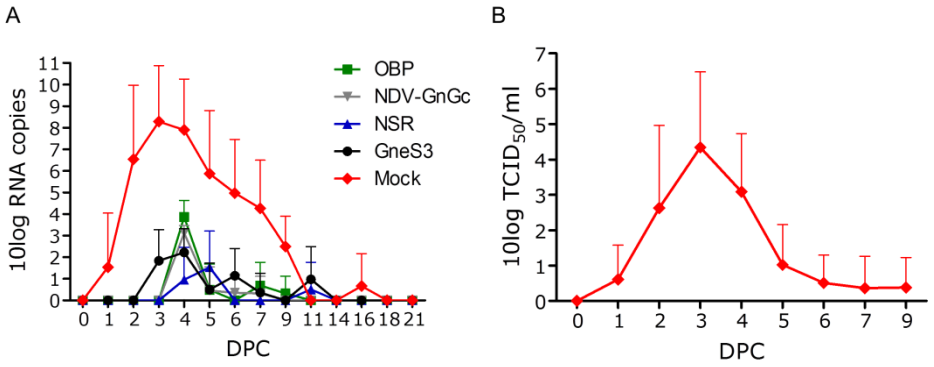

Figure.2. Monitoring of viremia in vaccinated and mock-vaccinated lambs. (A) Detection of viral RNA by qPCR in plasma samples of vaccinated and mock-vaccinated lambs obtained at different days post challenge (DPC) with RVFV. A repeated measures one-way ANOVA with Bonferroni's posttest was used to determine the statistical significance of differences in viral RNA levels (***; $p<0.0001$). Results obtained from vaccinated lambs are depicted as averages (n=6) with SD. The results obtained from mock-vaccinated lambs obtained after DPC 8 represent averages of 5 determinations, since one of the lambs died on this day. (B) Virus titres detected in plasma samples obtained from mock-vaccinated lambs at selected DPC. Virus isolations using plasma samples of vaccinated lambs were negative and are not depicted.

Because viral RNA levels in plasma samples of vaccinated lambs were very low as determined by qPCR, only selected samples with the highest PCR signals were used for virus isolation. These samples were either obtained on DPC 4 or 5 (Fig. 2A) and were tested in fourfold. No virus was isolated from these samples.

The body weights of the lambs were determined weekly. The lambs in the mock-vaccinated control group all displayed weight loss in the first two weeks after challenge (between days 18-25). Some weight loss, at a later time point (between days 25-32), was noted in five lambs vaccinated with NDV-GnGc and two lambs vaccinated

with the NSR vaccine (Fig. 3). Repeated measures ANOVA demonstrated that body weights determined between DPC -1 and 39 of mock-vaccinated lambs differed significantly from those of lambs vaccinated with the NSR vaccine ($p<0.05$).

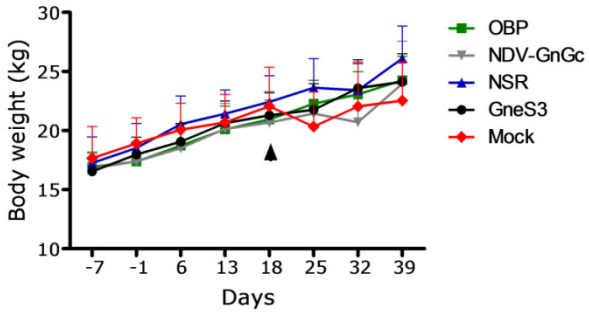

Figure.3. Body weights of lambs vaccinated once with the indicated vaccines. The moment of challenge infection is indicated by the arrowhead. Body weights of vaccinated lambs are depicted as averages (n=6) with SD. Body weights of mock-vaccinated lambs determined on days 32 and 39 are averages of 5 determinations since one lamb of this group died on day 27.

To investigate the occurrence of liver and renal damage, biochemical analysis was performed on serum samples using the Spotchem EZ dry chemistry analyzer. Statistical significance was tested by repeated measures one-way ANOVA with Bonferroni's correction. Hepatic dysfunction was assessed by sequential measurements of serum alkaline phosphatase (ALP), alanine transaminase (ALT) and total protein concentrations (TP) (Fig. 4). Total protein concentration is assumed to represent mostly albumin levels. In mock-vaccinated lambs, ALP and ALT levels were clearly increased when compared to vaccinated lambs and TP levels were clearly decreased. Statistical significance ($p<0.05$) was however only achieved when comparing ALP levels in plasma obtained from mock-vaccinated lambs (Mock) and NDV-GnGc-vaccinated lambs.

The concentrations of blood urea nitrogen (BUN) and creatinine were measured to assess renal function. BUN levels in serum obtained from mock-vaccinated lambs were clearly on average higher than levels detected in the serum of vaccinated lambs (Fig. 4). Statistical significance was however not achieved.

Creatinine levels in serum from mock-vaccinated lambs were significantly higher when compared to levels detected in serum from vaccinated lambs (OBP vaccine, $p<0.0005$; GneS3, $p<0.005$; NSR, $p<0.005$; NDV-GnGc, $p<0.05$).

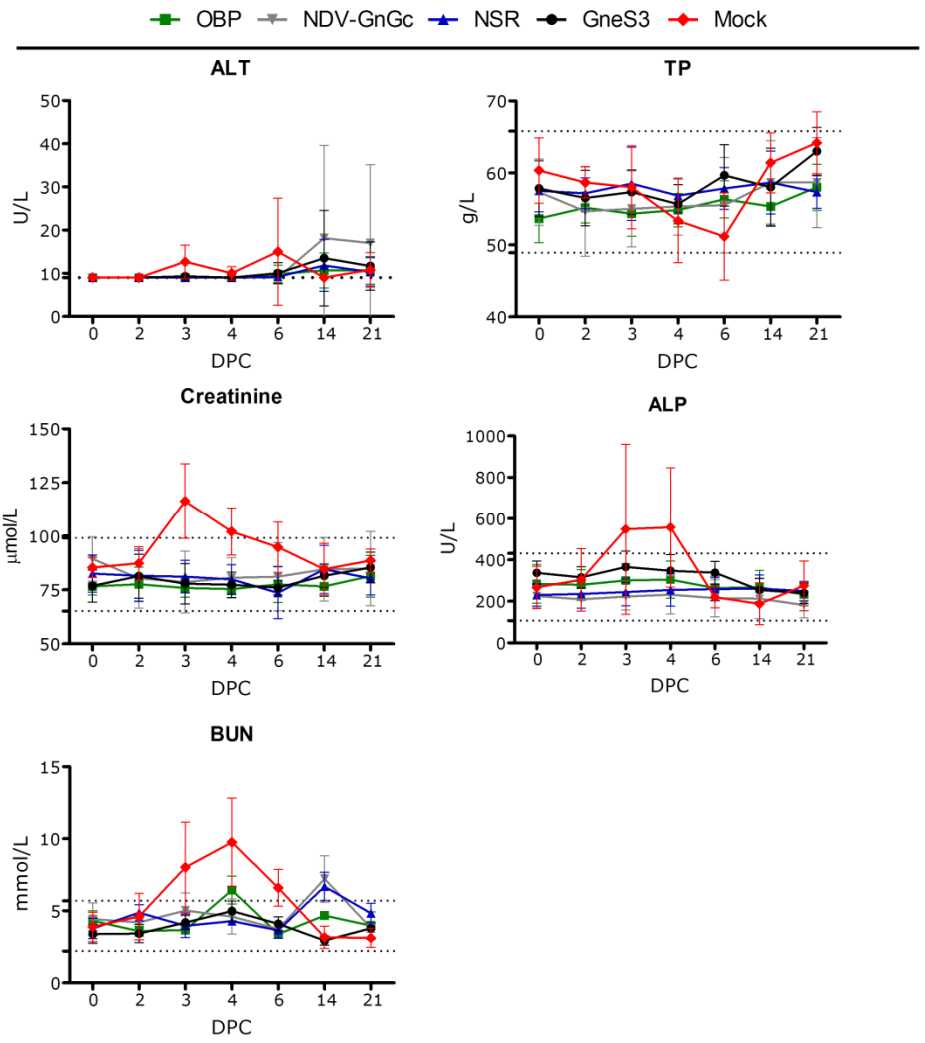

Figure.4. Biochemical analysis of serum samples obtained from lambs vaccinated with the indicated vaccines or mock-vaccinated lambs at different time points post challenge (DPC) with virulent RVFV. Alkaline phosphatase (ALP), alanine transaminase (ALT), creatinine, total protein (TP) and blood urea nitrogen (BUN) concentrations measured in serum of vaccinated lambs are depicted as averages (n=6) with SD. The results obtained with sera from mock-vaccinated lambs on DPC 14 and 21 are averages of 5 determinations, since one of the lambs died on DPC 8. Repeated measures one-way ANOVA with Bonferroni's posttest was used to determine the statistical significance of differences in biochemical values. The results of these analyses are described in the results section.

The liver of the one mock-vaccinated lamb that succumbed to the infection obtained at necropsy on DPC 8 was successfully used for virus isolation. No virus was isolated from any of the livers obtained from surviving lambs at the end of the experiment.

Antibody responses

Sera obtained on the day of vaccination (DPC -19) and at the day of challenge (DPC 0), which corresponds to 19 days post vaccination, were analysed for the presence of neutralizing antibodies by VNTs as previously described (24). Five out of six lambs in the group vaccinated with the OBP vaccine developed detectable levels of neutralizing antibodies (Fig. 5A). Neutralizing antibodies were also detected in two lambs vaccinated with the NDV-GnGc vaccine and four lambs vaccinated with GneS3. All six lambs vaccinated with the NSR vaccine developed neutralizing antibodies and the titers in this group were also the highest (Fig. 5A). No neutralizing antibodies were detected in mock-vaccinated lambs before challenge, but after challenge these sera contained the highest levels of neutralizing antibodies. The one mock-vaccinated lamb that did not display viremia also did not develop detectable levels of neutralizing antibodies. After challenge, neutralizing antibodies were detected in three lambs vaccinated with the OBP vaccine, five lambs vaccinated with NDV-GnGc or GneS3-vaccines and in all lambs vaccinated with the NSR vaccine.

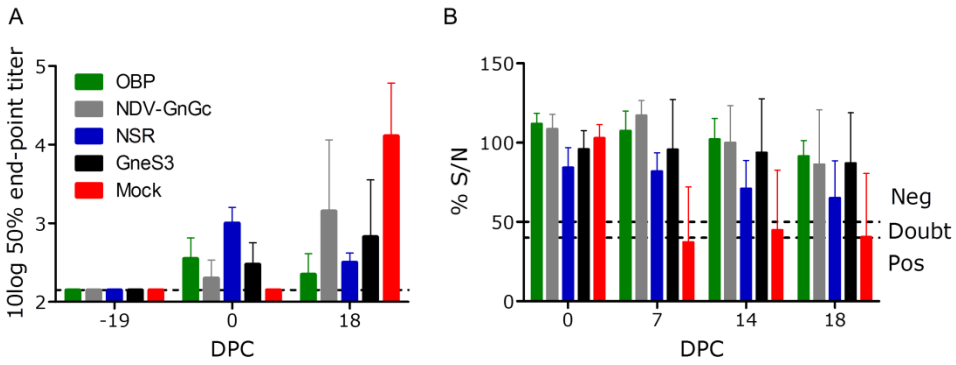

Figure.5. Virus neutralization test and N protein-specific antibody detection in sera obtained from lambs vaccinated with the indicated vaccines or mock-vaccinated lambs at different days post challenge (DPC). (A) Detection of neutralizing antibodies in sera obtained before vaccination (DPC -19), 19 days after the first vaccination (DPC 0) with the indicated vaccines and 18 days after challenge (DPC 18). Results obtained with plasma of vaccinated lambs are depicted as averages (n=6) with SD. The results obtained with plasma from mock-vaccinated lambs on DPC 18 are averages of 5 determinations, since one of the lambs died on DPC 8. The detection limit of the assay is depicted by the interrupted line. (B) Detection of N protein-specific antibodies in sera obtained from lambs vaccinated with the indicated vaccines or mock-vaccinated lambs at different days post challenge (DPC). S/N percentages less than or equal to 40% are considered positive, values between 40 and 50% are considered doubtful and values greater than 50% are considered negative. Results obtained with sera of vaccinated lambs are depicted as averages (n=6) with SD. The results obtained with sera from mock-vaccinated lambs on DPC 14 and 18 are averages of 5 determinations, since one of the lambs died on DPC 8.

All serum samples were also analysed by the commercial ID Screen® Rift Valley Fever Competition ELISA (ID-VET, Montpellier, France), which detects antibodies against the N protein of RVFV. All sera obtained from control lambs that developed viremia were scored positive in the ELISA (Fig. 5B). Sera obtained from lambs vaccinated with the OBP vaccine were all scored negative. In the groups vaccinated with GneS3, NDV-GnGc or NSR, one lamb in each group was scored positive after challenge.

DISCUSSION

We here report the efficacies of three novel RVFV candidate vaccines and a commercially available inactivated vaccine in sheep. The sheep in this study were vaccinated once at the age of six weeks and challenged 19 days later. Based on previous experimental work and field observations, RVFV infection in sheep of this age was expected to result in high viremia and fever, but only low mortality (14). In addition, it was anticipated that clinical signs in sheep that do not succumb to the infection remain unapparent. Considering this, the primary aim of our study was not to prevent morbidity and mortality, but instead to investigate if our vaccines can significantly reduce viremia. Reduction of viremia by vaccination is likely to decrease the chance of RVFV transmission by mosquito vectors and thereby reduce the number of livestock and human casualties during epidemics.

Analysis of plasma samples by qPCR and virus isolation demonstrated that all but one of the mock-vaccinated lambs developed high viremia. The reason why one of the lambs did not develop high viremia remains unclear. In mock-vaccinated lambs, RNA levels approaching 10^10 copies/ml were detected in plasma samples and virus isolations on these samples revealed titers of up to 10^6 $TCID_{50}$/ml. This result demonstrates that monitoring viremia by the M segment-based qPCR is much more sensitive than virus isolation. RNA levels in the blood were strongly reduced by vaccination with any of the vaccines and we were unable to isolate virus from plasma samples of vaccinated lambs. The results obtained from PCR analysis of plasma samples suggest that our vaccines do not provide sterile immunity within three weeks after vaccination but that vaccination does reduce viremia to levels undetectable by virus isolation. Recent studies suggested that vaccines based on live-attenuated RVFV can provide sterile immunity in sheep after a single vaccination. In contrast to the current work, titres of challenge virus in the blood of unvaccinated animals were not

reported in these studies (5, 13), making it difficult to appropriately qualify this suggestion.

Clinical disease was monitored by body weight measurements and biochemical blood analysis. Biochemical blood analysis revealed signs of liver and kidney damage in mock-vaccinated lambs in the first week after challenge and this correlated with a clear drop in body weight. Five lambs vaccinated with the NDV-GnGc vaccine and two lambs vaccinated with the NSR vaccine also revealed some weight loss in the second week after challenge. Although some variations in biochemical parameters were found in vaccinated animals, this did not correlate with weight loss and no viral RNA nor live virus was detected in the blood at these time points. The relevance of these findings therefore remain unclear.

Neutralizing antibodies are currently the only established correlate of protection against RVFV. A single vaccination with any of the vaccines evaluated in the current work resulted in a neutralizing antibody response within three weeks after vaccination. Interestingly, challenge infection of lambs vaccinated with the NDV-GnGc or GneS3 vaccine resulted in a clear boost of the neutralizing antibody response, whereas challenge infection of lambs vaccinated with either the OBP or the NSR vaccine did not boost this response (Fig. 5A). A boost in neutralizing antibodies is likely to result from replication of the challenge virus in the vaccinated animal, leading to the suggestion that vaccination with the OBP vaccine or the NSR vaccine resulted in the strongest immunity. Analysis of sera for antibodies against the N protein also provides insight into replication of the challenge virus in the vaccinated animals. Only one lamb in each group vaccinated with the candidate vaccines seroconverted for antibodies against N, again demonstrating that vaccination strongly reduced replication of the virus.

It was interesting to observe that neutralizing antibody levels in some animals declined somewhat within three weeks after vaccination (Fig. 5A). Similar findings were recently recorded during studies with the Clone 13 vaccine (39). The consequences of this finding remain speculative, particularly since we found that lambs even lacking detectable levels of neutralizing antibodies at the moment of challenge were protected from viremia and disease. Considering this, it will be valuable to elucidate the correlates of protection provided by the different candidate vaccines in future studies.

In conclusion, we here demonstrate efficacy of our vaccine candidates in the major natural target species after a single vaccination. Further evaluation of the

vaccines will include studies on minimum protective dose, onset and duration of immunity as well as safety and efficacy trials in gestating animals.

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Chapter 5

Heparan sulfate facilitates Rift Valley fever virus entry into the cell

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ABSTRACT

Rift Valley fever virus (RVFV), an emerging arthropod-borne pathogen, has a broad host and cell tropism. Here we report that the glycosaminoglycan heparan sulfate, abundantly present on the surface of most animal cells, is required for efficient entry of RVFV. Entry was significantly reduced by preincubating the virus inoculum with highly sulfated heparin, by enzymatic removal of heparan sulfate from cells and in cells genetically deficient in heparan sulfate synthesis.

Rift Valley fever virus (RVFV) belongs to the Phlebovirus genus of the Bunyaviridae family. Its negative-stranded tripartite RNA genome is encapsidated by nucleocapsid protein and is surrounded by a lipid-containing envelope which is derived from the trans-Golgi network (36). Two membrane-anchored viral glycoproteins, Gn and Gc, assemble into capsomers that cover the viral surface, following a T = 12 icosahedral symmetry (12, 19). The glycoproteins mediate host cell attachment of the virus and its subsequent entry into the cell (36). A 78-kDa glycoprotein of unknown function, which is an N-terminally extended version of Gn, has been reported as a third structural glycoprotein, present only in minute amounts in the viral envelope (21, 39).

RVFV is responsible for severe epidemics among ruminants in Africa and on the Arabian Peninsula, manifested by abortion storms and high mortality among young animals. The virus is transmitted by a wide variety of mosquito vectors. After introduction into the body by the bite of an infected mosquito, the virus can spread and infect different organs, including the brain (32). Humans can also be infected, and a small percentage develops severe disease (31, 36). Apart from mosquitoes, ruminants, and humans, a wide variety of animal hosts can be infected with RVFV, including nonhuman primates, rodents, and pets (11, 18). The virus also efficiently infects a large collection of different cell types in vitro (see Fig. S1 in the supplemental material). The broad host, tissue, and cell tropism of RVFV suggests the involvement of a common cell surface attachment factor to be utilized by RVFV to establish infection.

To initiate entry into the cell, viruses need to interact with a cellular receptor, which is sometimes preceded by binding to a primary attachment factor (30). The cell surface structures which facilitate entry of bunyaviruses remain largely unknown, although some receptors have been described. Beta3 integrins and nucleolin have been reported to be involved in attachment of hantavirus and Crimean-Congo hemorrhagic fever virus (genus Nairovirus), respectively (14, 42). DC-SIGN, a C-type lectin primarily restricted to interstitial dendritic cells and certain tissue macrophages (33), has been identified as a receptor for some phleboviruses, including RVFV (29). The broad cell tropism of RVFV, however, suggests that other receptors are important for virus entry into cells that lack DC-SIGN expression.

All eukaryotic cells are covered by a dense and diverse array of carbohydrates. These sugars are essential for many different biological processes (40). It is not surprising that many viruses have evolved to use these ubiquitous and accessible surface glycans as part of their strategy to infect cells (26). Two types of glycans, sialylated glycans (SGs) and glycosaminoglycans (GAGs), have been particularly noted

to play a role in virus entry. For example, influenza viruses specifically bind SGs, while dengue virus (7) and adenovirus (34) interact with GAGs to facilitate entry. Merkel cell polyomavirus has been reported to use both SGs and GAGs for entry (37).

We started to study the involvement of SGs and GAGs in RVFV entry by using a collection of Chinese hamster ovary (CHO) cell mutants with specific genetic deficiencies in glycan synthesis (see Table S1 in the supplemental material) (22). Thus, CHO lec1 and 15B (16, 38) mutants are incapable of synthesizing complex N-linked glycans, while the CHO lec2 mutant cells express sialic acid free N- and O-linked glycans (9). The CHO pgsA-745 cell mutant (10) is deficient in the synthesis of GAGs. To facilitate our studies, we made use of the recently developed nonspreading RVFV (here referred to as $RVFV_{ns}$) (25). In contrast to wild-type virus, $RVFV_{ns}$ can be handled outside biosafety level 3 facilities, while the presence of the enhanced green fluorescent protein (eGFP) gene in the viral genome enables infection to be easily monitored. The mutant lec1 and 15B CHO cells and, to a somewhat lesser extent, the CHO lec2 cells were as efficiently infected with $RVFV_{ns}$ as the parental wild-type cells (pro5 and K1), suggesting that N- and O-linked SGs play a minor role in virus infection. In contrast, infection of CHO psgA-745 was dramatically reduced, indicating that GAGs are important for $RVFV_{ns}$ infection (Fig. 1).

GAGs are linear polysaccharides that can be attached to proteins to form proteoglycans. There are five classes of GAGs, heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate, and hyaluronic acid (28). Of these GAGs, HS has been identified as an attachment factor for a number of viruses and, unlike other GAGs, is abundantly expressed on most cell types (28). We first evaluated whether $RVFV_{ns}$ infection could be inhibited by inclusion of soluble heparin, a GAG analogue of HS, as a competitor in the inoculum (23). As a control virus, we used a nonspreading vesicular stomatitis virus (here referred to as VSV_{ns}), a VSV-ΔG/GFP recombinant virus pseudotyped with its authentic fusion glycoprotein G (5). Preincubation of $RVFV_{ns}$ with heparin reduced infection of CHO K1 cells in a dose-dependent manner, whereas no such effect was observed for VSV_{ns} (Fig.2A).

