Pathogen-specific resistance to Rift Valley fever virus infection is induced in mosquito cells by expression of the recombinant nucleoprotein but not NSs non-structural protein sequences

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Rift Valley fever virus (RVFV) is an arbovirus of the Bunyaviridae family, causing recurrent disease outbreaks in Africa. Natural vertebrate hosts include cattle and humans. Several mosquito species belonging to the Aedes and Culex genera act as vectors of this phlebovirus. To test whether pathogen-derived resistance against RVFV could be induced by expressing genomic sequences in mosquito cells, as has been shown for La Crosse and dengue 2 viruses, we generated various recombinant Semliki Forest viruses expressing the S segment (or its genes) in the genomic or antigenic sense. Expression of the N but not the NSs gene interfered with the production of RVFV in mosquito cells and this phenomenon was RNA-dependent. These results raise questions on the molecular mechanisms involved in virus resistance.

Rift Valley fever virus (RVFV) is an arbovirus responsible for recurrent disease epidemics and epizootics in Africa (Meegan & Bailey, 1989; Peters & Linthicum, 1994), the most recent of which occurred in Egypt in 1993, Madagascar in 1990–1991, Kenya, Somalia and Tanzania in 1997–1998 and Mauritania in 1998. Natural vertebrate hosts of RVFV include cattle and humans. Mosquito species of the Aedes and Culex genera were reported to be the main vectors in Kenya and Egypt, respectively (Gonzalez-Scarano & Nathanson, 1990). RVFV (family Bunyaviridae, genus Phlebovirus) has a tri-segmented RNA genome of negative (L and M segments) or ambisense (S segment) polarity (Giorgi, 1996; Schmaljohn, 1996). The L and M segments code, respectively, for the RNA-dependent RNA polymerase and for a polypeptide which is cleaved during translation, thus generating the envelope glycoproteins G1 and G2 and the nonstructural proteins 14K and 78K. The S segment utilizes an ambisense strategy: the 5′ halves of the antigenomic and genomic strands contain, respectively, ORFs encoding the nucleoprotein N and the nonstructural protein NSs.

Homologous interference has been described for several arboviruses: togaviruses (Karpf et al., 1997; Stollar & Shenk, 1973), flaviviruses (Schmaljohn & Blair, 1977), bunyaviruses (Beaty et al., 1983; Elliott & Wilkie, 1986) and RVFV (Turell et al., 1990). More recently, B. J. Beaty’s group has developed recombinant Sindbis virus-based expression systems to produce intracellular immunity or pathogen-derived resistance against La Crosse virus or dengue virus in mosquitoes and in mosquito cells (Gaines et al., 1996; Olson et al., 1996; Powers et al., 1994, 1996). Using recombinant Semliki Forest virus (SFV) replicons as a vector, we investigated the possibility of producing mosquito cells resistant to RVFV and showed that homologous interference was induced by N but not NSs sequences and was RNA-mediated. This raises questions on the molecular mechanisms involved in virus resistance.

SFV replicons carrying partial or complete sequences of the RVFV MP12 strain S segment were prepared by inserting the viral sequences into pSFV-1 at the unique SpeI site and electroporated into BHK-21 cells together with the Helper 2-RNA to produce suicide particles, as described by Liljestrom & Garroff (1991, 1995).

To examine the effect of RVFV nucleoprotein sequences on replication of homologous virus we constructed pSFV-N, containing the sequence of the N ORF, and infected Aedes pseudoscutellaris (Ap61) cells with the recombinant SFV-N particles. Infection was monitored by immunofluorescence assay using the anti-SFV nsp3 or an anti-N monoclonal antibody and a fluorescein-labelled secondary antibody. In cells infected at an m.o.i. of 5 and examined at day 2 post-infection (p.i.), more than 80% of the cells exhibited a bright cytoplasmic fluorescence corresponding to the specific distribution of the RVFV N or SFV nsp3 proteins (Fig. 1A, B). Of the infected cells 50% were still positive on day 7 and 20% on day 12 p.i. (not shown), but staining was less intense, indicating that the replicons persisted in the cells but were diluted or became inefficient for protein expression as cells proliferated.