To confirm the involvement of HS in RVFV entry, CHO K1 cells were treated prior to infection for 1 h at 37°C with different heparinases or chondroitinase to remove HS or CS/DS, respectively, from the cell surface (Fig. 2B). Enzymatic treatment of CHO K1 cells with heparinase caused a marked increase of infection with VSV_{ns} of more than 2-fold. In contrast, independently of the different heparinases used, infection of heparinase-treated cells with $RVFV_{ns}$ was reduced to about 50%. No effect of chondroitinase treatment was observed. The reduced $RVFV_{ns}$ infectivity of

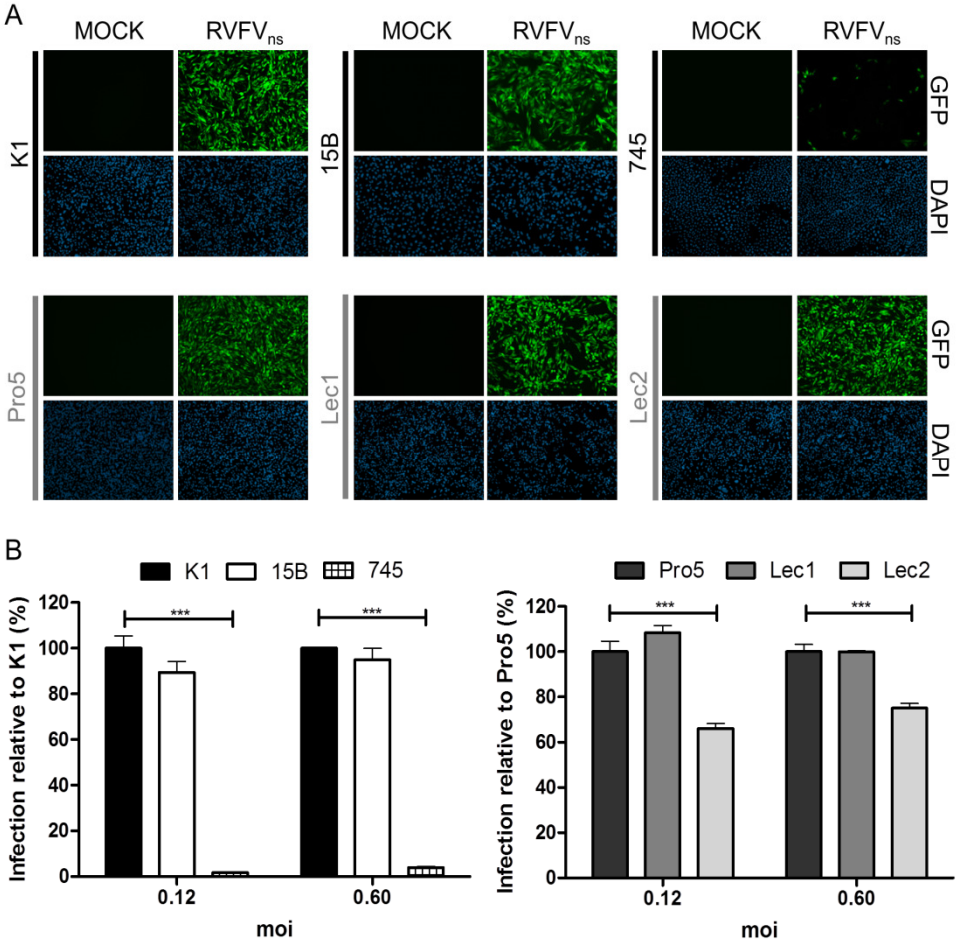

Fig.1. RVFV$_{ns}$ infection is drastically reduced in the absence of GAGs. The CHO 15B and CHO 745 mutant cells derived from the CHO K1 cell line and the CHO lec1 and CHO lec2 mutant cells derived from the CHO Pro5 cell line were cultured in Ham's F-12K medium (Invitrogen) supplemented with 10% fetal calf serum (FCS). Subconfluent monolayers were infected with RVFV$_{ns}$ at different multiplicities of infection (moi's of 0.12 and 0.6). At 20 hours postinfection (h.p.i.), the cells were washed once with phosphate-buffered saline (PBS) and prepared for fluorescence microscopy (A) or fluorescence-activated cell sorter (FACS) analysis (B). (A) Cells were fixed with 3.7% formaldehyde–PBS for 20 min at room temperature, and representative pictures were taken using an Evos fl microscope (AMG) (magnification, X4; data shown refer to infections at an MOI of 0.6). Nuclei were counterstained with 4',6'diamidino-2-phenylindole (DAPI). MOCK, mock-infected cells. (B) Cells were trypsinized and fixed with 3.7% formaldehyde–PBS for 20 min at room temperature, and RVFV$_{ns}$ infected (GFP-positive) cells were quantified by FACS (FACSCalibur). Graphical data shown are normalized to the infectivity of CHO K1 or CHO Pro5 cells and are representative of the results of two independent experiments performed in triplicate. Significant differences between conditions are indicated (analysis of variance [ANOVA]-Bonferroni); ***, P <0.001. Error bars represent standard deviations (SD).

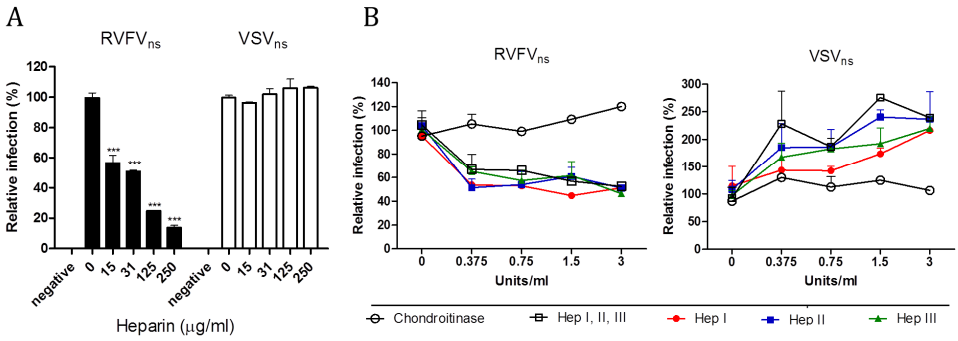

Fig.2. RVFV$_{ns}$ infection is decreased in the presence of heparin and after enzymatic removal of heparan sulfate from the cell surface. (A) RVFV$_{ns}$ and VSV$_{ns}$ were incubated with different concentrations of soluble heparin (MPBio) for 10 min at room temperature in culture medium, prior to infection of CHO K1 cells. At 8 (VSVns) or 20 (RVFV$_{ns}$) h.p.i., infection was quantified by FACS analysis as described for Fig. 1. The data shown correspond to the results of a representative set of two independent experiments performed in triplicate. (B) GAGs were enzymatically removed from the cell surface of CHO K1 cells. Chondroitinase ABC (specific for chondroitin and dermatan sulfate), heparinase I (specific for heparin and highly sulfated domains), heparinase II (specific for heparin and heparan sulfate), and heparinase III (specific for heparan sulfate), all purchased at Sigma, were dissolved in resuspension buffer (20mM HEPES [pH 7.5], 50 mM NaCl, 4 mM CaCl2, 0.01% bovine serum albumin [BSA]). Dilutions were prepared in digestion buffer (20Mm HEPES [pH 7.5], 150 mM NaCl, 4 mM CaCl2, 0.1% BSA). CHO K1 cells were treated for 1 h at 37°C with heparinase I, II, or III, with a combination of them, or with chondroitinase at the indicated concentrations. The cells were washed twice with culture medium and then incubated with RVFV$_{ns}$ or VSV$_{ns}$ for 30 min at 37°C. The cells were washed twice with culture medium and further incubated in culture medium at 37°C for 8 (VSV$_{ns}$) or 20 (RVFV$_{ns}$) hours, after which infection was quantified by FACS analysis as described for Fig. 1. Data were obtained from two independent experiments performed in duplicate. Significant differences between conditions are indicated (ANOVA-Bonferroni); ***, P<0.001. Error bars represent SD.

heparinase-pretreated cells was confirmed using six different cell lines, while the susceptibility of these cells to VSV$_{ns}$ was not affected (see Fig. S2 in the supplemental material).

To further characterize the interaction between RVFV and highly sulfated HS polysaccharides, we analyzed RVFV$_{ns}$ infection of CHO K1 cells that were subjected to passage in the presence of 50 mM sodium chlorate ($NaClO_3$). $NaClO_3$ is known to inhibit the addition of *O*-sulfate groups to GAGs (1, 35). Importantly, we did not observe any apparent changes in growth rate or cell morphology of CHO K1 or A549 cells cultured for 7 days in the presence of up to 70 mM NaClO3 (data not shown). Infection by RVFV$_{ns}$ of CHO K1 or A549 cells maintained in the presence of $NaClO_3$ was dramatically reduced (Fig. 3A and B), in contrast to infection by VSV$_{ns}$, suggesting that *O*-sulfation of HS is necessary for efficient RVFV$_{ns}$ infection of both cell lines.

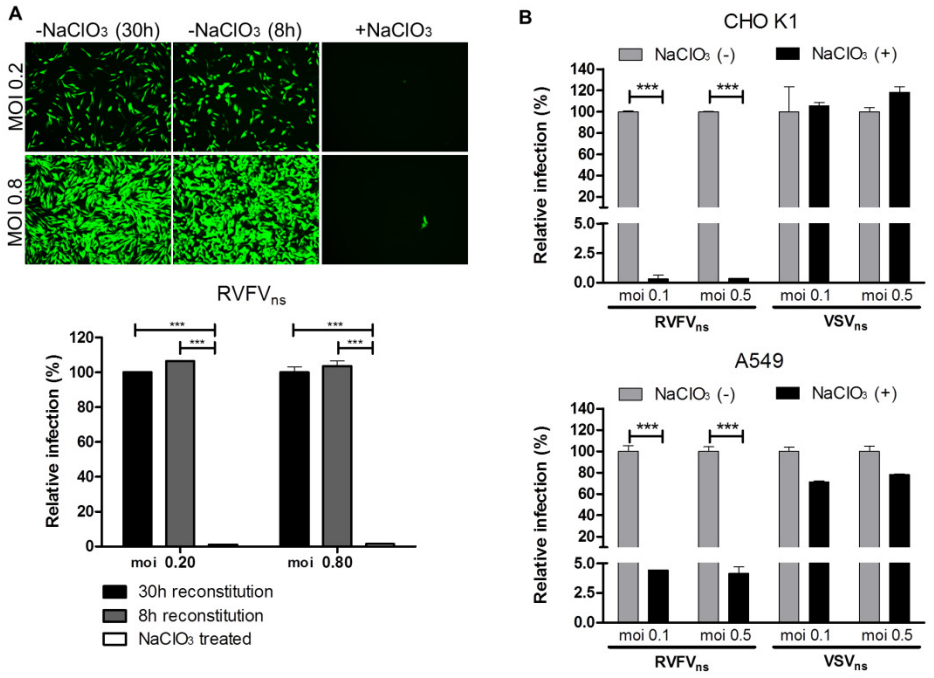

Fig.3. RVFV$_{ns}$ infection strongly depends on sulfation of heparan sulfate. (A) CHO K1 cells were subjected to two passages in culture medium containing 50 mM NaClO3 (Sigma) and subsequently cultured in the presence of 50mM sodium chlorate, or in chlorate-free culture medium for 30 or 8 h prior to infection, to reverse the chlorate effect. Twenty hours postinfection, cells were analyzed by fluorescence microscopy or FACS analysis as described for Fig. 1. Graphical data shown are normalized and are representative of the results of two individual experiments performed in triplicate. (B) A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS). CHO K1 or A549 cells were subjected to two passages in culture medium containing 50 mM NaClO3 (Sigma) and subsequently cultured in the presence of 50 mM sodium chlorate [NaClO3 (+)] or in chlorate-free culture medium [NaClO3 (-)] for 8 h prior to inoculation with RVFV$_{ns}$ or VSV$_{ns}$ at the indicated moi. At 8 (VSV$_{ns}$) or 20 (RVFV$_{ns}$) h.p.i., cells were analyzed by fluorescence microscopy or FACS analysis as described for Fig. 1. Significant differences between conditions are indicated (ANOVA-Bonferroni; ***, P<0.001). Error bars represent SD.

Next we tested the susceptibility of CHO pgsD-677 cells (CHO HS[-]), which are deficient in HS synthesis (27), to RVFV$_{ns}$ and VSV$_{ns}$ infection. Compared to infection of the parental CHO K1 cells with RVFV$_{ns}$, infection of CHO HS[-] cells was greatly reduced (>97%), whereas VSV$_{ns}$ infection of these cells was enhanced (Fig. 4). To confirm HS dependency of RVFV, an autonomously replicating virus was included in this experiment. This virus expresses the eGFP reporter from its genome in a manner similar to that seen with RVFV$_{ns}$ and was rescued as previously described (25). Also, this virus displayed significantly reduced infectivity on CHO HS[-] cells. Altogether, the observations strongly support the idea of an important role of HS for RVFV infection.

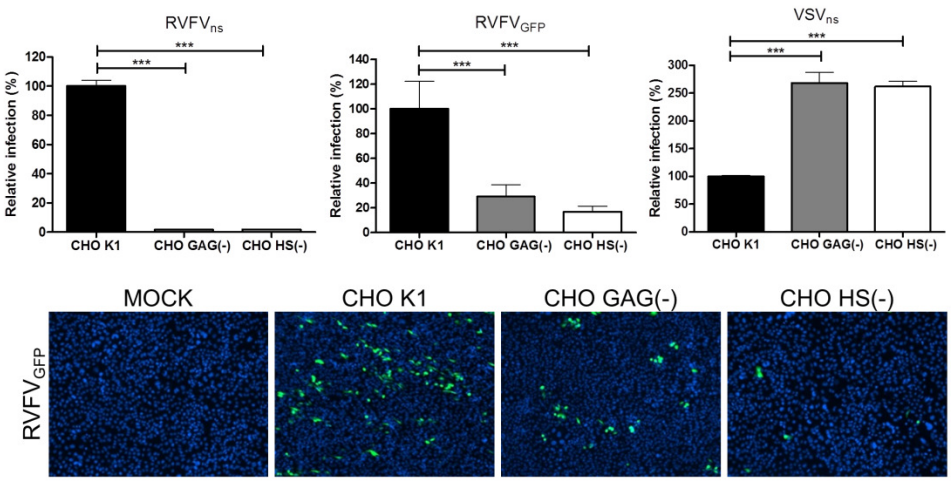

Fig.4. Entry of RVFV$_{GFP}$ into GAG-deficient CHO cells is inefficient due to the lack of heparan sulfate. Mutant CHO pgsD-677 cells (HS[-], able to express all GAGs except for heparan sulfate) and pgsA-745 cells (CHO GAG[-], deficient in expression of all GAGs) and the parental CHO K1 cells were inoculated with RVFV$_{ns}$, VSV$_{ns}$, or RVFV$_{GFP}$. At 8 (VSV$_{ns}$), 16 (RVFV$_{GFP}$), or 20 (RVFV$_{ns}$) h.p.i., cells were analyzed by fluorescence microscopy and GFP-expressing RVFV-infected cells were quantified. Graphical data shown are normalized to the infectivity of CHO K1. Significant differences between conditions are indicated (ANOVA-Bonferroni); ***, $P < 0.001$. Error bars represent SD.

Many viruses have been reported to utilize HS for host cell attachment (reviewed in reference 28). Interactions of viruses with heparan sulfate are often based on electrostatic contacts between the negatively charged sulfate groups on HS and clusters of basic residues occurring in viral surface proteins. These clusters often comprise a BBXB or a BBBXXB motif (B, basic amino acid; X, any amino acid) (3). In analyzing the complete M segment-encoded polypeptide sequence of the RVFV used in this study (strain 35/74; GenBank accession number JF784387.1), we identified two overlapping BBBXXB HS binding motifs (116-**R**CE**RRR**DAK-124) in the pre-Gn region of the 78-kDa protein (where the boldface characters represent the first and the underlining represents the second motif), while no HS binding motifs were identified in the Gn or Gc protein sequence. The 78-kDa protein is considered to be a minor structural glycoprotein (39) and is apparently dispensable. RVFV recombinants lacking the pre-Gn region display wild-type growth kinetics in cell culture, calling into question whether the basic amino acid motifs in the protein indeed contribute to HS binding (15, 41). Alternatively, other linear or nonlinear arrangements of basic residues in Gn and/or Gc may create an HS binding motif in the tertiary structures of these glycoproteins (13, 17). Clearly, the identification of the HS binding site on the viral surface requires further study.

HS dependency has for some viruses been shown to be acquired after repetitive virus passage in cell culture through the acquisition of single or multiple amino acid substitutions in the surface glycoproteins, creating a positively charged HS binding motif (6, 8, 20, 24). The RVFV 35/74 strain was isolated from the liver of a sheep that died during an RVFV outbreak in the Free State province of South Africa in 1974. The virus was amplified in suckling mouse brain and subjected to three passages in BHK-21 cells (25). To study the possible acquisition of a HS-binding motif during these procedures, the M segment-encoded polypeptide sequence was aligned with those of four RVFV isolates that had been directly sequenced from serum or organ material of infected animals (2, 4). This analysis did not reveal the presence of additional basic amino acids in the 35/74 sequence (see Table S2 in the supplemental material), indicating that the requirement for HS for efficient entry of the RVFV used in this study is not likely the result of cell culture adaptation.

Although infection of RVFV in the GAG- and HS-deficient CHO cells was dramatically reduced, we observed residual infection of both cell lines. It remains to be determined whether this infection in the absence of HS is explained by the binding of RVFV to another, unidentified attachment factor or receptor present on these cells.

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SUPPLEMENTAL MATERIAL

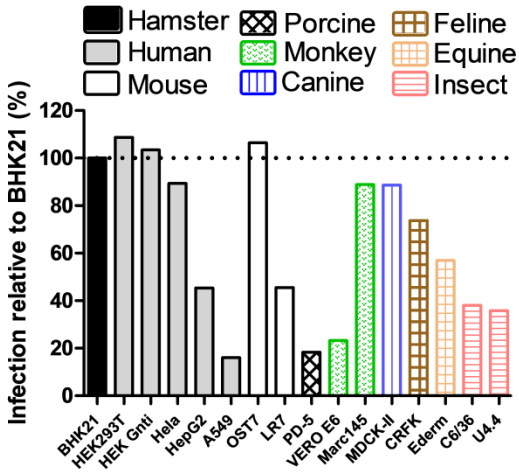

Fig.S1. Susceptibility of different cell lines for $RVFV_{ns}$. Subconfluent monolayers were inoculated with $RVFV_{ns}$ (moi 0.9; titer as determined on BHK-21 cells). Twenty hours post infection the percentage of GFP positive cells was quantified by FACS, as described for Fig. 1. Graphical data shown are normalized to the infectivity on BHK-21 cells.

Table.S2. Characteristics of the Chinese hamster ovary cell types used in this study

Cell line (reference)	ATCC #	Deficiency	Phenotype
CHO K1 (22)	CCL-61	***	Parent of pgsA-745, pgsD-677 and 15B
CHO pgsA-745 (10)	CRL-2242	Cells have a defect in xylosyltransferase I, the first sugar transfer in glycosaminoglycan synthesis.	Cells do not produce glycosaminoglycans.
CHO pgsD-677 (28)	CRL-2244	Cells lack both N-acetylglucosaminyltransferase and glucuronyltransferase activities.	Cells do not produce heparan sulfate (they do produce 3 to 4 fold more chondroitin sulfate then CHO K1).
CHO 15B (16)	Not available	Cells lack the processing enzyme N-acetylglucosaminyl (GlcNac) transferase I.	Cells are unable to form complex and hybrid N-linked glycans resulting in the expression of high-mannose N-linked oligosaccharides.
CHO Pro5 (38)	CRL-1781	***	Parent of lec1, lec2.
CHO Lec1 (38)	CRL-1735	Cells lack the processing enzyme N-acetylglucosaminyl (GlcNac) transferase I.	Cells are unable to form complex and hybrid N-linked glycans resulting in the expression of high-mannose N-linked oligosaccharides.
CHO Lec2 (9)	CRL-1736	Cells are defective in the cytidine monophosphate-sialic acid (CMP-Sia) transporter.	Cells are unable to translocate CMP-Sia, a substrate for the sialylation of proteins and lipids, across Golgi vesicle membranes, creating an asialo phenotype containing terminal galactosyl residues.

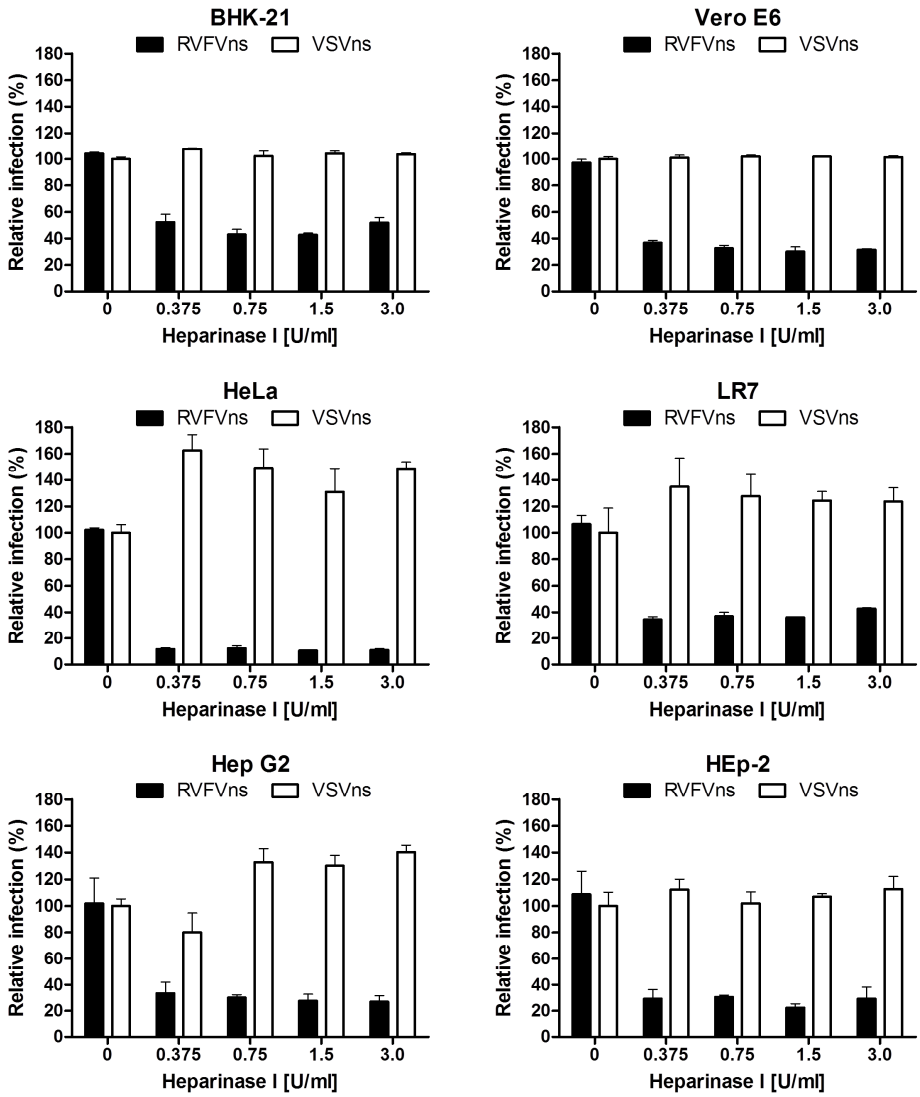

FIG. S2. $RVFV_{ns}$ infection of different cell lines is decreased after enzymatic removal of heparan sulfate from the cell surface. Vero E6, HeLa, HEp-2 (all derived from ATCC), Hep G2 (HPA Culture Collections) and LR7 cells (1) were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS). BHK-21 cells (ATCC) were cultured in GMEM (Invitrogen) supplemented with 4% tryptose phosphate broth, 1% MEM NEAA (Invitrogen), and 5% FCS. Cells were treated with heparinase I and infection was performed as described for Fig. 2. Infection (GFP-positive cells) was quantified by FACS as described for Fig. 1 at 8 (VSV_{ns}) or 20 ($RVFV_{ns}$) hours post infection. Data are representative of two independent experiments performed in triplicate. Error bars represent SD.