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Fig. 1. Detection of viral proteins and RNAs after infection of Ap61 with recombinant SFV. Cells were infected with SFV-N (A, B) or SFV-NSs (C) at an m.o.i. of 5 or mock-infected (D). After 48 h they were fixed with methanol, incubated with an anti-N (A) or anti-NSs (C) mouse monoclonal antibody (gifts of J. Smith) or rabbit anti-nsP3 serum (B) (gift of L. Kääriäinen), followed by incubation with fluorescein-labelled goat anti-mouse or rabbit IgG (Sigma) and counterstaining with Evans blue. (E) Northern blot analysis of cytoplasmic RNA extracted from Ap61 cells infected for 48 h with SFV-1, SFV-N, SFV-Nanti, SFV-NSs, SFV-S2, or SFV-S2ag at an m.o.i. of 5, or mock-infected. A 32P-labelled oligonucleotide complementary to the 5’ terminal sequence of the SFV 26S subgenomic RNA (5’ CAGAATTCTGTGTATTAACGCACC 3’) was used as a probe.
Table 1. Production of MP12 virus by day 4 p.i. in Ap61 cells pre-infected with various recombinant SFVs expressing RVFV sequences

<table>
<thead>
<tr>
<th>SFV recombinant*</th>
<th>Expt I [log₁₀ (p.f.u./ml)]†</th>
<th>Expt II [log₁₀ (p.f.u./ml)]†</th>
<th>Expt III [Δlog₁₀ (p.f.u./ml)]‡</th>
<th>Mean of n expts [Δlog₁₀ (p.f.u./ml)]‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>7·91</td>
<td>7·02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFV-1</td>
<td>7·19 (+ 0·05)</td>
<td>7·32 (+ 0·41)</td>
<td>7·08 (+ 0·06)</td>
<td>+0·27 ± 0·24, n = 6</td>
</tr>
<tr>
<td>SFV-fgal</td>
<td>7·08 (– 0·06)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFV-N</td>
<td>4·31 (– 2·83)</td>
<td>3·72 (– 3·19)</td>
<td>3·30 (– 3·72)</td>
<td>–3·01 ± 0·45, n = 6</td>
</tr>
<tr>
<td>SFV-N₅₉</td>
<td>3·97 (– 3·17)</td>
<td>3·88 (– 3·03)</td>
<td>3·78 (– 3·24)</td>
<td>–3·32 ± 0·35, n = 4</td>
</tr>
<tr>
<td>SFV-N_stop</td>
<td></td>
<td>2·38 (– 4·04)</td>
<td></td>
<td>–4·31 ± 0·47, n = 2</td>
</tr>
<tr>
<td>SFV-NS</td>
<td>7·43 (± 0·52)</td>
<td>6·87 (– 0·15)</td>
<td></td>
<td>+0·10 ± 0·37, n = 3</td>
</tr>
<tr>
<td>SFV-NS₅₉</td>
<td>7·38 (± 0·47)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SFV-NS_stop</td>
<td>7·36 (± 0·45)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFV-S₉</td>
<td>5·68 (– 1·49)</td>
<td></td>
<td>–2·26 ± 0·34, n = 4</td>
<td></td>
</tr>
<tr>
<td>SFV-S₉_stop</td>
<td>4·13 (– 3·01)</td>
<td></td>
<td>–1·26 ± 0·28, n = 2</td>
<td></td>
</tr>
</tbody>
</table>

* SFV-fgal, recombinant SFV for β-galactosidase; SFV-N and SFV-N₀₅₉, RVFV N protein sequence in sense or antisense orientation, respectively; SFV-NSs and SFV-NSs₅₉, RVFV NSs protein sequence in sense or antisense orientation, respectively; SFV-NSs_stop and SFV-N_stop, RVFV NSs or N protein sequence with the initiation codon mutated; SFV-S₉ and SFV-S₉_stop, RVFV S segment sequence in genomic or antigenomic orientation, respectively.
† Values in parentheses are the differences between MP12 titres (log₁₀) observed in SFV- and mock-infected cells.
‡ Differences between MP12 titres observed in SFV- and mock-infected cells ± standard deviations; results obtained in n experiments.