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Chapter 6

Acid-activated structural reorganization of the Rift Valley fever virus Gc fusion protein

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ABSTRACT

The entry of the enveloped Rift Valley fever virus (RVFV) into its host cell is mediated by the viral glycoproteins Gn and Gc. We investigated the RVFV entry process and, in particular, its pH-dependent activation mechanism using our recently developed nonspreading-RVFV-particle system. Entry of the virus into the host cell was efficiently inhibited by lysosomotropic agents that prevent endosomal acidification and by compounds that interfere with dynamin- and clathrin-dependent endocytosis. Exposure of plasma membrane-bound virions to an acidic pH (<pH 6) equivalent to the pH of late endolysosomal compartments allowed the virus to bypass the endosomal route of infection. Acid exposure of virions in the absence of target membranes triggered the class II-like Gc fusion protein to form extremely stable oligomers that were resistant to SDS and temperature dissociation and concomitantly compromised virus infectivity. By targeted mutagenesis of conserved histidines in Gn and Gc, we demonstrated that mutation of a single histidine (H857) in Gc completely abrogated virus entry, as well as acid-induced Gc oligomerization. In conclusion, our data suggest that after endocytic uptake, RVFV traffics to the acidic late endolysosomal compartments, where histidine protonation drives the reorganization of the Gc fusion protein that leads to membrane fusion.

INTRODUCTION

Rift Valley fever virus (RVFV) is an emerging pathogen that affects ruminants and humans and is transmitted between susceptible hosts by different species of mosquitoes. RVFV was first isolated in Kenya in 1930 and has since spread throughout the African continent and the Arabian Peninsula (12, 46). An outbreak of RVFV can have a devastating socioeconomic impact on the region (2, 18, 50) and is characterized by abortion storms in adult livestock and high mortality among newborns. Humans can also be infected through contact with infected tissues or via mosquito bites, typically causing a self-limiting febrile illness. A small percentage of human infections result in hemorrhagic fever or encephalitis with generally fatal outcome (41).

RVFV belongs to the *Phlebovirus* genus of the *Bunyaviridae* family, which comprises four additional genera (*Orthobunyavirus*, *Hantavirus*, *Nairovirus*, and *Tospovirus*). Members of the *Bunyaviridae* family are enveloped viruses of ~100 nm in size that have a tripartite negative-strand RNA genome which is replicated in the cytoplasm. The genomic segments are encapsidated by the nucleocapsid protein, forming the ribonucleoproteins (RNPs) (56). The lipid envelope surrounding the RNPs contains an ordered shell made up by units of two glycoproteins, Gn and Gc (20). These glycoproteins are responsible for entry into the host cell, but their precise functional roles in receptor binding and fusion are poorly understood.

Gn and Gc are type I membrane glycoproteins which form heterodimers after posttranslational processing of a glycoprotein precursor in the endoplasmic reticulum. By virtue of a Golgi localization signal in Gn, the Gn-Gc heterodimers are targeted to the Golgi apparatus (56, 65). Here, interaction between the Gn carboxyl-terminal cytoplasmic tail and the RNPs allows virus budding into the lumen of the Golgi cisternae (45, 47, 56). In the case of the RVFV virion, a 78-kDa protein consisting of pre-Gn and Gn regions has been identified as a third but minor structural component (27, 60). Cryoelectron microscopy studies of RVFV virions showed that the viral envelope comprises 720 heterodimers of Gn (54 kDa) and Gc (56 kDa), forming 110 cylinder- shaped hexamers and 12 pentamers according to a T = 12 icosahedral lattice (13, 24).

Enveloped viruses carry dedicated proteins in their envelope which mediate fusion between viral and cellular membranes, allowing translocation of the viral genome into the cytoplasm. This membrane fusion process is driven by structural rearrangements in the metastable viral fusion protein which are triggered by receptor

binding, proteolytic cleavage, or the acidic pH of endosomes, allowing fusion to occur at the right time and place (8). Viral fusion proteins have been divided into three classes (classes I, II, and III) based on their structural features (67). The Gc glycoprotein of bunyaviruses has been proposed to be a class II viral fusion protein (15, 53). Similar to class II fusion proteins of alphaviruses and flaviviruses, Gc is predicted to be mainly composed of β-sheet structures, is synthesized from a polyprotein downstream of a companion protein (Gn), and assembles into a heterodimer in the ER (15, 48, 62, 63). Common to all class II fusion proteins is that they utilize the low pH in the acidified endosome to activate their fusion process. Low pH triggers the dissociation of the glycoprotein dimeric state, resulting in the exposure and subsequent insertion of a highly hydrophobic stretch of amino acids. Insertion of this fusion peptide into the target membrane is followed by trimerization of the monomeric fusion proteins. A stable trimeric hairpin structure is subsequently formed which brings together the fusion peptide and transmembrane domain at one end of the molecule, mediating the fusion of the cellular and viral membrane.

While the acid induced conformational changes of class II viral fusion proteins have been well documented (for an comprehensive review, see reference 30), the mechanism of low-pH triggering is not well understood. Protonation of histidines has been suggested to act as a molecular switch that triggers the activity of pH-activated viral fusion proteins (4, 28). The rationale for this hypothesis is that histidine is the only amino acid residue with a side chain pKa value (pKa 6.4) within the pH range found in the endolysosomal pathway (pH 4.5 to 6.5) (28, 54). Evidence in support of a crucial role for histidine protonation in promoting fusion protein rearrangements leading to fusion has been obtained for a number of low pH-dependent viruses (3, 4, 6, 14, 26, 34, 36, 49, 57, 61).

Knowledge of the fusion mechanism of bunyaviruses is still in its infancy. In agreement with a class II virus classification, bunyaviruses appear to have an acid-dependent fusion machinery. Exposure of cells overexpressing RVFV Gn and Gc to low pH results in the formation of multinucleated cells (11). Acid-induced syncytium formation has also been reported for other members of the *Bunyaviridae* family, including Uukuniemi virus (UUKV, *Phlebovirus* genus), La Crosse virus (*Orthobunyavirus* genus), and Hantaan virus (*Hantavirus* genus) (1, 39, 42, 48). In accordance with the hypothesis of acid-induced fusion activation, bunyaviruses are sensitive to acidotropic agents which raise the pH of intracellular endocytic compartments (55, 58). In addition, acid exposure of plasma membrane-bound UUKV could bypass the endosomal acidic pH requirement needed for infection (39).

In this study, we have examined the entry mechanism of RVFV. Studies of natural RVFV strains have been hampered by the requirement of handling the virus under biosafety level 3 (BSL3) containment. We have taken advantage of our recently established nonspreading-RVFV ($RVFV_{ns}$) production system, which can be handled outside BSL3 containment (33). This system allows the efficient production of RVFV particles upon transfection of a single Gn/Gc expression plasmid into a replicon cell line which maintains the replication machinery of RVFV. The expression of the enhanced green fluorescent protein (eGFP) gene from the viral genome enables infection to be easily monitored. Most importantly, the system greatly facilitates Gn and Gc structure/function studies as long as particle assembly is not compromised. Using $RVFV_{ns}$, we have examined the endocytic uptake of the virus, the low-pH dependence and kinetics of virus entry, and the effects of low-pH treatment of the virus on infectivity and rearrangement of the glycoproteins, as well as the role of conserved histidines in Gn and Gc in virus entry.

MATERIALS AND METHODS

Cells and viruses

BHK cells were grown in Glasgow minimum essential medium (GMEM; Invitrogen) supplemented with 4% tryptose phosphate broth, 1% minimum essential medium nonessential amino acids (MEM NEAA; Invitrogen), and 5% (vol/vol) fetal calf serum (FCS; Bodinco). For maintenance of BHK-Rep2 cells (33), geneticin (G-418) was used at a concentration of 1 mg/ml. A549 and CHO K1 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Lonza Biowhittaker) and Ham's F-12K medium (Invitrogen), respectively, supplemented with 10% FCS. Cells were grown at 37°C and 5% CO_2. *Drosophila* Schneider (S2) cells (Invitrogen) were grown at 27°C in serum-free InsectXpress medium (Lonza). Recombinant RVFV ($RVFV_{rec}$) and $RVFV_{ns}$ have both been rescued from cDNA as described previously (33). VSV-ΔG/GFP/G* is a recombinant vesicular stomatitis virus (VSV) whose glycoprotein gene has been replaced by the GFP gene. The virus was pseudotyped with its authentic glycoprotein G as described previously (5).

Plasmids

The QuikChange XL site-directed mutagenesis kit (Stratagene) was used according to the manufacturer's protocol to exchange specific histidine for alanine codons in the pCAGGS-M expression plasmid (33) at amino acid positions 157, 540, 572, 580, 778,

836, 857, and 1087 of the RVFV M segment (GenBank sequence accession number JF784387). The recombinant plasmids were sequenced to confirm that only the desired mutations were present. To generate a stable *Drosophila* S2 cell line expressing the Gc ectodomain (Gce) of RVFV (GenBank sequence accession number JF784387), a truncated version of the Gn-Gc coding sequence lacking the transmembrane and cytoplasmic domains of Gc (GnGce; 153-AEDPHL/FGGPLK-1158) was cloned into the pMT/BiP/V5-HisA (Invitrogen) expression vector downstream from the BiP-encoding signal sequence. A quadruple Strep tag-encoding sequence separated by glycine linkers [DPTGWSHP QFEK(GGGSGGGSGGGSWSHPQFEK)3] was appended C terminally to allow purification of the secreted Gc ectodomain from the culture supernatant using Strep-Tactin Sepharose affinity chromatography (IBA GmbH). Stable S2 cell lines were generated according to the manufacturer's protocol (Invitrogen) with minor modifications. S2 cells were transfected with the expression vector and pCoBlast plasmid in a 1:19 ratio. Stable cell lines were subsequently selected at 27°C in serum-free InsectXpress medium containing 25 µg/ml blasticidin-S-HCl (Invitrogen) and maintained in this culture medium in the presence of 10 µg/ml blasticidin-S-HCl.

Production of polyclonal antiserum against Gc ectodomain

Polyclonal antibodies were raised against the Gc ectodomain after immunization of rabbits (Davids Biotechnologie). Rabbits were immunized at days 0, 14, and 21 with ~40 µg of Strep tag-purified Gc ectodomain. Total serum was collected at day 35.

Chemicals

Bafilomycin A1, dynasore, cytochalasin D, nystatin (Sigma), and dyngo-4a (Abcam) were prepared in dimethyl sulfoxide (DMSO). Chlorpromazine and ammonium chloride (Sigma) were prepared in distilled sterile water. Diethyl pyrocarbonate (DEPC; Sigma) was dissolved in 96% ethanol.

Fluorescence-activated cell sorting (FACS)

Flow cytometry was performed on a FACSCalibur flow cytometer (Becton, Dickinson Immunocytometry Systems). For the analysis of the data, Cyflogic 1.2.1 software was used.

Fluorescence microscopy

Fluorescence microscopy on living or fixed cells (fixed with 3.7% formaldehyde for 20 min at room temperature) was performed on the EVOS fl microscope (AMG). Nuclei of fixated cells were stained with 4'-6-diamidino-2-phenylindole (DAPI).

Production of $RVFV_{ns}$ particles

$RVFV_{ns}$ particles were produced as described previously (33). In short, BHK-rep2 cells (33) were seeded in Opti-Mem medium (Invitrogen) supplemented with 2% FCS and cultured in 175m^2 cell culture flasks (Greiner Bio-One B.V.). Cells were transfected (JetPEI; Polyplus) with pCAGGS-M (or derivatives thereof) in Opti-Mem containing 0.2% FCS. Twenty hours post transfection, the $RVFV_{ns}$ particles in the collected medium were concentrated and transferred into a phosphate-buffered saline (PBS) or HNE (5mM HEPES, 150 mM NaCl, 0.1mM EDTA) buffer, pH 7.4, using Amicon Ultra centrifugal filters (Millipore) with a molecular mass cutoff of 100,000 kDa (Millipore). Titers of $RVFV_{ns}$ stocks were determined by 50% tissue culture infective dose ($TCID_{50}$) analysis as described previously (33).

Inhibition of RVFV infection by pharmacological drugs

To analyze the effects of specific drugs on RVFV entry, BHK-21 cells were pretreated for 1 h with ammonium chloride, chlorpromazine (both dissolved in water), bafilomycin A1, cytochalasin D, dynasore, dyngo-4a, or nystatin (the latter five dissolved in DMSO). Cells were inoculated for 2 h with $RVFV_{ns}$ or VSV_{ns} (VSVΔG-GFP/G*) (multiplicity of infection [MOI] of ~0.4) at 37°C before the culture medium was replaced with medium containing 10 or 40 nM bafilomycin A1, respectively. Cells were further incubated at 37°C. The inhibitory effects of the compounds on virus replication were tested by the addition of the chemicals 2 h post infection (h.p.i.). After a period of 3 h, the drug-containing medium was replaced with culture medium containing bafilomycin (10 nM). $RVFV_{ns}$ or VSV_{ns} infection was quantified at 20 or 8 h after virus inoculation, respectively, by analysis of GFP-expressing cells using FACS or fluorescence microscopy. The viability of the drug-treated cells was measured using a WST-1 assay (Roche) according to the manufacturer's recommendations.

Ammonium chloride add-in time course

Infection was synchronized by allowing $RVFV_{ns}$ particles to bind to cells on ice for 1 h in serum free medium. The cells were washed with cold medium to remove unbound virus and subsequently transferred rapidly to a 37°C water bath after the addition of

serum-free prewarmed Roswell Park Memorial Institute 1640 medium (RPMI 1640; Invitrogen) adjusted at different pHs. Ammonium chloride (10 mM) was added to the medium at the indicated times. Infection (GFP-positive cells) was quantified by FACS and fluorescence microscopy at 18 h after warming.

Polyacrylamide gel electrophoresis, Western blotting, and dot blot analysis
Samples containing $RVFV_{ns}$ particles were heated for 10 min at 40 to 80°C in Laemmli sample buffer lacking β-mercaptoethanol (LSB[-]) and loaded onto NuPAGE bis-Tris gels (Invitrogen). After separation, proteins were blotted onto polyvinylidene fluoride (PVDF) membranes. Western blot analysis was performed using a rabbit Gc peptide antiserum (9), a rabbit polyclonal antiserum against Gce (see above), the 4-D4 mouse anti-Gn monoclonal antibody (provided by Connie Schmaljohn, USAMRIID) (29), or a sheep polyclonal antiserum against RVFV (32) as the primary antibody and an appropriate peroxidase-conjugated secondary antibody. For dot blot analysis, samples containing $RVFV_{ns}$ particles were heated for 20 min at 80°C and spotted onto nitrocellulose membrane (Whatmann). The membrane was blocked with PBS containing 0.05% Tween 20 (vol/vol) and 5% Protifar (wt/vol; Nutricia). $RVFV_{ns}$ particles were detected using the 4-D4 anti-Gn monoclonal antibody.

Fusion at the plasma membrane
$RVFV_{ns}$ particles were allowed to bind to a confluent layer of BHK-21 cells on ice for 2 h. Cell-bound virus was subsequently exposed to prewarmed RPMI 1640 medium for 3 min at 37°C (water bath). The pH of the culture medium varied from pH 7.4 to 5.0. The RPMI medium was replaced by culture medium containing bafilomycin A1 (20 nM), and cells were further incubated at 37°C, 5% CO2. Twenty hours after the 3-min pH shock, infection (GFP-positive cells) was quantified by FACS and fluorescence microscopy.

Low-pH treatment of $RVFV_{ns}$ particles
$RVFV_{ns}$ particles in HNE buffer, pH 7.4, were exposed to conditions ranging from pH 7.4 to 5.0 by the addition of a pretitrated volume of 100mM MES (morpholineethanesulfonic acid; pH 6.5 to 5.3) or 200mM acetic acid (pH 5.0). The particles were incubated for 3 min at the desired pH at 37°C before the samples were back-neutralized to pH 7.4 with 0.1 N NaOH. Samples were subsequently used to infect BHK-21 cells, and the infections were analyzed 20 h.p.i. by FACS and fluorescence microscopy. To analyze the effect of low-pH treatment on the structural

glycoproteins Gn and Gc, the pH of HNE-buffered $RVFV_{ns}$ particles was varied from pH 7.4 to 4.25 by the addition of MES (pH 6.5 to 5.3), 200 mM HAc (pH 5.0 to 4.75), or 500 mM HAc (pH 4.5 to 4.25). The $RVFV_{ns}$ particles were incubated for 0 to 10 min at 37°C, and the buffer was subsequently returned to pH 7.4 by adding a predetermined amount of 0.1 N NaOH or 0.5 N NaOH (pH 4.25). Samples were analyzed by Western blotting as described above.

DEPC treatment of $RVFV_{ns}$ particles

A diethylpyrocarbonate (DEPC) solution (1 M) in 96% ethanol was freshly prepared from a 1.12- g/ml commercial stock (Sigma). $RVFV_{ns}$ particles in PBS were incubated with various concentrations of DEPC as indicated for 10 min at 37°C. BHK-21 cells were inoculated with the treated virus for 20 h and assayed for infection (GFP-positive cells) by FACS and fluorescence microscopy.

Statistics

The data are representative of multiple independent experiments. Values are shown as means ± standard deviations (SD). Standard deviations were calculated using GraphPad Prism version 5.00 for Windows (GraphPad Software).

RESULTS

Entry of RVFV by endocytic uptake

We started our studies on RVFV cell entry with a limited pharmacological drug screen to investigate the uptake of the virus into cells using established inhibitors of cellular endocytic pathways. BHK-21 cells were pretreated for 1 h with different concentrations of drugs that interfere with dynamin-2- (dynasore), caveolin- (nystatin), clathrin- (chlorpromazine), or actin- (cytochalasin D) dependent endocytosis. Cells were then inoculated with nonspreading RVFV ($RVFV_{ns}$) at an moi of ~0.4 in the presence of the drugs and subsequently incubated without the drugs but in the presence of bafilomycin A1 to inhibit further infection (Fig. 1A). A nonspreading recombinant of VSV (VSVΔG-GFP/G* [VSV_{ns}]) was used as a control virus (5). Both viruses express GFP from the viral genome, facilitating quantification of infection. Treatment of BHK-21 cells with dynasore or chlorpromazine, but not with the other compounds tested, reduced RVFV infection considerably, more strongly even than they affected VSV infection, which is well known to be dependent on dynamin-2 and clathrin during entry (Fig. 1A). The presence of these drugs did not

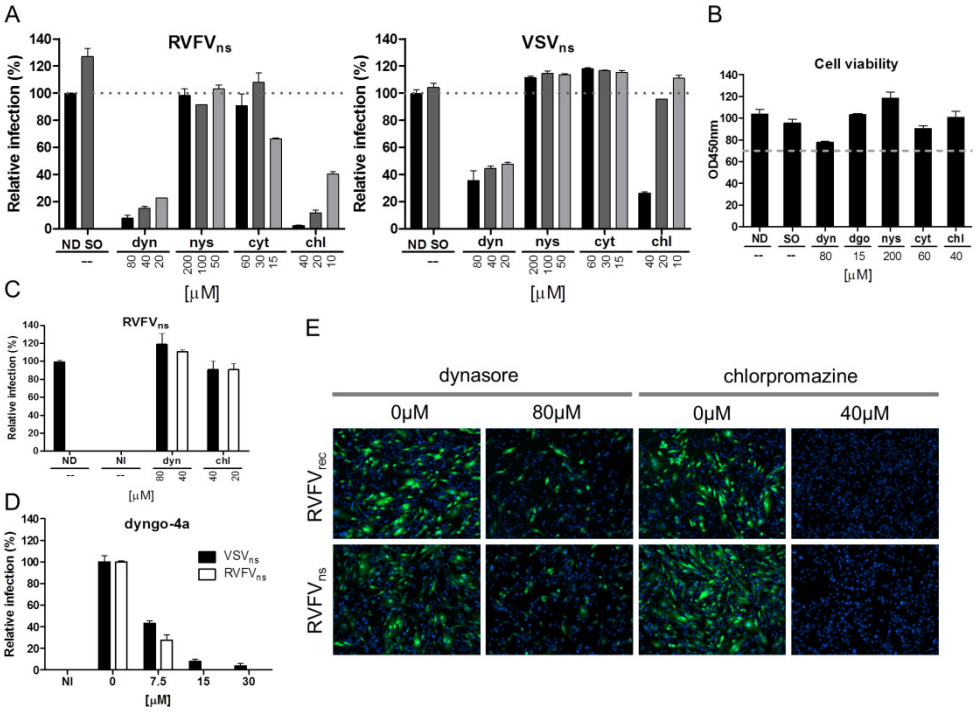

Figure.1. Rift Valley fever virus enters the cell via endocytosis. (A) BHK-21 cells were pretreated for 1 h with 2-fold dilutions of the indicated drugs and inoculated with $RVFV_{ns}$ (moi of ~0.1) or VSV_{ns} (moi of ~0.8) for 2 h in the continued presence of the drugs, after which the inoculum was replaced by culture medium containing bafilomycin A1 (10 to 40 nM) to inhibit further RVFV entry (see Fig. 2). Infection was quantified 20 h ($RVFV_{ns}$) or 8 h (VSV_{ns}) postinfection by measuring GFP-positive cells using FACS. The data are the means of three independent experiments done in duplicate. ND, no drugs; SO, solvent (1% DMSO); dyn, dynasore; nys, nystatin; cyt, cytochalasin D; chl, chlorpromazine. (B) BHK-21 cells were incubated with the indicated drugs for 3 h, after which medium containing the drugs was replaced with culture medium containing bafilomycin A1 (20 nM). Twenty hours later, the effect of the chemical compounds on the metabolic activity of the cells was determined using a spectrophotometric assay. Cells were considered viable if the metabolic activity of treated cells remained above 70% (indicated by the dashed line) relative to that of untreated cells (ND). The results shown are representative of two independent experiments performed in triplicate. OD450, optical density at 450 nm; dgo, dyngo-4a. (C) BHK-21 cells were infected with $RVFV_{ns}$ (moi of ~0.2) for 2 h, after which infection was continued for 3 h in the presence of 80 or 40 μM dynasore or 20 or 40 μM chlorpromazine. Cells were incubated overnight in culture medium containing bafilomycin A1 (20 nM). At 20 h postinfection, infected (GFP-positive) cells were quantified by FACS. Data shown are representative of two independent experiments performed in duplicate. NI, not infected. (D) Analysis of the effect of dyngo-4a on $RVFV_{ns}$ and VSV_{ns} infection (moi of ~0.4) of BHK-21 cells. Infection was performed and quantified as described for panel A. (E) Representative fluorescence pictures of BHK-21 cells infected with $RVFV_{ns}$ or $RVFV_{rec}$ in the presence of dynasore (80 μM) or chlorpromazine (40 μM). Infection was performed as described for panel A at an moi of ~0.2. Nuclei were stained with DAPI. Pictures are representative of two independent experiments performed in duplicate.

have a significant effect on the cell viability (Fig. 1B). Importantly, infection of $RVFV_{ns}$ was hardly affected if dynasore or chlorpromazine was added 2 h postinoculation, indicating that the drugs only affect the entry stage of infection (Fig. 1C). Similar effects of dynasore and chlorpromazine on $RVFV_{ns}$ infection were seen on A549 cells (data not shown).

We confirmed the involvement of dynamin-2 in $RVFV_{ns}$ entry on BHK-21 cells using a novel and potent inhibitor of dynamin-2 named dyngo-4a (23) (Fig. 1B and D). Dynasore and chlorpromazine also strongly inhibited entry of a recombinant RVFV, $RVFV_{rec}$, expressing GFP (Fig. 1E). These drug inhibition data indicate that entry of RVFV is sensitive to inhibitors which interfere with dynamin-2 and clathrin-dependent endocytosis.

RVFV entry depends on vacuolar acidification

Previously, it was described that low-pH incubation of cells overexpressing the RVFV glycoproteins resulted in extensive cell-cell fusion, suggesting that after endocytosis, RVFV fusion is activated by the acidic environment of endosomal compartments (11, 37). To explore the requirements of a low endosomal pH for virus entry, we analyzed the infection of $RVFV_{ns}$ in the presence of the lysosomotropic agents bafilomycin A1, an inhibitor of vacuolar-type H^+- ATPase proton pumps, and the lipophilic weak base ammonium chloride (NH4Cl), both of which prevent acidification of endosomal compartments. VSV_{ns}, known to be acid activated in early endosomes with a pH threshold of pH 6.1 (35, 68), was used as a control. The results demonstrate that $RVFV_{ns}$ entry is strictly dependent on vacuolar acidification, as infection of BHK-21 and A549 cells was inhibited by bafilomycin A1, as well as by NH4Cl (Fig. 2A). Compared to VSV_{ns}, $RVFV_{ns}$ appears to be more sensitive to the lysosomotropic agents, suggesting that the virus has a lower pH threshold for activation and therefore may be activated in more-acidic vacuoles beyond the early endosomal compartments.

To define the time of acid exposure after receptor binding, we used a previously described NH4Cl add-in experiment (40). The addition of NH4Cl to the medium is known to almost instantly raise the endosomal pH (43), allowing studies of the kinetics by which viruses pass the acid-requiring entry step after binding. We synchronized the infection of $RVFV_{ns}$ by allowing the virus to bind to cells in the cold. After removal of the unbound virus, we rapidly transferred the cells to 37°C and added 10 mM NH4Cl at different times after warming. About 90% of the virus experienced the acid-dependent step between 4 and 48 min after warming of the cells (Fig. 2B). The entry kinetics of $RVFV_{ns}$ are similar to those reported for the *Phlebovirus*

UUKV (39) and influenza viruses (40), which were both shown to fuse at low pH in late endosomal or lysosomal compartments (38, 39, 69).

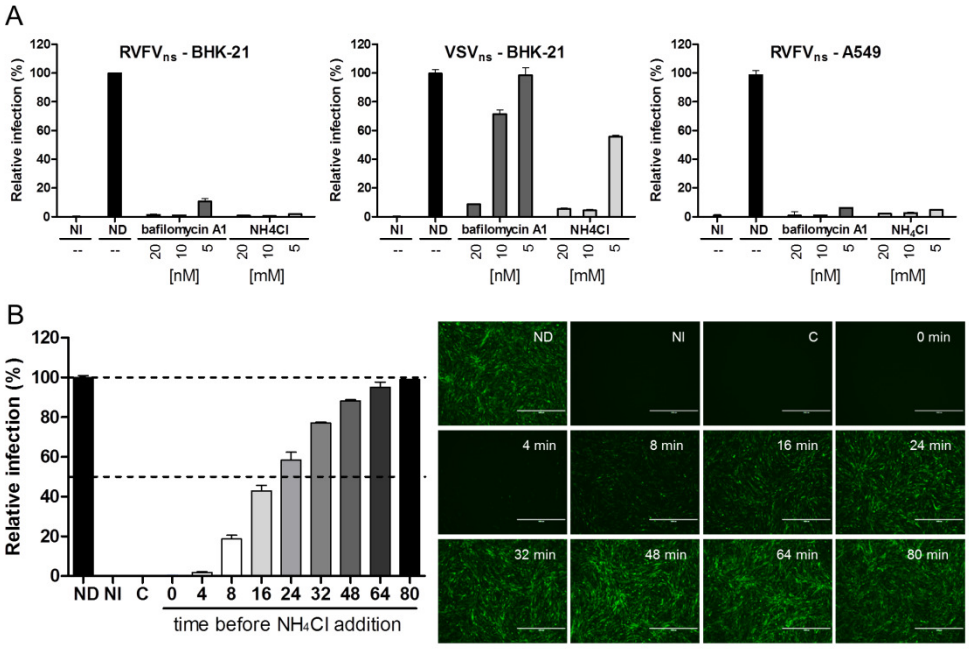

Figure.2. Entry of RVFV$_{ns}$ depends on vacuolar acidification. (A) BHK-21 or A549 cells were pretreated for 1 h with different concentrations of bafilomycin A1 or ammonium chloride (NH4Cl) and subsequently infected with RVFV$_{ns}$ (moi of ~0.1) or VSV$_{ns}$ (moi of ~0.8). Infection (GFP-positive cells) was quantified by FACS. Results shown are representative of three individual experiments performed in duplicate. NI, not infected; ND, no drugs. (B) RVFV$_{ns}$ was bound to BHK-21 cells for 1 h in the cold and subsequently warmed to 37°C. NH4Cl (10 mM) was added at indicated times to instantly raise the endosomal pH, thereby inhibiting further infection. Eighteen hours after warming, infection (GFP-positive cells) was analyzed by fluorescence microscopy (right panel; size bars represent 400 µM) and quantified by FACS (left panel). Untreated cells yielded ~70% infection. Graphical data shown are representative of two independent experiments performed in duplicate. C, NH4Cl control (not infected).

RVFV fusion occurs under acidic conditions that resemble the endolysosomal compartments

To determine the pH threshold for fusion of RVFV, we measured infection after low-pH induced fusion at the plasma membrane under conditions where acidification of endosomes —and normal virus entry— is blocked by bafilomycin A1. In this acid-mediated endocytosis bypass experiment, which was previously described for SFV and UUKV (19, 39), RVFV$_{ns}$ particles were bound to cells in the cold and subsequently exposed for 3 min at 37°C to media with various pHs. The medium was replaced with culture medium containing bafilomycin A1 to inhibit further infection via the

endocytic pathway. The results demonstrate that treatment of the cell-bound virus at or below pH 5.5 fully bypassed the requirement for vacuolar acidification (Fig. 3A). The bypass was less efficient at pH 5.7, and only limited infection was observed at pH 6 or higher, indicating that the pH threshold for RVFV membrane fusion is around pH 5.7. Exposure of free $RVFV_{ns}$ particles to low pH (pH 6.5 to 5) for 3 min did reduce virus infectivity, although virus inactivation was not complete (Fig. 3B).

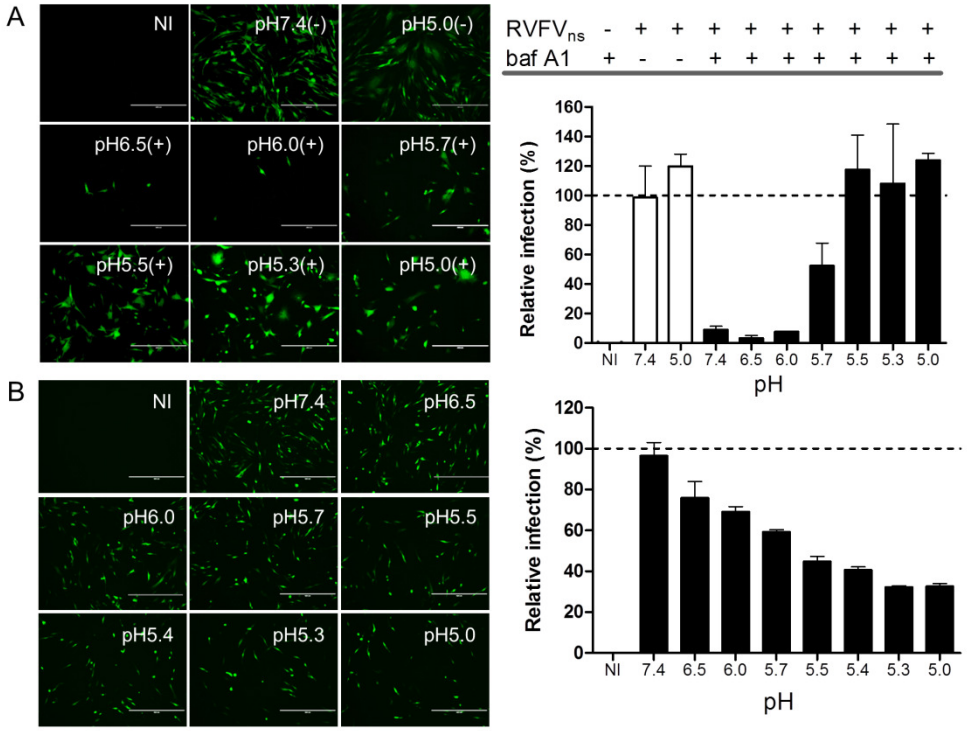

Figure.3. Low-pH-activated penetration of cell-bound $RVFV_{ns}$ and inactivation of unbound $RVFV_{ns}$ particles. (A) $RVFV_{ns}$ was allowed to bind in the cold to a confluent monolayer of BHK-21 cells for 2 h before the cell-bound virus was exposed for 3 min at 37°C to the indicated pH. Infection was continued in culture medium containing bafilomycin A1 (baf A1; 20 nM) to inhibit infection via the endocytic route. Infection (GFP-positive cells) was analyzed 20 h after warming by fluorescence microscopy and was quantified by FACS. Untreated cells (pH 7.4) yielded ~40% infection. The results shown are representative of two individual experiments performed in triplicate. (B) $RVFV_{ns}$ particles were incubated at the indicated pH for 3 min at 37°C. After neutralization of the medium, infectivity of the virus was assayed on BHK-21 cells. Infection (GFP-positive cells) was analyzed 20 h.p.i. by fluorescence microscopy (left panel; size bars represent 400 μM) and quantified by FACS (right panel). Virus incubated at neutral pH resulted in ~30% GFP-positive cells. Graphical data shown are representative of four individual experiments performed in duplicate. The controls in the experiments whose results are shown in panels A and B were cells that were not infected (NI).

Acid-induced rearrangement of the Gc into higher-order structures

Next, we examined the effects of exposure to low pH on the structural organization of the RVFV envelope glycoproteins. $RVFV_{ns}$ particles were incubated in buffers of low pH for 10 min. After neutralization of the buffers, viral proteins were analyzed by Western blotting performed under nonreducing conditions. The Gn and Gc glycoproteins, as well as the 78-kDa protein, were detected using a polyclonal RVFV antiserum, a Gn-specific monoclonal antibody, and a Gc-specific peptide antiserum (Fig. 4A). Incubation of $RVFV_{ns}$ under the low-pH conditions that activate virus entry (pH 6) (Fig. 3A) led to the disappearance of the Gc monomer and the concomitant appearance of a more slowly migrating protein moiety. Incubation at low pH did not influence migration of the Gn or the 78-kDa protein. The disappearance of the Gc monomer suggested that the slowly migrating band consists of an oligomer of Gc which can be detected by the polyclonal RVFV antiserum but, perhaps through epitope inaccessibility, not by the Gc peptide antiserum. Consistent with this notion, heating of acid-activated $RVFV_{ns}$ to 90°C resulted in the disappearance of the slowly migrating band and the simultaneous appearance of monomeric Gc (Fig. 4B).

To further study the identity of the presumed Gc oligomer, we produced a polyclonal antiserum by inoculating rabbits with the ectodomain of Gc produced using the *Drosophila* expression system. This antiserum efficiently recognized the monomeric and the more slowly migrating form of Gc on Western blots, confirming the identity of the Gc oligomer (Fig. 4C). Using this antiserum, we analyzed the kinetics of Gc oligomer formation. The conversion of Gc into SDS-stable oligomers slowly increased with time of pH treatment (Fig. 4C). Conversion became detectable already after 15 s and was nearly complete after 4 min of incubation at low pH. These results demonstrate that incubation of RVFV at the fusion-activating pH triggers a structural reorganization of Gc from an apparently metastable to a highly stable oligomeric conformation, reminiscent of the stable acid-activated trimeric class II fusion proteins of alpha- and flaviviruses (31, 59, 64).

RVFV entry is inhibited by substitution of a single histidine in Gc

Protonation of one or more histidines can be crucial for triggering conformational changes in viral fusion proteins (28). A first clue for a pH-sensing role of histidines in RVFV infection came from the observation that preincubation of $RVFV_{ns}$ with diethylpyrocarbonate (DEPC), a chemical which specifically modifies the aromatic ring of histidines, preventing its protonation (7), clearly inhibits RVFV infection of BHK-21 cells in a concentration-dependent manner (Fig. 5A). To further study the role of

histidine protonation in virus entry, we identified and replaced eight conserved histidines in Gn and Gc (Table 1 and Fig. 5B) with the small and nonpolar amino acid alanine.

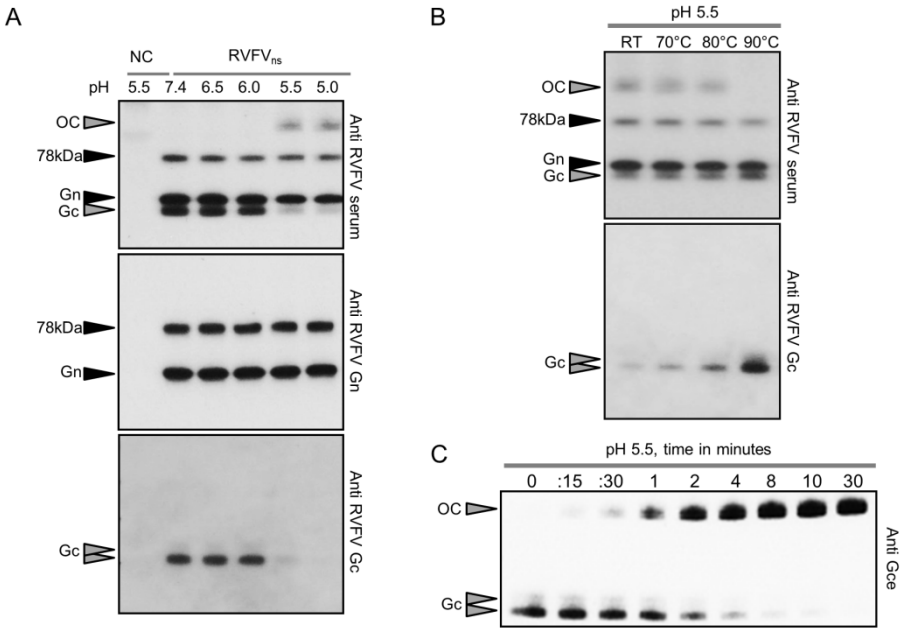

Figure.4. Acid exposure triggers the formation of an SDS- and temperature-resistant Gc oligomer. (A) RVFV$_{ns}$ particles were exposed to the indicated pHs for 10 min at 37°C, returned to neutral pH, and subsequently analyzed by Western blotting performed under nonreducing conditions. RVFV glycoproteins were detected with an RVFV antiserum, a monoclonal antibody against Gn, or a polyclonal anti-Gc peptide antiserum. In some cases, Gc migrates as a closely spaced doublet. (B) RVFV$_{ns}$ particles were exposed for 10 min to pH 5.5 at 37°C, returned to neutral pH, and heated for 20 min at the indicated temperatures. Samples were subsequently analyzed by Western blotting (nonreducing conditions) using an RVFV antiserum or an anti-Gc peptide antiserum. (C) RVFV$_{ns}$ particles were exposed to pH 5.5 at 37°C for the indicated times and analyzed by Western blotting (nonreducing conditions) using a polyclonal antiserum raised against the purified Gc ectodomain (Gce). OC, oligomeric complex; NC, negative control (medium from mock-transfected replicon cells); RT, room temperature.

RVFV$_{ns}$ particles carrying the histidine-to-alanine replacements were produced and secreted from BHK-21 replicon cells (33) with efficiencies resembling that of wild-type RVFV$_{ns}$, indicating that the mutations in Gn and Gc did not impair the assembly and release of virus particles (data not shown). The amounts of mutant and wild-type virus particles were normalized using a dot blot assay, and equal virus quantities were compared for their relative infectivity's (Fig. 5C). RVFV$_{ns}$ mutants with histidine-to-alanine substitutions in Gn were all viable, although their infectivity was slightly

reduced. In contrast, three of the four histidine-to-alanine substitutions in Gc either completely abolished (H857A mutant) or strongly impaired infectivity (H778A and H1087A mutants), to less than 5% relative to that of wild-type $RVFV_{ns}$.

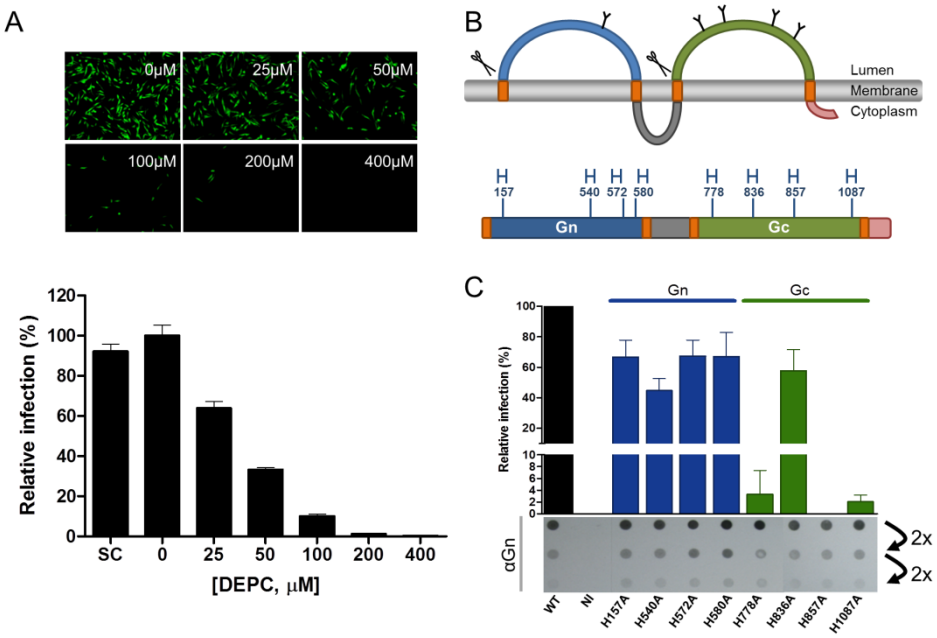

Figure.5. Role of histidines in $RVFV_{ns}$ infectivity. (A) $RVFV_{ns}$ was pretreated with different concentrations of DEPC for 10 min at 37°C and subsequently assayed for infectivity. Infectivity (GFP-positive cells) was analyzed 20 h post inoculation by fluorescence microscopy and quantified by FACS. BHK-21 cells inoculated with virus treated with 0 μM DEPC yielded ~20% infection. Graphical data shown are representative of two independent experiments performed in triplicate. SC, solvent control (0.2% ethanol). (B) Membrane topology of the RVFV M segment-encoded polyprotein starting from the fourth methionine (16). The predicted N-linked glycosylation sites (Y symbols) and signal peptidase cleavage sites (scissors) are indicated. The luminal domains of Gn and Gc and the cytoplasmic domains of Gn and Gc are colored in blue, green, gray, and pink, respectively. The orange blocks represent the transmembrane- spanning regions. The positions and amino acid numbering of conserved histidines (H), calculated from the first methionine (GenBank sequence accession number JF784387), are indicated in the linear diagram of the GnGc polyprotein. (C) Wild-type (WT) $RVFV_{ns}$ and mutants containing histidine- to-alanine substitutions, used in equal amounts as determined by a dot blot assay (2-fold dilutions of viruses are shown), were analyzed for their infectivity on BHK-21 cells. BHK-21 cells inoculated with wild-type $RVFV_{ns}$ particles resulted in ~20% infection. Infection (GFP-positive cells) was quantified by FACS. Noninfected (NI) BHK-21 cells were included as a negative control. The data shown represent the combined results for three independently produced batches of wild-type and mutant $RVFV_{ns}$ particles tested in independent experiments, each performed in triplicate.

H857 is essential for acid-induced rearrangement of Gc into higher-order structures

Next, we analyzed the effects of the histidine-to-alanine mutations on the formation, stability, and conversion kinetics of the Gc oligomer. Acid exposure of similar amounts of mutant and wild-type $RVFV_{ns}$ particles resulted in the formation of the SDS-resistant Gc oligomer in all mutants, with the exception of the H857A mutant (Fig. 6A,

Table.1. Conservation of the selected histidines in the RVFV Gn and Gc glycoproteins among members of the *Phlebovirus* genus of the *Bunyaviridae* family as determined by ClustalW alignment.