At 48 h p.i. cultures infected with SFV-N were superinfected with the RVFV MP12 strain and the virus released into the extracellular medium 4 days later was plaque assayed, a method which titrated only RVFV since the recombinant SFV suicide particles did not produce any infectious virus. Preliminary experiments indicated that the kinetics of MP12 virus production by Ap61 cells showed an exponential curve and reached a plateau 4–5 days p.i. (not shown). When compared to the titre obtained in the mock-infected cells or in cells infected with SFV-1 or SFV-fgal, the yield of MP12 virus in Ap61 cells infected with SFV-N was inhibited more than 1000-fold (Table 1). In addition, less than 5% of these superinfected cells exhibited positive staining by immunofluorescence assay (using a mixture of anti-G2 and anti-NSs RVFV-specific monoclonal antibodies on day 4 p.i.) whereas all the control cells were positive (not shown). Optimal conditions for interference were achieved when recombinant SFV and MP12 were used at m.o.i.s of 5 IU (infectious unit) and 0·01 p.f.u. per cell, respectively. Decreasing the m.o.i. of SFV-N to 1 did not significantly reduce the interference level. However, when superinfection with MP12 was carried out at an m.o.i. of 1, the phenomenon still occurred but virus production was inhibited only 10-fold. Interestingly, SFV-1 without insert or SFV-fgal induced a positive interference toward RVFV, which was more pronounced during the first days after infection (Table 1).

To determine whether interference was due to protein or RNA, pSFV-N was mutated into pSFV-N_stop by changing the initiation codon of the N ORF into a stop codon. Inhibition of MP12 production in Ap61 cells infected with SFV-N_stop was similar to that caused by SFV-N or even more (Table 1). Similar interference was observed in cells infected with SFV-N₅₉, a replicon expressing the N sequence in the antisense orientation (Table 1). These results strongly suggest that the N sequence in sense or antisense orientation but not the protein is responsible for the inhibitory effect.

Because the S segment encodes two proteins in opposite orientations, we investigated the possible effect of the NSs sequences and constructed replicons SFV-NSs and SFV-NSs₅₉ containing the NSs sequences in the sense and antisense orientation, respectively. Fluorescent nuclear filaments, as observed in RVFV infection, were seen in Ap61 cells infected with SFV-NSs (Fig. 1C). However, although the amounts of recombinant genomic and subgenomic RNAs estimated by Northern blot analysis were equivalent to those present in SFV-N-infected cells (Fig. 1E), expression of NSs sequences in Ap61 cells did not induce any homologous interference (Table 1). To eliminate a possible antagonistic effect of NSs protein toward putative inhibition induced by the NSs nucleotide sequence, we mutated the AUG of the initiation codon of SFV-NSs, generating replicon SFV-NSs_stop. Infection with this virus did not induce any protection to superinfection by MP12 (Table 1), confirming that the NSs gene possesses no interfering potential.

Since the 3’ and 5’ halves of the S segment induced different effects on intracellular immunity, we tested the effect of complete S segment expression by generating SFV-S₉ and...
SFV-SS\textsubscript{ag}. Cells infected with SFV-SS\textsubscript{ag} were resistant to MP12 infection to the same degree as cells infected with SFV-N but interference was significantly lower in cells expressing the S segment in the genomic orientation (Table 1). The degree of inhibition induced by SFV-S\textsubscript{g} and SFV-S\textsubscript{ag} was not due to different amounts of RNAs synthesized by the recombinant SFVs, as revealed by Northern blot (Fig. 1E); increasing the m.o.i. of SFV-S\textsubscript{g} to 20 IU per cell had no effect.

To address the question of whether the observed interference due to the N sequence of RVFV was restricted to the homologous virus or could be extended to other viruses, we challenged SFV-N-infected Ap61 cells with the heterologous RVFV Clone 13, the related virus Toscana phlebovirus, and the unrelated Germiston bunyavirus and yellow fever flavivirus (17D strain). A similar experiment with SFV-N\textsubscript{anti} led to similar results. Pre-infection with SFV-N inhibited Clone 13 replication although to a lesser extent than MP12 (Table 2). Interestingly, the sequences encoding the N protein of Clone 13 and MP12 share 97.8\% identity [16 mutations over 736 nucleotides (Müller et al., 1995)]. If intracellular immunity involves hybridization between the inducer and challenge RNAs, the fact that Clone 13 partially overcomes the resistance induced by the N sequences is likely to be due to an increased level of replication compared to MP12, a property repeatedly observed in cells infected with Clone 13. The sequences of RVFV N protein did not induce resistance toward Germiston or yellow fever viruses but resulted in a slightly reduced yield of Toscana virus (Table 2), the progeny of which contained a considerable amount of a small plaque variant when compared to the progeny grown in control cells. This result indicates that the related Toscana virus, sharing 59\% identity, was inhibited to some extent by the RVFV N sequences. A similar observation was reported by Powers et al. (1996) who showed that La Crosse bunyavirus sequences, too, had an effect on the production of the related Trivittatus virus (62\% identity).

In summary, expression of RNA molecules derived from the S segment of the RVFV MP12 genome in Ap61 cells showed that N, but not NSs, RNA molecules interfere with replication of the homologous virus. Interference was induced by RNA molecules in both orientations and was independent of protein expression. Using recombinant Sindbis virus to induce resistance against La Crosse virus, Powers et al. (1994, 1996) reported a major effect of the N sequences and, concerning the role of the NSs sequence, the antisense orientation was found to interfere with the virus yield but the sense orientation could not be tested because expression of the protein was toxic for the cells. This is in contrast with the situation observed with the RVFV NSs sequences which did not interfere in any orientation. It is noteworthy that the S organization of bunyaviruses and phleboviruses is different: the N and NSs ORFs are in the same orientation and overlap in bunyaviruses whereas they are in opposite orientations and do not overlap in phleboviruses. Thus, since the N sequence of La Crosse virus inhibits virus production, the specific region corresponding to NSs would also be expected to interfere unless it is too short, a situation reported for tospoviruses where the inducer RNA must have a minimal size (Jan et al., 2000). Although some sequences of La Crosse virus M segment (Powers et al., 1996) or the L segment of RVFV (not shown) were also inefficient for interference, the fact that 5‘ and 3‘ regions of the S segment lead to a different effect is intriguing and raises questions on the molecular mechanisms involved in resistance. Since, when it occurs, the phenomenon is RNA-mediated, RNA duplexes between the RNA inducer and the targeted viral RNAs might be formed, blocking translation, replication, transcription or RNP formation, depending on the sense of the expressed RNA. If RVFV NSs is an accessory protein as suggested (Kohl et al., 1999; Vialat et al., 2000), inhibition of its translation by SFV-NS\textsubscript{anti} would not have major consequences and would not cause interference. However, the NSs RNA could also hybridize with the genome (or with the antigenome in cells expressing SFV-NSs and SFV-

### Table 2. Heterologous interference in cells expressing MP12 RVFV sequences via infection with SFV-N or SFV-N\textsubscript{anti}