Phlebovirus (GenBank accession no.)	Amino acid at indicated position[a] in:							
	Gn protein				Gc protein			
	157	540	572	580	778	836	857	1087
Rift Valley fever virus (JF784387)	H	H	H	H	H	H	H	H
Sandfly fever Naples virus (YP089671)	H	H	H	H	H	H	H	H
Toscana virus (ABS85173)	H	H	H	H	H	H	H	H
Massilia virus (ACI24011)	H	H	H	H	H	H	H	H
Echarate virus (HM119411)	H	Y	Y	H	H	H	H	H
Turuna virus (HM119432)	H	H	N	H	H	H	H	H
Chandiru virus (HM119408)	H	H	N	H	H	H	H	H
Mucura virus (HM119420)	H	H	Q	H	H	H	H	H
Ariquemes virus (HM119405)	H	H	S	H	H	H	H	H
Morumbi virus (HM119423)	H	H	T	H	H	H	H	H
Alenquer virus (HM119402)	H	H	K	H	H	H	H	H
Itaituba virus (HM119417)	H	H	K	H	H	H	H	H
Serra Norte virus (HM119429)	H	Y	K	H	H	H	H	H
Nique virus (HM119426)	H	H	N	H	H	H	H	H
Oriximina virus (HM119435)	H	Y	H	H	H	H	H	H
Jacunda virus (HM466935)	H	Y	Y	H	H	H	H	H
Sandfly fever Sicilian virus (AAA75043)	H	H	E	H	R	H	H	H
Punta Toro virus (ABD92923)	H	H	H	H	H	H	H	H
Severe fever with thrombocytopenia syndrome virus (HM745931)	-	H	E	Y	R	R	P	S
Uukuniemi virus (AAA79512)	-	R	A	F	H	R	Y	H

[a]Amino acid numbers correspond to the RVFV M-segment encoding polyprotein. [-], ClustalW alignment displays a gap at this position.

upper left). The Gc oligomers of the H778A, H836A, and H1087A mutants were not stable at 80°C, in contrast to those of the wild type and the Gn histidine mutants (Fig. 6A, lower left). To analyze their thermostability in more detail, the Gc oligomers were subjected to heating at different temperatures prior to Western blot analysis. Compared to the wild-type oligomer, the Gc oligomers of the H778A, H836A, and H1087A mutants appeared slightly less thermostable and dissociated at 80°C but not at 70°C (Fig. 6A, right).

Next, we used Western blotting to assess the effects of the mutations on the kinetics of the Gc conversion into the SDS-stable oligomer upon acid exposure (Fig. 6B). The conversion kinetics of the Gc H778A, H836A, and H1087A mutants upon pH treatment appeared grossly similar to that of the wild type (Fig. 6B). Clearly, acid exposure of the Gc mutant carrying the lethal H857A substitution even up to 30 min did not allow the formation of the SDS-resistant Gc oligomer (Fig. 6B), suggesting that the formation of the stable Gc oligomer induced by protonation of histidine 857 is essential for virus entry into the host cell. Finally, to assess a possible shift in the pH activation threshold, we analyzed the Gc conversion for the H857A Gc mutant and wild-type $RVFV_{ns}$ particles at pH values below pH 5.5. Incubation under these acidic conditions for 10 min at 37°C resulted in the formation of the stable oligomer in wildtype $RVFV_{ns}$ particles. In contrast, no stable Gc oligomer was observed after treatment of $RVFV_{ns}$ particles containing the H857A mutation (Fig. 6C).

DISCUSSION

It is well established that bunyaviruses require the acidic pH found in endosomes to activate fusion, but it remains unclear how fusion is mediated at the molecular level. In this study, we demonstrate that exposure of RVFV particles to acidic pH converts the viral Gc protein into a highly stable oligomer. The pH required for this major conformational change correlates with the pH threshold required to activate fusion of virus particles at the plasma membrane. Mutagenesis of conserved histidines in the viral glycoproteins identified a single histidine in Gc that was strictly required for entry, as well as for the acid-induced conversion of Gc into its stable oligomeric form. These data provide new insight into the molecular basis of acid-activated bunyavirus fusion.

Most bunyaviruses studied to date utilize clathrin-mediated endocytosis to gain access to the cell interior (22, 25, 38, 39, 55, 58). For UUKV, a clathrin-independent pathway has been suggested as an alternative route (39).

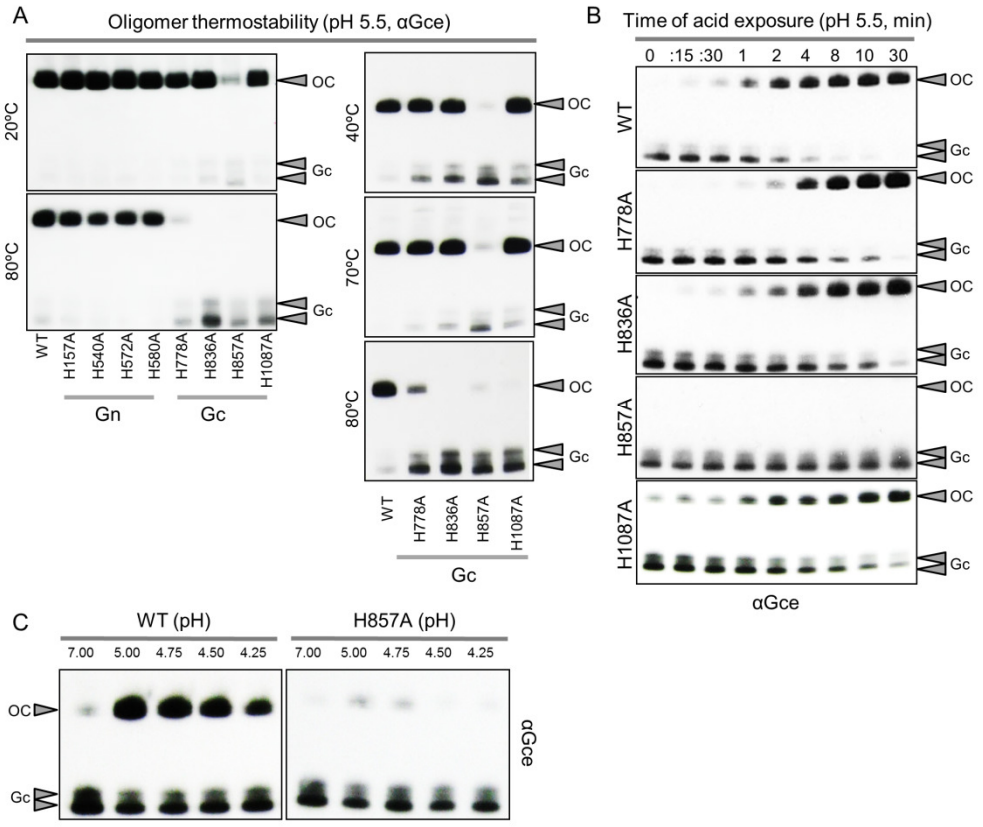

Figure.6. Effects of histidine substitutions in RVFV glycoproteins on the formation and stability of the Gc oligomer. (A) Equal amounts of wild-type and mutant $RVFV_{ns}$ particles were exposed for 10 min to pH 5.5 at 37°C, returned to neutral pH, heated for 20 min at the indicated temperatures, and subsequently analyzed by Western blotting (nonreducing conditions) using a polyclonal antiserum raised against the Gc ectodomain (Gce). In some cases, Gc runs as a closely spaced doublet. The thermostability of the acid-induced Gc oligomer is indicated for the wild-type and Gn- and Gc-His mutant $RVFV_{ns}$ particles at 20°C and 80°C (left) or for the wild-type and the Gc-His mutant $RVFV_{ns}$ particles at 40°C, 70°C, and 80°C (right). (B) Equal amounts of wild-type and mutant $RVFV_{ns}$ particles containing histidine-to-alanine mutations in Gc were exposed to pH 5.5 at 37°C for the indicated times and analyzed by Western blotting (nonreducing conditions) using a polyclonal antiserum raised against the Gc ectodomain. OC, oligomeric complex; WT, wild-type. (C) Equal amounts of wild-type and H857A mutant $RVFV_{ns}$ particles were exposed for 10 min at 37°C to the indicated pH. The Gc conversion was analyzed by Western blotting as described for panel B.

We demonstrate here that RVFV entry is sensitive to drugs perturbing the function of clathrin and dynamin, which is consistent with entry via a clathrin-mediated endocytic pathway. Further studies are required to confirm this assumption.

Upon endocytosis, RVFV particles are transported deep into the cell through maturing endosomes. The pH threshold (pH <6) required for virus fusion at the plasma membrane and rearrangement of Gc suggests that fusion of RVFV occurs in late endosomes. This late penetration is consistent with the time at which 50% of the total amount of infection has been reached (half-time) of RVFV of ~20 min during which the virus undergoes its acid-activating step. Similar pH thresholds and penetration kinetics have been observed for UUKV, another *Phlebovirus* (39). Our data indicate that RVFV can be classified as a late-penetrating virus (38).

Exposure to low pH converts the $RVFV_{ns}$ Gc protein from an apparently metastable to a highly stable oligomeric conformation. Similar pH-induced rearrangements have been reported for envelope glycoproteins of other bunyaviruses. These conformational changes have variously been shown to lead to dissociation of the Gn-Gc heterodimer (Hantavirus), to changes in the Gc antigenic structure, or to alteration in the cleavage pattern after proteolytic processing of the Gc protein (UUKV and La Crosse virus) (16, 21, 52). The low-pH-induced changes in the glycoprotein shell of UUKV virions have been visualized nicely by electron microscopy, revealing the flattening of the glycoprotein capsomers (44, 52). Low-pH incubation of La Crosse virus and UUKV leads to virus aggregation (44, 66). Activation of fusion proteins generally triggers the exposure of a hydrophobic "fusion peptide" which normally functions by inserting into the host membrane and initiating fusion, which might explain the observed acid-induced virus aggregation. Typically, fusion proteins are trimeric in this fusion active state (8, 17). Hence, we assume that the RVFV Gc stable oligomer is also made up of three Gc molecules, but further research is needed to confirm this hypothesis.

The acid-induced Gc oligomer displays a remarkable resistance to temperature dissociation and SDS detergent denaturation (10). In agreement with these observations, back-neutralization of the acid-exposed virus particles did not reverse the transition of Gc into a stable oligomer. In general, viral fusion proteins convert to a stable form during fusion, thereby generating the energy needed for membrane coalescence. For class I and II viral fusion proteins, this transition was shown to be irreversible (51). Intriguingly, exposure of $RVFV_{ns}$ to the activating pH in the absence of target membranes also fully converts Gc into the stable oligomer; however, although a reduction is seen, it does not completely inactivate virus infectivity even after incubation times of up to 16 min (data not shown). It remains to be seen whether the stable Gc oligomer represents the final postfusion form of the Gc fusion protein or represents a crucial intermediate which awaits further reorganization. In the latter

situation, alternative factors (e.g., interaction with the target membrane) may be required to drive the conformational rearrangements to completion.

In the RVFV virion, Gc is organized as a heterodimer together with Gn (24). The Gn-Gc heterodimer is the building block of 110 hexamers and 12 pentamers containing six and five of the Gn-Gc heterodimeric units, respectively. Together, these 122 capsomers make up the icosahedral shell of the virion, following a T = 12 triangulation (13, 24). Exposure of virions to the activating pH will lead to rearrangement of the metastable Gn-Gc heterodimer and concomitantly enforce the interactions between Gc subunits, resulting in the formation of a Gc homooligomer. Exposure of virions to the acidic pH of 6 did not have any impact on the structure of RVFV particles as shown by cryoelectron tomography, consistent with the fusion-activating pH threshold of below 6 that we observed (24). It will be interesting to investigate how the Gc protomers in the virions —organized as pentamers and hexameric Gn-Gc heterodimers— collectively undergo the structural transition toward the (presumably) trimeric Gc oligomer after exposure to the activating pH.

Protonation of histidines in Gc may play a key role at different stages of the fusion process. The inability of the H857A mutant to form stable Gc oligomers, in combination with its abrogated entry function, suggests that Gc oligomerization is essential for virus entry and that protonation of H857 plays a crucial role in this process. In addition, the substitution of H778 and H1087 in Gc has a severe effect on virus entry, yet Gc oligomerization still occurred and the temperature stability was only marginally affected. This suggests that formation of the Gc oligomer, although required, may not be sufficient for inducing fusion. We speculate that the single mutations may impair a later step in the fusion reaction (36). The H778, H857, and H1087 histidines in Gc may collectively contribute to pH sensing, initiation, and propagation of conformational rearrangements toward the postfusion structure. Further interpretation of the consequences of the histidine substitutions awaits the elucidation of the high-resolution structures of the Gc protein in its pre- and postfusion states.

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Chapter 7

General Discussion

PREFACE

Since its discovery in 1930, during an epidemic among sheep at a farm near lake Naivasha in the Rift Valley province of Kenya, RVFV has spread across the African continent and to the Arabian Peninsula (37, 114). RVFV outbreaks are characterized by abortion storms and high mortality among young animals. Adult animals are also affected but mortality in these animals is considerably lower. Although sheep, cattle and goats are the most severely affected, also other species of ruminants, such as camels and buffalo, are susceptible to disease. Humans can be infected by exposure to blood or other body fluids of viremic animals or via mosquito bite. RVFV has the potency to spread across large distances by as yet unknown mechanisms, explaining the need for established contingency plans in currently unaffected areas.

At the start of our research two veterinary RVFV vaccines were available, which are both still used to control RVF in African countries with recurrent outbreaks. Outside Africa, however, no registered vaccines are available to control future outbreaks. In this thesis we aimed to develop control measures to protect livestock against RVFV infection.

In this chapter we discuss the development and characterisation of different experimental vaccines, based on glycoprotein subunits, live-attenuated virus, viral vectors expressing the glycoproteins or RVFV replicon particles that can be used to protect livestock and, potentially, humans against RVFV infection. Next we will discuss our observations and ideas on RVFV attachment, endocytic entry and membrane fusion and the function of the glycoproteins in these processes. Selected findings and unpublished observations will be discussed and critically projected against current knowledge on bunyavirus biology and vaccine development.

TOWARDS SECOND AND THIRD GENERATION VACCINES

The classical RVFV vaccines

Two classical vaccines are currently used in Africa. The first is based on inactivated whole virus and can safely be applied in animals of all ages. The major disadvantages of this vaccine are the high cost of production and the revaccination and annual booster vaccinations that are required to obtain optimal protection (2, 3). The second vaccine is based on the live-attenuated Smithburn neurotropic strain (138). A single

vaccination with this vaccine can provide long-lasting, immunity. Due to residual virulence, however, this vaccine can cause abortions and foetal malformations (11, 20, 139). With the objective to develop a live-attenuated RVFV vaccine that can safely be applied in animals of all ages, the virulent RVFV isolate ZH548 was attenuated by passage of the virus in the presence of the mutagen 5-fluorouracil (16). The resulting virus, named MP-12, was shown to contain mutations on each of the genome segments, however, the mutations on the S segment were shown not to be involved in attenuation of the virus (6). Since the individual mutations responsible for attenuation were never determined, it is possible that a minimum of only two nucleotide reversions results in a virulent virus (100). Additionally, although the MP-12 virus can safely be applied in young animals, the virus was shown to cause abortions and teratogenic effects (65, 101).

Second generation vaccines

Recently, a third vaccine has become commercially available, called Clone 13. The Clone 13 virus was isolated from a human febrile case and was demonstrated to contain a large in-frame deletion in the S segment (102). This vaccine virus is highly attenuated due to a defective NSs gene, which encodes a multifunctional suppressor of the host innate immune system. This renders this vaccine virus inherently more safe than the live-attenuated Smithburn and MP-12 vaccines. Although Clone 13 is highly attenuated, neurological disorders and paralysis have been reported to occur in mice (156). A more recent study with Clone 13 in pregnant ewes however did not show any clinical signs nor abortions, indicating that under these experimental conditions the vaccine appeared to be safe (28).

THIRD GENERATION VACCINE CANDIDATES

Vaccines based on live-attenuated RVFV

Vaccines based on attenuated RVFV generally provide swift protection after a single vaccination. The possibility of reversion to virulence of these vaccine viruses must however be minimized (66). To further increase safety, a reassortant virus, named R566, was developed that contains the S segment of the Clone 13 virus and the M and L segments of MP-12 (12, 148). Considering the increased attenuation of the R566 virus, it would be interesting to compare vaccine efficacy of this virus with Clone 13.

By making use of reverse-genetics, a vaccine candidate was developed that not only lacks the NSs gene, but also the pre-Gn region of the M segment, which

encodes the NSm protein (7). The absence of both NSm and NSs (156, 164) renders the recombinant virus highly attenuated *in vivo* (7). A first published report about the safety and efficacy suggests that this ΔNSs/ΔNSm vaccine is effective and can safely be applied in gestating sheep (8). These preliminary data are promising and warrant more extensive safety trials.

Subunit vaccines

The vaccines described in **chapter 2** are based on subunits, rendering these vaccines completely safe, since neither production nor vaccination involves a replicating virus. Since studies have indicated that antibodies to Gn alone are capable of neutralizing the virus (46, 130, 157) we first evaluated the vaccine efficacy of a soluble form of the Gn ectodomain (referred to as Gn-e or GneS3), which was expressed and affinity-purified from insect cell culture supernatant. We demonstrate that a single immunization of sheep with soluble GneS3 provides full protection against RVFV (**chapter 4**). Since soluble protein antigens are generally poorly immunogenic, we administrated this vaccine in the presence of adjuvant (**chapter 2 and 4**). The drawback of the GneS3 subunit vaccine formulation is the mild to moderate swelling that occurs at the site of inoculation (**chapter 4**).

The second subunit vaccine that we developed is composed of virus-like particles (VLPs), which were generated by co-expression of both glycoproteins in insect cells (**chapter 2**). VLPs resemble the virus structurally and seemed more immunogenic since a single non-adjuvanted vaccination with VLPs in mice induced neutralizing antibodies, whereas two vaccinations with the adjuvanted Gn-e vaccine were required to induce these antibodies (**chapter 2**). We, and others (95, 103), demonstrated that vaccination with VLPs protects mice and rats from a lethal RVFV challenge (**chapter 2**). Although vaccination with VLPs seemed promising in the mouse model, development of a cost-effective vaccine for large-scale use in livestock seems challenging due to inefficient production and laborious purification procedures. To develop cost-effective glycoprotein-based RVFV vaccines, replicon-based vaccines and vector vaccines based on live viruses are being evaluated.

Replicon-based vector vaccines

The first replicon-based vector vaccines that were developed for the control of RVFV are constructed from the alphaviruses Venezuelan equine encephalitis virus (VEEV) or Sindbis virus (SINV). One important advantage of alphavirus replicons is the ability to produce these replicons at an escalating scale as a tri-component genome virus (32). A

single vaccination with a VEEV replicon expressing Gn protected mice from a lethal RVFV challenge, whereas vaccination with a SINV replicon did not provide protection (46). Although these first results obtained from experiments with VEEV-based replicons are promising, the authors reported that Gn expression interferes with replicon packaging, which would be problematic for large-scale manufacturing. Interestingly, despite the aforementioned disappointing results with SINV replicons, Heise *et al.* developed a SINV replicon expressing both Gn and Gc, which protected mice against a lethal dose of RVFV and induced neutralizing antibodies in sheep (54).

Another replicon-based approach makes use of adenovirus. A replication-defective adenovirus type 5 (Ad5) named CAdVax-RVF was designed to express human codon-optimized Gn and Gc under control of the human cytomegalovirus (CMV) promoter (59). A single intraperitoneal vaccination with a dose of 10^8 plaque-forming units (PFU) of this vaccine virus completely protected mice against a lethal dose of RVFV. Since pre-existing immunity against Ad5 exists in the human population, which might interfere with efficacy of Ad5-based vector vaccines when used in humans, an efficacy experiment in mice was performed mimicking this situation. To this end, mice were pre-immunized with a heterologous CAdVax vaccine construct followed 10 weeks later by vaccination with a low dose (2x10^6 PFU) or high dose (2x10^8 PFU) of the CAdVax-RVF vaccine. This vaccination was repeated after 15 weeks and mice were challenged two weeks later. In the group of mice vaccinated with a low dose only 25% of the mice survived the RVFV challenge, whereas in the group vaccinated with the high dose 75% of the mice survived. When extrapolated to humans, this suggests that pre-existing immunity may have a significant negative effect on efficacy of the AdV5-based RVFV vaccines, which should be taken into consideration. Application of Ad5-based replicon vaccines in ruminants is, however, unlikely to be compromised by pre-existing immunity in the field.

Although the results reported from experiments with alphavirus and adenovirus-based replicons warrant further experiments in sheep, the recently developed RVFV replicon particles described in **chapter 4** are likely to be more effective. Although RVFV replicon particles do not produce the structural glycoproteins *in vivo*, high levels of neutralizing antibodies targeting these proteins are induced by a single vaccination. Furthermore, similar replicon particles later developed by Dodd *et al*., were shown to induce broad antiviral and chemokine gene expression already 12 hours post vaccination, which was attributed to RVFV genome replication *in vivo* (26). It is plausible to assume that these unique properties of RVFV

replicons will contribute to both early onset and duration of immunity and render them superior to alternative replicon-based RVFV vaccines.

In **chapter 4**, we demonstrate that a single vaccination of lambs with RVFV replicon particles provides complete protection from viremia, pyrexia and mortality. Interestingly, similar systems can possibly be developed for other members of the bunyavirus family, like the recently emerged severe fever with thrombocytopenia syndrome virus (4) and Schmallenberg virus (169).

Vector vaccines based on live viruses

The use of poxviruses as live vector vaccines is widely explored due to their strong safety and stability profile. The first poxvirus that was used to express RVFV proteins was lumpy skin disease virus (LSDV), a capripox virus of cattle (158). The attenuated South African Onderstepoort LSDV vaccine strain was used to express Gn and Gc, resulting in the rLSDV-RVFV vaccine (157). The use of LSDV as a vector of RVFV antigens is aimed to result in a vaccine that protects ruminants not only from RVFV, but also from LSDV, sheep pox virus (SPPV) and goat pox virus, a feature that could be a major advantage to overcome the general reluctance to vaccinate during RVF interepidemic periods. A prime-boost vaccination protected mice from a lethal dose of RVFV and a similar vaccination schedule seemed to protect sheep against both RVFV and SPPV. Both challenge infections were however relatively mild, precluding definite conclusions (157).