<table>
<thead>
<tr>
<th>Pre-infection with:*</th>
<th>Infection with:†</th>
<th>RVFV MP12</th>
<th>RVFV C13</th>
<th>Toscana virus</th>
<th>Germiston virus</th>
<th>Yellow fever virus</th>
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<tbody>
<tr>
<td>Mock</td>
<td></td>
<td>6:08</td>
<td>7:08</td>
<td>4:78</td>
<td>4:77</td>
<td>5:60</td>
</tr>
<tr>
<td>SFV-1</td>
<td></td>
<td>7:15</td>
<td>7:48</td>
<td>6:11</td>
<td>5:30</td>
<td>5:43</td>
</tr>
<tr>
<td>SFV-N</td>
<td></td>
<td>3:28</td>
<td>5:49</td>
<td>4:30</td>
<td>5:45</td>
<td>5:60</td>
</tr>
<tr>
<td>SFV-N\textsubscript{anti}</td>
<td></td>
<td>3:28</td>
<td>5:36</td>
<td>4:30</td>
<td>5:38</td>
<td>5:32</td>
</tr>
</tbody>
</table>

* Cells were infected with the recombinant SFV at an m.o.i. of 20.
† Cells were challenged at 48 h p.i. with recombinant SFV at an m.o.i. of 0:01 for RVFV MP12 and C13, 1 for Toscana and Germiston viruses and 0:1 for yellow fever virus. These m.o.i.s yielded 80–100\% immunofluorescent positive cells. Virus produced by day 4 was plaque-assayed.
NSs\textsubscript{stop} and be expected to block transcription/replication or encapsidation. Obviously, this is not the case and it is difficult to understand why this inhibition occurs with the N sequences and not with the NSs sequences. As data accumulate on virus resistance and on post-transcriptional gene silencing in plants, fungi and higher organisms, it would be tempting to assume that interference in mosquito cells results from similar mechanisms (Baulcombe, 1999; Cogoni & Macino, 1999; Fire, 1999; Hunter, 2000; Waterhouse et al., 1998). As in plants, synthesis of recombinant RNA may trigger degradation of the target viral RNA through the possible action of double-stranded targeted RNases or synthesis of RNases similar to the vertebrate RNase L, directly or after amplification by an RNA-dependent RNA polymerase. At the moment, we cannot explain why NSs sequences have no effect. In plants also, there exist sequences such as the 5' region of the \( \beta \)-glucuronidase gene (English \textit{et al.}, 1996) or the cowpea mosaic virus movement protein (Sijen \textit{et al.}, 1996) which do not induce gene silencing or virus resistance. Numerous reports have emphasized the importance of double-stranded RNA, injected into cells [Fire (1999) and references cited], generated by transgenes containing sense and antisense sequences in foldback configuration (Waterhouse \textit{et al.}, 1998), or synthesized as replicative forms during replication of recombinant RNA viruses (Angell & Baulcombe, 1997; Dalmay \textit{et al.}, 2000), in the induction of gene silencing in plant and higher organisms. Here, the replicative forms synthesized via the SFV-NSs replicon might not be efficient enough to provoke interferences. To test a possible role of an RNA duplex, we co-infected cells with the recombinants SFV-NSs and SFV-NS\textsubscript{anti} but did not find any resistance (not shown), indicating that the double-stranded RNA is not a sufficient signal. Further experiments will be necessary to determine the mechanism involved in induction of resistance to RVFV.

In their studies, B. J. Beatty and colleagues utilized recombinant Sindbis viruses to express La Crosse or dengue virus sequences and showed that mosquito cells as well as mosquitoes were resistant to homologous infection (Olson \textit{et al.}, 1996; Powers \textit{et al.}, 1996). Here, for biosafety we used SFV suicide particles to orally infect \textit{Aedes aegypti} mosquitoes but unfortunately, we were unable to detect replicon-driven expression probably because a very small number of cells had been infected. A replicative vector efficient for oral infection would be necessary to test the interfering effect in mosquitoes.

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References


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