A similar approach makes use of the KS-1 strain of LSDV (140). First evaluation in mice yielded only partial protection, but this was explained by the poor replication of the KS-1 strain in mice. A subsequent experiment in sheep demonstrated that a single vaccination induced neutralizing antibodies against both RVFV and SPPV which were boosted by a second vaccination 28 days later. Sheep were divided over two groups and either challenged with RVFV or SPPV 28 days after the second vaccination. Three out of five control sheep challenged with RVFV developed viremia and all five developed fever whereas only one of the vaccinated sheep displayed fever and none displayed viremia. All sheep challenged with SPPV were protected from SPPV-induced pathology, suggesting that the rKS-1/RVFV vaccine provides protection against SPPV and, at least partial, protection from RVFV.

Other poxviruses that are being explored, perhaps more focused on human application, are the replication deficient Modified Vaccinia Ankara virus (31, 80) and the attenuated replication-competent Vaccinia virus (rVACV, 110). A single vaccination with MVA expressing Gn and Gc provided full protection in mice, whereas

only partial protection was elicited after one (maximally 50%) or two (maximally 90%) vaccinations with rVACV. Nevertheless, vaccination of baboons by scarification, followed by intramuscular booster on day 28, induced neutralizing antibodies already after the first vaccination (110), a finding that holds promise for human application.

Considering the seeming suboptimal performance of available live vector vaccine candidates, we explored the use of Newcastle disease virus (NDV) for this approach. The use of NDV as a vaccine vector offers several advantages. First, being an exclusive pathogen of birds, vaccine efficacy of NDV when applied in mammals is not likely to be compromised by pre-existing immunity in the field. Another advantage of this approach is the inherent safety of NDV when applied in mammals (13, 14, 25). The host-range restriction of NDV is explained by the species specificity of the "V" protein, an antagonist of type-I interferons (111, 112). Furthermore, we made use of the LaSota vaccine strain, which is used worldwide for the control of NDV in poultry. This NDV strain is completely avirulent, even in its natural target species. Additionally, NDV LaSota as well as the recombinant NDV-GnGc vaccine virus can be propagated in embryonated eggs, yielding titers exceeding 10^9 egg infectious doses. We demonstrate that a single vaccination protects sheep against viremia and clinical signs (**chapter 4**). The vaccine efficacy, lack of pre-existing immunity in livestock and cost-effective production of this vaccine highlight its potential as a vector vaccine for the control of RVFV in livestock (77). Registration trials with the NDV-GnGc vaccine are currently being performed.

Detection of infection in vaccinated animals

At present, RVFV is confined to Africa and the Arabian Peninsula, but the pathogen poses a risk to nations outside the current area of distribution (52, 62). Outbreaks following first incursions of RVFV in a given area can result in explosive epidemics, exemplified by the epidemics that followed the first introduction of RVFV in Egypt in 1977 and the Arabian peninsula in 2000 (1, 41). In order to control a RVFV outbreak, various immediate actions are recommended including vector control, ban of animal transport and vaccination (18, 31, 107). According to OIE guidelines *(Office International des Épizooties),* countries with RVFV outbreaks are restricted from export of livestock animals (107), which may take up to four years before the certified disease free status can be regained. Ironically, it is questionable if the disease free status can be obtained sooner when vaccination with conventional vaccines is applied. The disease free status can only be declared after an OIE-prescribed surveillance program has demonstrated no evidence of RVFV infection in humans, animals or

mosquitoes (107). Vaccination with conventional vaccines will induce overall RVFV-specific antibodies and can thereby delay the freedom of disease declaration. This paradox is particularly relevant when countries bordering epidemic areas, presumed to be disease free, want to prevent an epidemic by vaccination. Large-scale vaccination in these countries will result in economic losses due to the imposed trade restrictions. DIVA vaccines ("Differentiating Infected from VAccinated individuals" or, in our view more appropriately spelled out as "Detection of Infection in Vaccinated Animals", 151) provide an elegant solution to this problem. DIVA vaccines lack an immunogenic protein that is present in the pathogen, a so-called "negative marker" (152). When a DIVA vaccine is used to vaccinate animals in the field and an accompanying DIVA ELISA is used to screen animals before they are transported to disease free areas, the abovementioned OIE recommendation regarding trade restrictions can possibly be adapted.

It has been suggested that vaccines that lack the NSs protein, such as the Clone 13, R566 and ΔNSs/ΔNSm vaccines, can be used as DIVA vaccines when accompanied by an NSs-specific serological assay (8, 67). Since NSs is poorly immunogenic and anti-NSs antibodies are not consistently produced upon infection, it is preferable to use alternative viral proteins for DIVA diagnostics (35, 86). Considering that the N protein is the most immunogenic and antigenically conserved virus component (129), this protein is a preferred target for DIVA diagnostics. Conveniently, highly sensitive and specific N protein-based ELISAs are commercially available (68, 78, 153). Several promising third generation RVFV vaccines can be applied as DIVA vaccines in conjunction with these N-ELISAs (chapter 2, 3 and 46, 54, 59, 67, 110, 153, 157). Although the added value of DIVA vaccines in countries where RVFV is currently endemic is questionable, using such vaccines in previously unaffected areas is, without a doubt, extremely valuable.

In order to respond to RVFV outbreaks quickly, epidemiological surveillance systems and control measures should be implemented in countries at risk. Large-scale vaccinations may limit the size of an epidemic and eliminate the source of human infections. To ensure adequate vaccination coverage a global RVF vaccine stockpile for immediate use in emergency vaccination campaigns must be established (31). We propose the NDV-GnGc vaccine as the most promising candidate for a vaccine stockpile since this vaccine can be used in both endemic regions and previously unaffected areas. The NDV-GnGc vaccine combines multiple safety features, single dose efficacy and cost-effective production by established industrial methods (**chapter 3**). The NDV-GnGc vector vaccine furthermore enables DIVA (151), as

recommended by the Food and Agriculture Organization (FAO) of the United Nations (31). A number of innovative vector and replicon vaccines that could fulfill the DIVA criterion have also been developed, but all seem to have their shortcomings (31, 67).

Towards a RVFV vaccine for use in humans

Veterinary RVFV vaccines are used to limit virus amplification and circulation in livestock and thereby also reduce human infections (9). Nevertheless, there is a strong and urgent need for a safe and effective human vaccine as well. Two vaccines for application in humans have been extensively tested. A formalin inactivated vaccine, named "Salk Institute-Government Services Division (TSI-GSD) 200" vaccine (123), requires a series of three vaccinations and a booster dose six months later. Unfortunately, due to complications in production, this vaccine is no longer available (12, 116, 123). The MP-12 vaccine, a candidate vaccine for use in livestock, is also considered as a human vaccine. MP-12 was evaluated for potency and safety in 20 volunteers in a phase II clinical trial and did not reveal any serious adverse reactions (31).

Another human vaccine candidate, of which the safety is beyond debate, is the purified Gn ectodomain that we describe in **chapters 2** and **4**. Apart from its inherent safety, based on vaccination-challenge experiments in sheep, a single vaccination may provide protection also in humans. Besides the GneS3 subunit vaccine, the vaccine based on nonspreading RVFV (NSR) ought to be evaluated as a human vaccine (79), which may optimally combine efficacy with safety. These potential human vaccines should be evaluated extensively in the nonhuman primate model, which is now available (137).

RVFV ATTACHMENT

Recently it was shown that phleboviruses exploit DC-SIGN as a receptor (93). DC-SIGN is a C-type lectin that is expressed mainly on interstitial dendritic cells and certain tissue macrophages (118). The ability of RVFV to infect cells lacking DC-SIGN makes clear that alternative receptors exist. In **chapter 5** we demonstrate that heparan sulfate (HS) is required for efficient entry of RVFV into cells. HS is a glycosaminoglycan (GAG) that is abundantly present on the cell surface of most animal cells and was shown to be involved in infection of cells by a variety of viruses (82, 89). HS was long considered as a non-specific receptor that interacts with viruses via motif*s* composed of basic amino acids (96), but elaborate studies have revealed

that these complex structures might specifically dock virus particles to the cell surface assisting receptor binding and virus infection (89, 149).

In general, virus-HS interactions are weak (142), meaning that the virus may constantly associates and dissociates from the abundantly expressed HS chains (27, 128). These oscillating interactions allow the virus to adhere to, but also move along a cell surface. This lateral movement may increase the chance of encountering a secondary receptor, to which the virus binds with high affinity. In accordance with this, we found that $RVFV_{ns}$ binding to the surface of cells is highly inefficient and therefore difficult to properly quantify (unpublished data). In an attempt to quantify attachment efficiency we used a cell-based ELISA to detect cell-bound virions. The sensitivity of this technique in our hands was however insufficient to allow quantification of virion attachment (unpublished data). We also analyzed virus binding by employing radiolabelled particles that were produced by the replicon cells (**chapter 4**). Yet, despite much effort, we were unable to efficiently quantify the binding of ^{35}S-cysteine-radiolabelled $RVFV_{ns}$ particles, presumably due to the relative low quantity of radiolabelled particles generate during the chase period (unpublished data). Previous studies, with the non-pathogenic *Phlebovirus* Uukuniemi virus (UUKV), demonstrated that, independent of relative high moi's used, only 7-9% of the virus particles adhered to the cell surface (94). When the inoculum of the initial experiment was used for a subsequent binding experiment, radioactivity measurements were similar, suggesting that the unbound particles were capable of cell association. In all probability, these sub-optimal binding efficiencies also apply to other *Phlebovirus* members, including RVFV.

The role of heparan sulfate in RVFV infection

In **chapter 5** we report that infectivity of $RVFV_{ns}$ and a replication-competent recombinant RVFV ($RVFV_{GFP}$) is reduced upon removal of GAGs from the cell surface. Also, we show that pre-incubation of the virus with highly-sulfated heparin, a heparan sulfate analogue, as well as enzymatic removal of heparan sulfate, from a variety of cells by heparinases, dramatically reduces $RVFV_{ns}$ infectivity. Virus infectivity was also significantly decreased in cells genetically deficient in heparin sulfate synthesis and cells that lack *O*-sulfation. The combined data point towards an important role for heparan sulfate in RVFV infection. Interestingly, although $RVFV_{ns}$ infectivity of completely GAG- and HS-deficient CHO cells (30, 85) was dramatically reduced, some residual infection was always observed (**chapter 5**). This suggests that HS binding is not strictly essential for virus entry. It is unlikely that this residual infection

represents a virus subpopulation that infects cells independent of HS binding, since the structural glycoproteins present in $RVFV_{ns}$ particles are produced from plasmid and thus are precisely defined and genetically homogeneous. More likely, and in addition to the possibility that alternative attachment factors are involved, direct binding of $RVFV_{ns}$ to a secondary high affinity receptor explains the observed residual infection.

Association of viruses to HS involves electrostatic interactions between the negatively charged sulfate groups on HS and clusters of basic residues present in viral surface proteins (17). In **chapter 5** we report a potential HS binding cluster in the pre-Gn region of the 78-kDa protein. Interestingly, the 78-kDa protein was previously proposed to be present in minute amounts in the viral envelop (73, 145). Unless other linear or non-linear arrangements of basic residues on the genomic M segment may create a HS binding motif in the tertiary structures of these glycoproteins (42, 57), HS binding is likely to be mediated by this HS binding motif. To pursue this hypothesis we have recently generated $RVFV_{ns}$ particles lacking the pre-Gn region ($RVFV_{ns}$/Δpre-Gn). We first compared the $RVFV_{ns}$/Δpre-Gn particle infectivity with that of wildtype particles using equal amounts of particles (Fig. 1A). Intriguingly, $RVFV_{ns}$/Δpre-Gn particles appeared to have a nearly 2 log lower titer compared to wildtype particles (unpublished data). In order to investigate the role of the 78-kDa protein in HS dependent binding and subsequent entry we compared the infectivity of wildtype $RVFV_{ns}$ and $RVFV_{ns}$/Δpre-Gn particles on different (mutant) CHO cell lines (Fig. 1B). Compared to parental CHO cells, infection of wildtype $RVFV_{ns}$ (**chapter 5**) was clearly diminished on GAG- and HS-deficient CHO cells. Intriguingly, no clear difference was observed in the infectivity of the $RVFV_{ns}$/Δpre-Gn virus on the three cell lines. These experiments suggest a role for the 78-kDa protein in HS-dependent infection. The reduced specific infectivity of $RVFV_{ns}$/Δpre-Gn may be explained by its inability to bind HS on the cell surface. In contrast, others have shown that deletion of the pre-Gn region does not notably affect virus infectivity (44, 163). However, Kreher *et al*. (81) reported that recombinant viruses that lack expression of the pre-Gn region by mutagenesis of the first three AUG start codons demonstrated impaired *in vitro* growth kinetics and reduced plaque size. Compensating mutations were observed that restored expression of the 78-kDa protein, demonstrating the importance of this protein for virus proliferation.

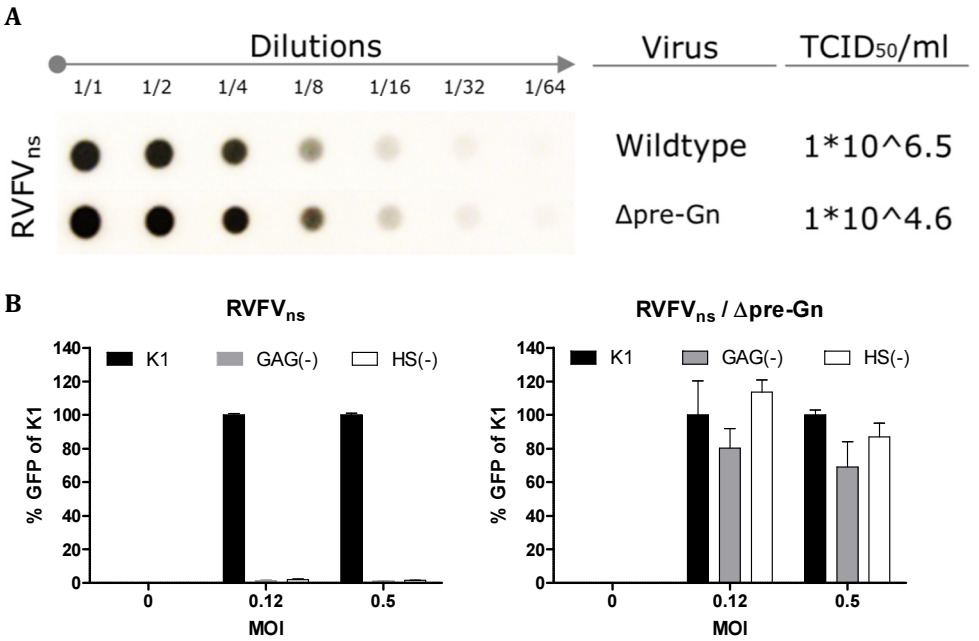


Fig.1. Infectivity and HS dependency of RVFV$_{ns}$ lacking the pre-Gn region. (A) RVFV$_{ns}$ particles were generated on replicon cells by transient expression of the M segment encoding polyprotein starting from the first (RVFV$_{ns}$) or fourth AUG codon (RVFV$_{ns}$/Δpre-Gn). Particles were normalized by dot blot using the 4-D4 αGn monoclonal antibody as described in chapter 6 and viral titers were determined by end-point titration. (B) Infectivity of RVFV$_{ns}$ and RVFV$_{ns}$/Δpre-Gn particles on CHO wildtype and mutant cell lines. GAG- and HS-deficient CHO cells and the parental cell (K1) were inoculated with RVFV$_{ns}$ and RVFV$_{ns}$/Δpre-Gn particles at different moi. At twenty hours post inoculation the percentage of infected (GFP-positive) cells was quantified by FACS.

It is important to note that the proportion of the 78-kDa protein relative to Gn is consistently higher in RVFV$_{ns}$ compared to authentic virus particles (**chapter 6**; 73, 145. A similar observation was made by Habjan *et al*. (48). RVFV$_{ns}$ particles are produced upon transfection of replicon cells with an expression plasmid encoding the M segment polyprotein of RVFV. In contrast to the viral genome, the first start codon of the M segment ORF in the expression plasmid has a near optimal translational initiation Kozak sequence (ACCATGT vs TAAATGT) likely leading to a more efficient translation. This may explain the unnatural high amounts of 78-kDa protein into RVFV$_{ns}$ particles and, as a consequence, the more pronounced HS dependency of RVFV$_{ns}$ compared to wildtype virus (**chapter 5**). To further investigate the role of the pre-Gn region in HS binding and infection, additional studies need to be performed that better quantify the presence of the 78-kDa protein in purified virions and RVFV$_{ns}$

particles. Furthermore, through mutational analysis, the role of the HS binding motif in the pre-Gn region for virus entry ought to be investigated.

Entry of virus by binding to heparan sulfate containing proteoglycans

Two possible mechanisms can be proposed by which HS facilitates virus entry. The virus may directly bind to HS found on proteoglycans on the cell surface facilitating the interaction with a possible second receptor to which it binds with higher affinity. Alternatively, RVFV may bind to HS-containing proteoglycans that become soluble through a natural process of heparan sulfate proteoglycan (HSPG) shedding, which could facilitate entry via an alternative route similar to what recently has been shown for human papillomavirus (HPV). HPV seems to enter the cell via a novel mechanism by which the virus usurps a normal cellular process (147). In this model, HPV binds to the heparan sulfate side-chains of soluble or cell surface-associated syndecans, which are a family of four highly conserved type-I transmembrane HSPG. Syndecans are continuously cleaved off cell surfaces, mainly, by matrix metalloproteinase peptidases and participate in many different cellular activities like cell growth, wound healing and cell-cell adhesion (5, 19, 33).

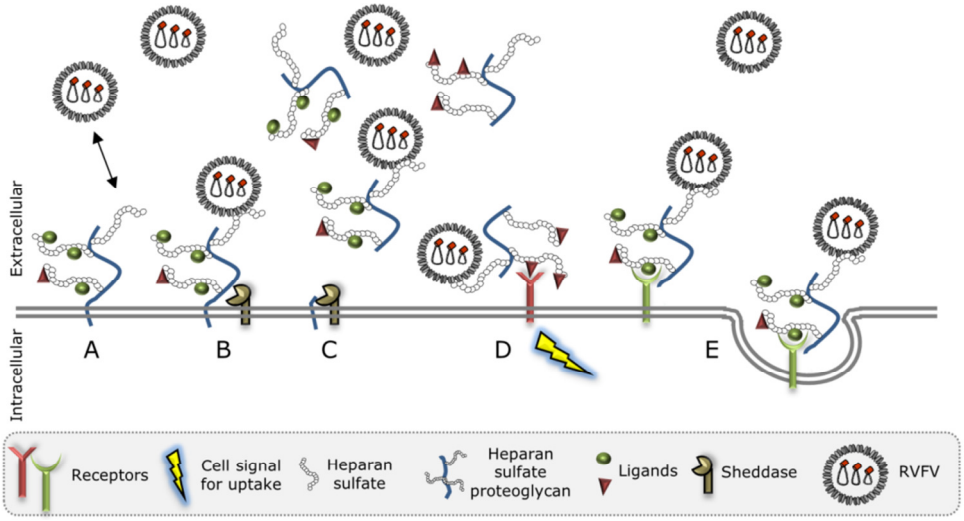


Fig.2. The role of heparan sulfate proteoglycans in RVFV infection: A hypothetical model. (A) RVFV interacts with cell-associated heparan sulfate proteoglycans (HSPG). These HSPGs are decorated with a variety of cellular ligands. (B) As a natural process HSPGs are cleaved by sheddases (like metalloproteinase peptidases), releasing the HSPG-virus complex. (C) Virus can also interact with soluble HSPG ectodomains in the extracellular environment. HSPG-virus complexes move towards specific uptake receptors on host cells. (D) The ligands interact with their cognate receptors, activating signaling pathways. (E) The virus takes advantage of this cellular process and is internalized in complex with the HSPG.

In addition to heparan sulfate side-chains, syndecan ectodomains are decorated with ligands including growth factors (GFs), cytokines, chemokines or extracellular matrix proteins. After cleavage, the soluble syndecan ectodomains move through the extracellular space presenting their ligands to corresponding receptors. Receptor binding may trigger a signaling transduction pathway that mediates uptake of the HPV-HSPG-ligand complex. This model might explain why, despite intensive research, the secondary high-affinity receptor of HPV has never been elucidated. In a broader sense, RVFV and other HS-dependent viruses might use a similar mechanism for entry, consequently gaining access to a variety of cells (Fig. 2). Obviously, further investigations are needed to reveal the exact role of HS in RVFV entry.

Heparan sulfate-mediated actin- and myosin II–driven transport

Glycosaminoglycans, and particularly HSPG, are key components of cell surfaces and the extracellular matrix regulating embryonic development and tissue homeostasis (47, 72, 144). No evidence, so far, has been reported for a role of HS in transport of ligands or adhesion molecules. Such a transport mechanism could be exploited by viral pathogens to move along the cell surface or migrate from cell-to-cell.

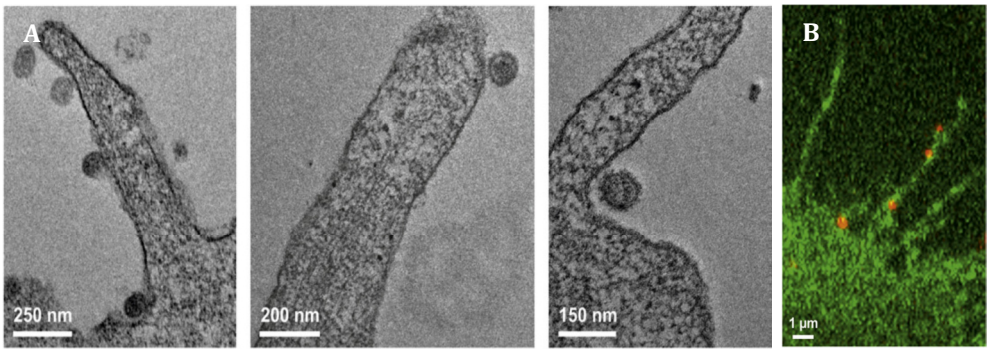


Fig.3. Binding of UUKV to filopodia. (A) Electron microscopy analysis of UUKV association to A549 cells. Pictures show UUKV association with filopodia. (B) UUKV particles coupled to a thiosulfate-activated red fluorescent dye (TS-link Bodipy-TR) associated with filopodia of BSC40 cells expressing eGFP. Samples were analyzed by confocal microscopy. Reprinted from Publication Entry of Bunyaviruses into Mammalian Cells, Volume 7, Issue 6, Lozach PY, Mancini R, Bitto D, Meier R, Oestereich L, Overby AK, Pettersson RF, Helenius A. Cell Host & Microbe, p488-499, Copyright (2010) with permission from Elsevier.

In several recent studies, the interactions of viruses with cell-associated filopodia were investigated. These studies suggested that the well-studied process of retrograde F-actin flow is involved in the transport of ligands, possibly via heparan sulfate containing glycosaminoglycans (105, 127). Retrograde F-actin flow is a myosin motor-driven process in which filamentous actin (F-actin) is transported from far

ends of cellular extensions (filopodia) towards the proximal end of the extension (155). The retrograde F-actin flow is involved in cell motility, supports myosin-driven movement of proteins from the cell periphery inward (23, 38, 87) and directs the formation of lamellipodia and filopodia. Filopodia express HS on the surface and are involved in cell migration, cone growth of neuronal cells and wound healing (99). Likewise, dendritic cells (DC) have extensions that have the appearance of filopodia. Dendritic cells also express heparan sulfate (160) and HS might therefore assist in binding of bunyaviruses to the DC-SIGN receptor.

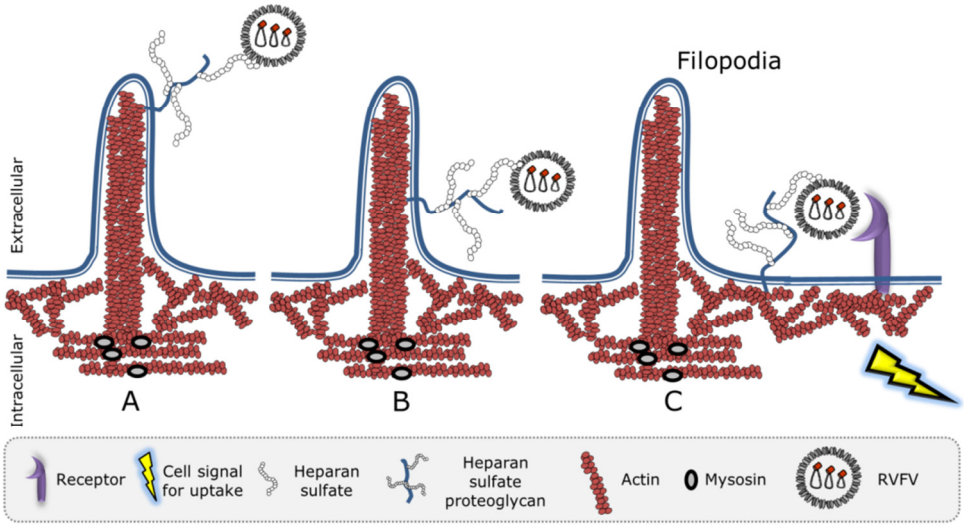


Fig.4. Hypothetical model of RVFV filopodia "surfing". (A) Binding of RVFV to heparan sulfate proteoglycans via the heparan sulfate side-chains. (B) After binding the HSPG-virus complex moves laterally along the filopodia surface, also referred to as "surfing", into the direction of the cell body. This "surfing" is mediated by the underlying actin cytoskeleton and driven by myosin motors. (C) Movement of the HSPG-virus complex along the cell surface increases the chance to encounter its cognate receptor. Receptor binding triggers uptake of the particle into the cell by endocytosis.

Indications that HS is involved in active ligand trafficking was first reported for murine leukemia virus (MLV). After binding, MLV seems to laterally "surf" along filopodia in a highly ordered movement towards sites that seem to promote virus uptake. This "surfing" is mediated by the actin cytoskeleton and is driven by myosin motors (127). In a study by Myung-Jin Oh (105) a link between virus-filopodia adhesion and the engagement of retrograde F-actin transport, after binding to heparan sulfate, was demonstrated. UUKV was also observed to associate with filopodia (94) (Fig. 3). In thin-section EM and fluorescent microscopy it was shown

that, after exposure of cells to UUKV, half of the observed particles were associated with filopodia or located at their base. In the future, it will be interesting to study if bunyaviruses also use filopodia "surfing" to facilitate entry (Fig. 4). Secondly, identification of the cellular link that connects the virus to the actin filaments could provide a novel target for the development of antiviral drugs.

RVFV ENTRY BY ENDOCYTOSIS

After virus attachment most viruses require endocytic uptake before they enter the cytosol by penetration. In **chapter 6** we confirm by using pharmaceutical drugs that RVFV is taken up by the cell through endocytosis. In particular, RVFV infection was inhibited in the presence of dynasore or chlorpromazine, which interfere with dynamin-2 and clathrin dependent endocytosis, respectively. These drugs only interfered with RVFV infection when administrated early in infection. These preliminary findings are in agreement with the suggested clathrin mediated entry (CME) route of related bunyaviruses (58, 71, 92, 94, 126, 134). For UUKV, entry via clathrin-coated vesicles was observed by electron microscopic analysis, though a clathrin-independent alternative pathway was suggested to be the dominant entry route (94). Very recently, it was reported that RVFV is able to enter the cell via a dynamin–dependent and caveolae-mediated mechanism (50). It is however unclear how RVFV particles, which are ~100 nm in size, can be transported via these 60-80nm sized caveolae, which generally facilitate entry of viruses half the size of RVFV particles (98, 113). Clearly, additional experiments are needed to confirm the entry pathway(s) of RVFV.

In context of the puzzling data, when studying virus cell entry *in vitro*, one should consider that a particular entry pathway may be favored by the virus simply due to different conditions of virus inoculation (24 and references therein). Techniques like 3D single particle tracking in living cells will enable virus entry to be examined in great detail (29). In the near future, these and other novel techniques will expand our knowledge about existing entry routes as well as those that are yet to be discovered.

A detailed study on UUKV entry clearly indicated that the virus, after endocytic entry, is trafficked into the degradative branch of the endosomal pathway before it undergoes membrane fusion (94). Using a variety of techniques these authors convincingly show that the virus undergoes membrane fusion only upon arrival in late endosomal or lysosomal compartments where the pH is optimal for

fusion activation (94). Similar to UUKV, we have indications that RVFV also fuses in late endo/lysosomal vacuoles. The slow entry kinetics (halftime of ~20 min, **chapter 6**) of the virus after attachment is congruent with the speed of normal cargo delivery into these vacuoles. In addition, the activating pH of <6 for RVFV membrane fusion is found in late endosomes and lysosomal compartments. These data suggest that phleboviruses are transported to the perinuclear region of the cell before they enter the cytosol by penetration.

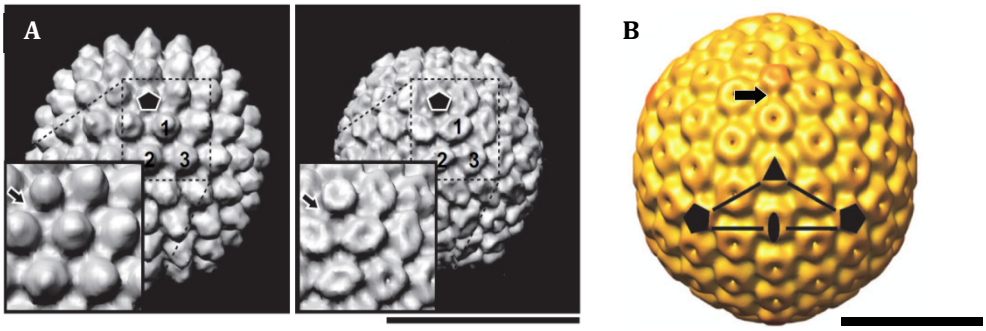
RVFV MEMBRANE FUSION

After endocytic uptake viruses respond to cues which activate virus entry. These cues trigger conformational changes in the virus leading to host cell penetration. It has been well established that RVFV membrane fusion is triggered by low pH (36, 90). At the start of our studies, however, the fusion process of bunyaviruses was largely unexplored. In the study described in **chapter 6** we provide new insights into the RVFV membrane fusion process. We demonstrate that highly stable oligomers of the Gc glycoprotein are formed after exposure of RVFV particles to acidic pH. Protonation of histidine residues in Gc, and not in Gn, seem to catalyze this conformational change. A single histidine-to-alanine substitution in Gc was found to abrogate acid-induced stable Gc multimere formation as well as entry of the virus into the cell.

Cryotomography analysis of the *Phlebovirus* virion structure

The structure of the RVFV virion has been studied using electron cryotomography analyses at neutral (pH 7.4) and slightly acidic pH (6.0). At both pH conditions the RVFV glycoproteins form cylindrical hollow spikes that cluster into distinct capsomers (39, 64). In contrast to RVFV, cryotomography analysis of UUKV particles revealed pH-dependent structural differences (108) (Fig. 5A, left). At pH <7, structural changes in the UUKV glycoproteins as well as aggregation of particles was observed (108) (Fig. 5A, right). Aggregation is probably caused by interactions of the acid-induced and exposed hydrophobic fusion loops within Gc. Previously, pH-dependent conformational changes in UUKV glycoproteins were reported to occur at pH 6.2 (122), however, to complete membrane fusion a more acid pH is required (92). Membrane fusion is commenced at pH <5.8 and the optimal pH for membrane fusion is pH ~5.4 for both phleboviruses (92) (**chapter 6**). This suggests that the reported glycoprotein rearrangement of UUKV virions at pH 6-7 represents an intermediate state. Our biochemical and functional analyses indicate that the pH threshold for RVFV

fusion protein activation is pH 5.7 (**chapter 6**) explaining the lack of conformational changes revealed by cryotomography after pH 6 exposure of the virions. The putative differences in the virion structures of UUKV and RVFV are intriguing and require further investigation.



UUKV pH 7 UUKV pH 6 RVFV

Fig.5. Particle morphology of UUKV and RVFV, determined by cryotomography at neutral or low acidic conditions. (A) Glycoprotein spike organization. Virions with spikes in extended conformation (pH 7, left) and in flat conformation (pH 6, right). Insets show close-ups of the area indicated with dashed lines. Bridging densities (indicated by arrows) are present between the spikes at every position and in both conformations (Scale bar, 100 nm). Reprinted by permission from Proceedings of the National Academy of Sciences. Overby AK, Pettersson RF, Grünewald K, Huiskonen JT. Insights into bunyavirus architecture from electron cryotomography of Uukuniemi virus. 19;105(7):2375-9, Copyright (2008) National Academy of Sciences, USA. (B) Shaded isosurface representation of RVFV MP-12 reconstructed from tilt series data at a 7.5-nm resolution. The structure was generated by averaging data for 46 individual particles extracted from three tomograms. Glycoprotein spikes are organized into flat "doughnut" shaped structures. Bridging densities are indicated by arrow. The icosahedral asymmetric unit is indicated by the black triangle. (Scale bar, 50 nm). Reprinted by permission from American Society for Microbiology Publisher of Journal of Virology (Freiberg, A. N., M. B. Sherman, M. C. Morais, M. R. Holbrook, and S. J. Watowich. 2008. Three-dimensional organization of Rift Valley fever virus revealed by cryoelectron tomography. J Virol 82:10341-10348.), copyright (2008).

Classification of the Bunyavirus fusion protein

Viral fusion proteins can be grouped in one of three classes based on shared post fusion structural motifs (22, 161). Previously, computational analysis suggested that bunyavirus Gc proteins belong to the family of class II fusion proteins (43). The authors proposed a class II fold with high β-sheet content similar to structures of the fusion proteins of alphaviruses and flaviviruses. Primary sequence homology and predicted secondary structural similarities between bunyavirus Gc and alphavirus E1 (43) support this prediction. The alpha- and flavivirus membrane fusion proteins are the prototype class II fusion proteins (75). Additional common features are shared

between the class II fusion proteins and the bunyavirus Gc glycoprotein (154). First, Bunyavirus glycoproteins are translated from a polyprotein precursor with the fusion protein located downstream of a companion glycoprotein (Gn). Second, bunyavirus glycoproteins assemble into heterodimers in the ER after which they are trafficked to the virus assembly site. In addition, after budding, the glycoprotein heterodimers are arranged into an ordered scaffold at the virion surface, completely (or partially in the case of hantavirus, 63) covering the viral membrane. In summary, these data implicate a strong homology between bunyavirus Gc and alpha- and flavivirus class II fusion proteins.

This assumption is further supported by recent modeling studies (124). Since the icosahedral structure of RVFV virions has been solved by electron cryotomography analysis (39, 64, 131), molecular modeling allows prediction of the glycoprotein structure and arrangement in the RVFV particle (124). Based on these modeling studies, the structure and prefusion arrangement for RVFV Gc was again proposed to share similarity with alphavirus class II fusion protein E1 (76). Until today these models have not been verified by crystal structure analysis.

Acid-driven reorganization of the Gc fusion protein

We report in **chapter 6** that membrane fusion of cell surface-bound $RVFV_{ns}$ is initiated at a pH below 5.7. Similar observations were reported for UUKV (94). This suggests that after receptor binding, no cellular cues other than low pH are required for entry of UUKV and RVFV. We also demonstrate that low pH incubation of $RVFV_{ns}$, in the absence of target membranes, results in formation of SDS- and heat-resistant Gc oligomers and a concomitant decrease in viral infectivity. These Gc oligomers were shown to be stable at temperatures as high as 80ºC. We speculate that the stable acid-activated Gc oligomer represents a membrane fusion intermediate or post fusion conformation and that the energy that is released upon rearrangement is applied for the merger of viral and host cell membranes. In order to determine the stoichiometry of the Gc oligomeric complex we performed gel filtration chromatography analysis with purified Gc ectodomain (Gce) at pH 7 and below (unpublished results and **chapter 6**). Unfortunately, due to heavy aggregation of Gce, we were unable to draw any clear conclusions. We propose that the Gc stable oligomer represents a postfusion conformation with trimeric stoichiometry, similar to the stoichiometry of all known viral fusion proteins in their post-fusion state (51). Such an organization of Gc suggests that the Gn/Gc heterodimers undergo at least a partial dissociation to allow trimerization of the Gc fusion protein. This would imply major and concerted

conformational rearrangements of the glycoproteins in the virus structure. It would be fascinating to reveal the details of bunyavirus particle reorganization upon acid-activation using high-resolution electron-microscopic techniques.

The role of conserved histidine residues in Gc

Our work indicates that histidines in Gc may serve as sensors of the acidic endosomal environment. Histidines are uncharged at neutral pH but become protonated and thereby positively charged at pH 6.0 or below (74). This is consistent with the pH threshold (pH <5.7) of $RVFV_{ns}$ fusion activation and Gc stable oligomer formation. By using alanine substitutions we identified three highly conserved histidines (H778, H857 and H1087) that are essential for virus entry. One of these substitutions (H857A) abrogated virus entry as well as the acid-induced formation of the stable Gc oligomer (**chapter 6**). The histidine at position H857 may therefore function as a *bona fide* pH sensor, similar to what has been reported for the flavivirus tick borne encephalitis virus and hepatitis C virus (10, 40). Intriguingly, the other two histidine substitutions (H778A and H1087A) did abrogate entry but not the pH-triggered conformational change in Gc, although the Gc oligomers were slightly less temperature stable. These results indicate that these histidines may have differential effects on distinct stages of membrane fusion (83). To unravel the precise role of these histidines in the membrane fusion process, assays need to be developed which can discriminate between the different steps of membrane fusion, in this case fusion peptide exposure and membrane insertion, hairpin formation, hemifusion, fusion pore formation and fusion pore expansion.

Besides histidine protonation, additional factors may drive membrane fusion. Viral fusion proteins often contain a stem region found upstream of its membrane anchor that comprises a short highly conserved motif enriched in aromatic amino acids. This juxtamembrane region may support virion assembly (84) but has also been implicated to play a role in the final stage of membrane fusion (21, 22, 61, 69, 70, 125, 146, 162). A predicted stem region of RVFV is positioned upstream of the C-terminal transmembrane region at position 1137-SGSWNFFDWFSGLMSWFGGPLK-1158. The importance of this domain in the membrane fusion process can be studied by alanine scanning mutational analysis.

How to overcome premature activation?

After their biogenesis, low-pH dependent viral fusion proteins have to avoid premature activation when passing the acidic environments of the Golgi-complex and

secretory vesicles. At least three different mechanisms can be distinguished to overcome this problem.

Influenza viruses express M2 proteins of which the ion channel activity helps to maintain a neutral pH in the *trans*-Golgi and secretory vesicles preventing inactivation of its class I HA fusion protein. Another elegant strategy is used by vesicular stomatitis virus (VSV) and baculoviruses. Low pH induced conformational changes in the class III fusion protein "G" of VSV and "gp64" of baculoviruses appear to be reversible, with pre- and postfusion forms existing in a thermodynamic equilibrium (97, 120, 121, 171).

The class II viral fusion proteins of alpha- and flaviviruses are protected by a peptide clamp (53, 135). In the case of alphaviruses the E2 precursor (p62 protein) assists in folding and transport of the p62-E1 heterodimer. During the maturation process the p62-E1 complex is processed by cellular furin-like proteases into E3/E2-E1 late in the secretory pathway (34, 170). The E3 peptide clamp stays associated with the E2-E1 complex suppressing the low-pH sensitive fusion protein from activation in the late and acidic parts of the exocytic pathway (15). E3 only dissociates after exposure to the neutral conditions outside the cell, priming the viral spikes for fusion (136). For flavivirus glycoproteins a similar mechanism has been reported. First the fusion protein E dimerizes with prM in the endoplasmic reticulum (ER) assisting folding (88). Budding occurs in the ER followed by trafficking through the trans-Golgi network (TGN). The acidic TGN induces a dramatic conformational change in the virus particle altering the glycoproteins from a spiky into a herringbone-like arrangement (167, 168). The acid-induced structural change allows furin to cleave the prM protein. The pr peptide remains bound and protects the fusion protein E from premature activation in the acidic environment of the late secretory pathway (168). The pr part is released upon exposure to neutral conditions of the extracellular environment (167) rendering the virus infectious.

Bunyavirus glycoproteins endure the mild acidic trans-Golgi (49, 91, 165, 166) and acidic content of export vesicles (15, 106) before they are released from the infected cell (104). We and others have shown that low pH followed by neutral pH incubation of bunyaviruses, leads to virus inactivation, which excludes a reversible activation mechanism (55, 56, 60, 119). In contrast to most class II viral fusion proteins, bunyavirus glycoproteins – with the exception of nairoviruses – do not undergo proteolytic maturation by Golgi-resident proteolytic enzymes.

Protection against premature inactivation may be closely associated with virus maturation. The maturation process of phleboviruses is unclear, whereas

maturation of the related *Orthobunyavirus* bunyamwera virus is well-studied. This maturation process involves three virus particle states, which are required to render the virus infectious (104). The first state represents particles with an annular morphology and a light core. Upon transition through the trans-Golgi network, these particles transform into more compact electron-dense particles. This process seems to be triggered by modification of glycans on the viral glycoproteins by *trans*-Golgi-resident enzymes (104). The particles undergo a final maturation when exiting the cell rendering them infectious. Inhibition of one of these crucial maturation stages severely decreases the infectivity of the virus. Importantly, no proteolytic processing, phosphorylation, or sulfation has been described for any of the *Orthobunyavirus* structural proteins.

It will be interesting to see whether this maturation process also applies to phleboviruses and how structural maturation of the virus particle is related to the priming of the fusion protein (133). In conclusion, it still remains to be seen how bunyaviruses prevent low pH inactivation of their fusion proteins in the acidic late secretory pathway.

CONCLUDING REMARKS

RVFV glycoproteins,

The RVFV genome encodes seven viral proteins that allow the virus to invade a variety of mammalian and insect hosts. The two surface glycoproteins Gn and Gc make up the virion icosahedral shell and are responsible for host cell attachment and entry. We have developed highly effective vaccines based on these structural glycoproteins and studied their role in virus entry and fusion.

Key to entry…

At the start of this study, little was known about the mechanism of bunyavirus entry and the role of the glycoproteins in this process. Recent studies have however revealed new insights into the entry pathway and the membrane fusion function of the Gc protein (**chapter 5, 6** and 50, 55, 92, 94, 117, 124, 132, 141, 150). Much less is known about the Gn glycoprotein, besides its putative functions in RNP packaging (109, 115, 143) and Gc folding and localization (45, 159). Recently it was reported that RVFV Gn contains a receptor binding domain similar to the influenza virus hemagglutinin protein (124). It will be of great interest to unravel the precise function of the bunyavirus Gn protein in both mammalian and insect cell entry.

Currently, no X-ray crystallography structure of any bunyavirus glycoprotein is available. The availability of pre- and postfusion structures will allow us to map the histidine residues identified in our studies providing further insight into the fusion process. It will be of particular interest to model the stages of conversion of the Gn/Gc metastable heterodimer towards the stable Gc oligomer.

…and control.

The RVFV glycoproteins induce neutralizing antibodies, currently the only established correlate of protection. In recent years there has been a strong increase in the development of novel RVFV vaccines that induce these antibodies (67). Due to our efforts and those of others, veterinary vaccines are expected to come to market in the next five to ten years that are superior over traditional vaccines with respect to combining DIVA, safety and efficacy.

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Nederlandse Samenvatting,

VOOR NIET INGEWIJDEN

Virussen en virologie, een introductie

Virussen zijn microscopisch kleine infectieuze agentia (Fig. 1) die levende cellen nodig hebben om zich te vermenigvuldigen. Met andere woorden, virussen zijn obligaat intracellulaire parasieten. Virussen zijn in staat alle levensvormen, dus ook andere micro-organismen zoals bacteriën, te infecteren. Wanneer een virus een gastheer binnendringt betekent dit niet dat deze ook ziek wordt. Virussen kunnen in harmonie met hun gastheer leven of worden als indringer herkend en direct opgeruimd door het interne verdedigingsmechanisme, ook wel immuunsysteem genoemd. Virulentie beschrijft het ziekteverwekkend vermogen van een pathogeen (ziekteverwekker). Hoog virulente virussen veroorzaken ziekte in een groter deel van de geïnfecteerde gastheren en veroorzaken ernstiger ziekte dan virussen met een lagere virulentie. Virologie is de medische wetenschap die zich bezighoudt met het bestuderen van virussen en het onwikkelen van middelen voor het voorkomen of behandelen van virale infecties. Iemand die zich gespecialiseerd heeft in de virologie noemt men een viroloog.

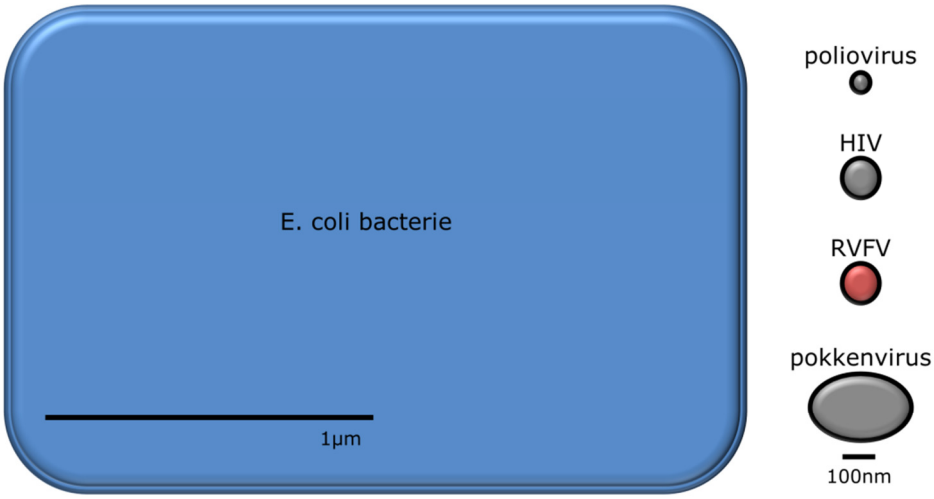

Figure.1. Illustratie van de grootte van een virus ten opzichte van een bacterie. De *Escherichia coli* bacterie (in blauw) leeft in ons darmstelsel en is op schaal ongeveer 500 keer kleiner dan deze punt →. Het RVFV virus daarentegen is weer vele malen kleiner. Eén centimeter (cm) = 10.000 micrometer (µm) = 10 miljoen nanometers (nm). RVFV; Rift Valley fever virus, HIV; humaan immunodeficiëntie virus.

Het virusdeeltje

Virussen bevatten een klein beetje genetisch materiaal (DNA of RNA). Dit is de blauwdruk van het virus waarop beschreven staat hoe in de geïnfecteerde gastheercel nieuwe virusdeeltjes gemaakt moeten worden. Het genetisch materiaal van het virus wordt beschermd door een omhulsel van eiwitmoleculen, dit noemt men ook wel het capside (Fig. 2). Sommige dierlijke virussen, waaronder het in dit onderzoek beschreven Rift Valley fever virus, hebben nog een extra beschermende buitenlaag die het envelop wordt genoemd. In het envelop zitten glycoproteïnen verankerd. Deze eiwitmoleculen zijn verantwoordelijk voor de interactie tussen het virus en de gastheer.

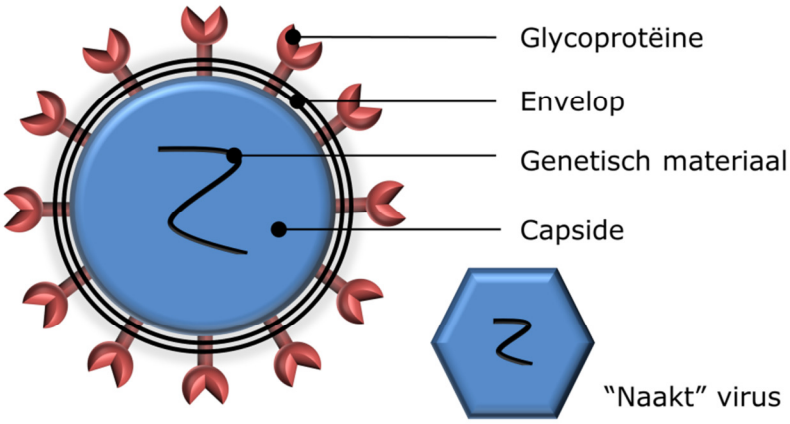

Figure.2. Schematische illustratie van een envelop omhuld virus en een "naakt" virusdeeltje. Het genetisch materiaal (genoom) van een virus wordt beschermd door een capside (blauw), welke in sommige gevallen omringt is door een envelop. Zogenaamd "naakte" virussen hebben geen envelop. Een virusdeeltje wordt ook wel virion genoemd.

Rift Valley fever virus

RVFV (een afkorting van het Engelse Rift Valley fever virus) behoort tot de virus familie genaamd *Bunyaviridae.* Virussen uit deze familie hebben een envelop, een RNA genoom en zijn ongeveer 100nm in doorsnede (Fig. 1). Het genoom van RVFV bestaat uit drie RNA-moleculen genaamd **S**mall, **M**edium en **L**arge (Fig. 3), welke samen coderen voor zeven virale eiwitten. Het virusdeeltje is opgebouwd uit vier van deze eiwitten. De andere drie eiwitten zijn nodig voor het efficiënt produceren van nieuwe virusdeeltjes in de gastheer.

Het RVFV is in 1930 ontdekt in de Rift vallei in Kenia tijdens een epidemie onder schapen. Naast schapen kan het virus ook runderen, geiten, kamelen en nog vele andere dieren infecteren. Een typisch kenmerk van een RVF uitbraak is grootschalige abortus.

RVF is een infectieziekte die van dieren overgedragen kan worden op mensen. Een ziekte met deze eigenschap noemt men een zoönose. Mensen kunnen geïnfecteerd worden door beten van besmette muggen of door contact met bloed van geïnfecteerde dieren. De symptomen in mensen zijn te vergelijken met griepachtige verschijnselen. Bij een klein percentage van de mensen kan de RVFV infectie leiden tot ernstige symptomen en complicaties, zoals hemorragische koorts (spontane bloedingen) of encefalitis (hersenvliesontsteking). Mensen met deze symptomen kunnen overlijden aan de gevolgen van de infectie.

De eerste grote uitbraak van RVFV deed zich voor in 1950-1951 in Kenia. Bij deze uitbraak overleden naar schatting 100 000 schapen aan de gevolgen van een RVFV infectie. In Afrika is RVFV voornamelijk te vinden in de regio's waar schapen en runderen worden gehouden zoals Kenia, Sudan en Senegal, maar het virus is ook te vinden in andere delen van Sub-Sahara Afrika inclusief Madagaskar. In 2000 is RVFV voor het eerst buiten Afrika geïdentificeerd in Saoedi-Arabië en Jemen. Er is een reële kans dat dit virus zich ook zal gaan verspreiden naar andere delen van de wereld, zoals Europa of America.

Een uitbraak van RVF in voorheen onaangetaste gebieden brengt grote sociale en economische schade met zich mee, zo is gebleken bij de uitbraken in Egypte (1977-78) en het Arabische schiereiland (2000). Daarom is het belangrijk om ons voor te bereiden op een mogelijke uitbraak, door het virus te bestuderen en beschermende maatregelen te nemen. De nog grotendeels onvoorspelbare en explosieve aard van RVFV uitbraken vereist dat RVFV vaccins een snelle bescherming bieden na éénmalige vaccinatie. Deze vaccins moeten niet alleen beschikbaar zijn voor landen waar RVFV momenteel endemisch is, maar ook voor landen buiten deze gebieden. Momenteel zijn er nog geen volledig veilige vaccins voor mens en dier beschikbaar die na éénmalige vaccinatie langdurige bescherming geven.

Het binnendringen van de gastheercel

Een virus kan zijn specifieke gastheer op verschillende manieren binnendringen. Dit kan zijn door de lucht of door middel van (in)direct contact. RVFV behoort tot de groep van arbovirussen (arthropod borne). Arbovirussen hebben gemeen dat ze door een geleedpotige vector kunnen worden overgedragen.

Het binden van virus aan een specifieke cellulaire receptor (molecuul voor aanhechting) kan worden beschouwd als de eerste stap van het infectieproces (Fig. 3A). Virussen die zijn omgeven door een envelop hechten zich aan de cel via de glycoprotëinen. RVFV heeft twee glycoproteïnen genaamd Gn en Gc (Fig. 3).

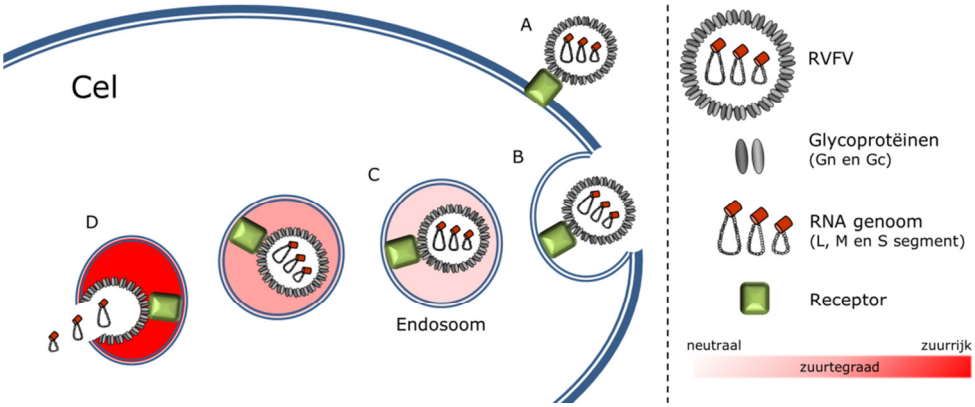

Figure.3. Hypothetisch model: de infectieroute van Rift Valley fever virus. (A) Het virus "plakt" aan het celoppervlak nadat de envelopeiwitten een specifieke cellulaire receptor herkennen en binden. (B) De receptor binding zorgt voor opname van het virus door de cel. Het virus wordt opgenomen in blaasjes genaamd endosomen. (C) Tijdens transport door de cel verzuurt de inhoud van de endosoom. Deze verzuring zorgt voor een verandering in de structuur van de envelopeiwitten. (D) De structuur verandering zorgt ervoor dat het membraan van het virus en de endosoom versmelten. Door deze fusie wordt het virale genoom in de cel afgeleverd. De cel zal in dienst van het virus nieuwe virusdeeltjes produceren en deze naar buiten transporteren. De cyclus zal dan weer bij (A) beginnen.

Nadat het virus zich aan de cel gehecht heeft kan een membraan omhuld virus zijn genetisch materiaal op twee verschillende manieren in de cel brengen. Door directe samensmelting tussen het virus en de celmembraan of na opname van het complete virusdeeltje door de cel in een blaasje genaamd endosoom. De inhoud van de endosoom verzuurt in de tijd (Fig. 3). Verschillende virussen gebruiken deze verzuring als startsein voor een structurele aanpassing van het virusdeeltje. Conformationele veranderingen in de glycoproteïnen kunnen een fusieproces initiëren waarin het virus en de endosomale membraan samensmelten (Fig. 3D). Voor RVFV is de route van binnendringen nog niet geheel bekend, maar verzuring van endosomen lijkt wel belangrijk te zijn voor infectie (Fig. 3).

Het virale genoom komt door de membraanfusie in contact met de inhoud van de cel. In opdracht van het virus zal de celmachinerie van de gastheer nieuwe virusdeeltjes maken die, nadat ze vrijkomen, weer nieuwe cellen kunnen infecteren. De infectiecycles begint vervolgens weer van voren af aan.

Vaccinatie

Bij een virus infectie van mens of dier zal in de meeste gevallen het immuunsysteem (ook afweersysteem genoemd) van de gastheer de infectie opmerken en de potentiële ziekteverwekker vernietigen. Er worden bijvoorbeeld antilichamen (antistoffen) aangemaakt die aan het virus binden. Vervolgens wordt het virus door de gastheer opgeruimd. Het virus wordt dan zogezegd, geneutraliseerd. In sommige gevallen wordt het virus laat of slecht herkend of is het afweersysteem niet direct in staat om de pathogeen te neutraliseren. Het virus kan zich dan vrij bewegen en vermenigvuldigen in de gastheer.

Vaccinatie heeft als doel specifieke immuniteit (bescherming in de vorm van antilichamen) tegen een pathogeen op te wekken door een kunstmatig verzwakte vorm of onderdeel van de ziekteverwekker toe te dienen. Het gevaccineerde dier is nu voorbereid op een infectie door dit specifieke pathogeen en is in de meeste gevallen beschermd voor langere tijd. Tegen sommige virusinfecties zijn antivirale geneesmiddelen beschikbaar die, in tegenstelling tot vaccins, alleen direct na toediening helpen om infecties te bestrijden door de virusinfecties af te remmen. Antibiotica zijn medicijnen voor het bestrijden van bacteriën en werken niet tegen virussen.

Nieuwe inzichten die gebruikt kunnen worden voor de preventie van virusinfecties kunnen alleen tot stand komen doormiddel van wetenschappelijk onderzoek. Nieuwe ontdekkingen kunnen gebruikt worden voor de ontwikkeling van nieuwe medicijnen en vaccins die bescherming bieden tegen een virusinfectie. Daarom is het belangrijk om de infectiecyclus van een virus te ontrafelen en interacties tussen het virus en de gastheer te bestuderen.

Het onderzoek

Het doel van het werk beschreven in dit proefschrift is de ontwikkeling van middelen die gebruikt kunnen worden voor de bescherming van mens en dier tegen RVFV. De RVFV glycoproteïnen bevinden zich aan het oppervlak van het virus en zijn daarom de belangrijkste doelen voor het immuunsysteem. Antilichamen gericht tegen de Gn en Gc glycoproteïnen kunnen het RVFV neutraliseren en de gastheer beschermen tegen een infectie. Binnen dit project zijn verschillende vaccins ontwikkeld en gekarakteriseerd die gebaseerd zijn op de RVFV glycoproteïnen. Deze kandidaat-vaccins worden als veilig beschouwd voor dieren van alle leeftijden. De werkzaamheid is geëvalueerd in muis- en schaapdiermodellen. Daarnaast is de infectieroute van

RVFV onderzocht waarbij bijzondere aandacht is besteed aan het fusieproces (Fig. 3). De resultaten van deze studies staan beschreven in de volgende hoofdstukken:

In de **hoofdstukken 2** en **3** is de ontwikkeling van drie experimentele vaccins beschreven. In **hoofdstuk 2** worden twee vaccins geëvalueerd die volledig op de RVFV glycoproteïnen zijn gebaseerd, zogenaamde subunit vaccins. Het eerste vaccin bestaat uit het gedeelte van het Gn glycoproteïne dat aan de buitenkant van het virusdeeltje zit, het zogenaamde ectodomein. Het Gn ectodomein (Gn-e) werd geproduceerd in insectencellen en opgezuiverd uit het medium waarin de cellen groeien. Het tweede vaccin bestaat uit virusachtige deeltjes zonder genetisch materiaal. Deze lege virusdeeltjes of VLPs (in het Engels virus-like particles) bestaan volledig uit de glycoproteïnen Gn en Gc en zijn wederom geproduceerd en uitgescheiden door insectencellen. In **hoofdstuk 3** wordt de beschermende werking van een recombinant Newcastle disease virus (NDV) vaccin beschreven. Dit recombinant NDV-GnGc virus produceert de RVFV structurele glycoproteïnen in het gavaccineerde dier. Een vaccin gebaseerd op deze techniek wordt een vector vaccin genoemd.

De werkzaamheid van de drie experimentele vaccins werd in een muisdiermodel getest. In dit diermodel beschermt vaccinatie met elke vaccin-kandidaat de muizen tegen een letale dosis van RVFV. Daarnaast werden lammeren gevaccineerd met het NDV-GnGc vector vaccin. Deze gevaccineerde lammeren ontwikkelden neutraliserende antilichamen tegen het RVFV.

In **hoofdstuk 4** wordt de effectiviteit van drie kandidaat-vaccins na éénmalige vaccinatie van lammeren beschreven. Deze studie omvatte het Gn-e subunit vaccin, het NDV-GnGc vector vaccin en een nieuw ontwikkeld vaccin, gebaseerd op RVFV replicondeeltjes. De replicondeeltjes lijken structureel op het virus, maar missen een gedeelte van de genetische code. Replicondeeltjes kunnen hun genoom nog wel repliceren in de geïnfecteerde cel, maar zijn niet in staat om nieuwe virusdeeltjes te maken en zich te verspreiden.

Deze studie liet zien dat na een eenmalige vaccinatie met elk van deze vaccins een neutraliserende antilichaam respons wordt geïnduceerd. Na vaccinatie zijn de lammeren geïnoculeerd met RVFV. Alle gevaccineerde lammeren waren beschermd tegen koorts, viremie (aanwezigheid van een virus in het bloed) en mortaliteit.

In het **vijfde hoofdstuk** wordt de rol van heparaansulfaat bij RVFV infectie besproken. Het glycosaminoglycaan (lange ketens van suikermoleculen) heparaansulfaat draagt bij aan een efficiënte RVFV infectie van verschillende cellijnen. Heparaansulfaat is veelvuldig aanwezig op het oppervlak van de meeste dierlijke

cellen en de betrokkenheid van deze structuur bij RVFV infectie kan mogelijk het brede gastheertropisme (affiniteit voor bepaald organisme) van het virus verklaren.

Het onderzoek in **hoofdstuk 6** beschrijft de bevindingen over de infectieroute van RVFV. Virusdeeltjes kunnen door de cel opgenomen worden via verschillende routes. Met behulp van farmaceutische drugs is de manier waarop RVFV een cel infecteert onderzocht. De resultaten van dit onderzoek moeten in toekomstig onderzoek bevestigd worden met behulp van alternatieve technieken.

De volgende stap in het infectieproces is membraanfusie. Onze studies bevestigen dat RVFV membraanfusie wordt geïnitieerd door lage pH (verzuring) zoals beschreven in het model van figuur 3. Incubatie van RVFV bij lage pH leidt tot veranderingen in de glycoproteïnestructuur van Gc. De structuurverandering initieert oligomerisatie (eiwitclustering van één soort molecuul) van het Gc glycoproteïne. Deze resultaten zijn in overeenstemming met de membraanfusie eigenschappen van glycoproteïnen van andere virussen. Specifieke histidine aminozuren in het Gc glycoproteïne (een eiwit is opgebouwd uit verschillende aminozuren) die belangrijk zijn voor de vorming van de Gc oligomeer zijn geïdentificeerd. Deze specifieke histidines spelen hoogstwaarschijnlijk een belangrijke rol in het membraanfusieproces.

Samenvattend

Epidemieën van RVF hebben in het verleden grote socio-economische schade veroorzaakt op het Afrikaanse continent en het Arabische Schiereiland. In dit proefschrift is een gedeelte van de levenscyclus van RVFV onderzocht om inzicht in de verschillende stadia van de infectie te krijgen. Klassieke moleculaire analyses en nieuw ontwikkelde technieken zijn toegepast om de Gn en Gc glycoproteïnen te bestuderen. Verschillende vaccinkandidaten werden geconstrueerd met als doel optimale effectiviteit te combineren met optimale veiligheid. Mede door de inspanningen beschreven in dit proefschrift is het mogelijk een veilig en effectief vaccin binnen enkele jaren beschikbaar te hebben dat gebruikt kan worden voor de bescherming van landbouwshuisdieren wereldwijd.

Dankwoord

DANKWOORD

Op 14 april 2008 ben ik met veel enthousiasme begonnen aan het onderzoek beschreven in dit proefschrift. Het einde van mijn promotietraject leek zo ver weg maar ik hier zit ik dan. Ik leg de laatste hand aan de afronding van uiteindelijk vier-en-een-half jaar werk.

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Martijn, wij hebben een speciale band. We zijn samen begonnen aan de MAVO in Apeldoorn en de MLO in Deventer welke ik na 1 jaar heb verlaten om verder te studeren in Arnhem. Desalniettemin kwam ik jou en je broertje weer tegen in Utrecht waar we drie jaar samen hebben gewerkt. Nu pakte jij de biezen en vertrok naar Nijmegen. Maar dat was van korte duur, want **Frank van Kuppeveld** nam je mee terug naar Utrecht. Welkom terug! We mogen de komende jaren weer samenwerken in de groep van **Frank**. Ik heb jou en mijn dierbare broertje **Jochem** gevraagd om samen mijn paranimfen te willen zijn. Fijn dat jullie beide deze “taak” hebben aangenomen.

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"Doe dingen waar je van houdt,
maar vooral hou van de dingen die je doet."

Curriculum Vitae

CURRICULUM VITAE

Matthijn de Boer werd geboren op 8 maart 1980 te Apeldoorn. In 1996 behaalde hij het MAVO diploma aan de Christelijke Scholengemeenschap de Heemgaard te Apeldoorn. In datzelfde jaar begon hij met de Middelbare Laboratorium Opleiding (MLO) aan het ROC Aventus in Deventer. Het daaropvolgende jaar werd de MLO studie voortgezet in Arnhem aan het Rijn IJssel College. Na stages bij het CPRO te Wageningen en het IRAD te Yaounde in Kameroen werd het MLO diploma "Biologische Laboratoriumtechniek" met de uitzonderlijke aantekening "uitmuntend" behaald.

De studie werd vervolgt in Velp waar hij begon aan de bachelor studie "Plant Biotechnology" aan het International Agricultural College Larenstein. Deze studie werd afgerond met stages bij Keygene N.V. te Wageningen en Plant & Food Research te Lincoln in Nieuw Zeeland. Na het behalen van zijn "Bachelor of Science" diploma in 2003 werd de studie voortgezet in Wageningen.

In augustus van 2003 is hij begonnen aan de opvolgende master aan de Universiteit van Wageningen en heeft deze opleiding twee jaar later afgerond. Tijdens deze studie heeft Matthijn stage gelopen aan de University of Berkeley, California onder begeleiding van Prof. Dr. Andy Jackson en Dr. Jennifer Bragg. Zijn afstudeerstage werd verricht bij de vakgroep Virologie aan de Universiteit van Wageningen onder begeleiding van Etienne Bucher, Dr. Marcel Prins en Prof. Dr. Rob Goldbach.

Alvorens Matthijn is begonnen aan zijn promotieonderzoek heeft hij 2 jaar als moleculair biologisch analist gewerkt bij het Centraal Veterinair Instituut van Wageningen UR te Lelystad (CVI-WUR), onder begeleiding van Dr. Ben Peeters. In april 2008 is hij aangesteld als assistent in opleiding bij de afdeling Virologie van het CVI-WUR en de afdeling Virologie van de faculteit Diergeneeskunde aan de Universiteit Utrecht. Onder begeleiding van Prof. dr. Rob Moormann, Prof. dr. Peter Rottier, Dr. Berend Jan Bosch en Dr. Jeroen Kortekaas werd het onderzoek verricht zoals beschreven in dit proefschrift.

Vanaf 1 januari 2013 zal Matthijn werkzaam zijn als post-doctoraal onderzoeker op de afdeling Virologie van de faculteit Diergeneeskunde te Utrecht, onder begeleiding van Prof. dr. Frank van Kuppeveld.

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