Persistent infection of mammalian cells by Rift Valley fever virus

Agnès Billecocq, Pierre Vialat and Michèle Bouloy

Institut Pasteur, Laboratoire des Bunyaviridés, 25 rue du Dr Roux, 75724 Paris Cedex 15, France

Infection of mammalian cells with Rift Valley fever virus (RVFV) leads generally to the production of virus and cell death. In this paper we examined the fate of Vero cells infected with three strains of RVFV and observed that, while a large proportion of cells exhibited a clear cytopathic effect (CPE), a small but significant fraction did not undergo a lytic infection but was able to proliferate and establish a persistent infection. Several independent RVFV persistently infected cell lines have been established and passaged for more than 1 year after infection with a virulent strain (ZH548) and two attenuated strains (C13 and MP12). Although the viruses used for the primary infection were plaque-purified, we do not know whether defective-interfering particles were responsible for the establishment of the persistent infection. The persistently infected cells became resistant to superinfection with RVFV but not with other viruses and shed low amounts of infectious, lytic and non-lytic virus during a limited number of passages. In all the passages tested, the three genomic segments or related products were synthesized as well as the structural nucleoprotein N and glycoproteins G1 and G2. Abnormal defective RNAs were detected, migrating faster or slower than their respective counterparts. The faster-migrating RNAs were internally deleted, some of them possessing only the very terminal part of the 5' genomic end.

Introduction

Rift Valley fever virus (RVFV) is an arbovirus causing haemorrhagic fevers and affecting animals and humans in Africa. The virus was first isolated in 1930 in Kenya. Since then several epidemics have occurred, the most important ones being reported in Egypt (1977–1978), Mauritania (1987) and Madagascar (1990–1991). After a silent period the virus reappeared in Egypt in 1993 (For a review see Peters & Linthicum, 1993).

Rift Valley fever virus belongs to the family Bunyaviridae (genus Phlebovirus) and possesses a trisegmented, single-stranded, negative-sense RNA genome (For reviews see Elliott, 1990; Bouloy, 1990; Schmaljohn, 1996). The three genomic segments have been sequenced: the L segment codes for the L protein (Müller et al., 1991), the M segment for the precursor to the glycoproteins G1 and G2 and the two non-structural proteins 78 kDa and 14 kDa (Collett et al., 1985; Kakach et al., 1988) and the S segment, which has an ambisense strategy, codes for the N nucleoprotein in the antigenomic sense and for the non-structural NSs protein in the genomic sense (Giorgi et al., 1991). The function of the non-structural proteins NSs, 78 kDa and 14 kDa are still unknown. The NSs protein is phosphorylated and forms filamentous structures observed in the nucleus (Struthers et al., 1984). The presence of the protein in the nucleus remains unexplained since viral replication takes place entirely in the cytoplasm and budding occurs at the membranes of the Golgi apparatus (Murphy et al., 1973; Anderson & Smith, 1987) or in some cases at the plasma membrane (Anderson & Smith, 1987).

Except for hantaviruses, which are transmitted by rodents, and tospoviruses, which infect plants, many members of the Bunyaviridae infect and replicate in vertebrates and invertebrates. Whereas it provokes a clear CPE in vertebrate cells, infection is non-lytic in invertebrate cells which continue to produce virus and become persistently infected (for a review see Gonzalez-Scarano & Nathanson, 1996). Only the phlebovirus Toscana (Verani et al., 1984), and nairovirus Dugbe (David-West & Porterfield, 1974) have been reported to establish a persistent infection in mammalian cells. In both cases, the viral inoculum seems to have been enriched in defective-interfering (DI) particles.

Here, we report that infection of mammalian cells (Vero) with RVFV gave rise to persistent infection: whereas most of the infected cells exhibited a clear CPE and died, a small but
significant fraction remained viable and became persistently infected. We do not know what the factors responsible for the establishment of persistence are, but, although the viruses used for the primary infection were plaque-purified, it cannot be excluded that the inoculum contained minor amounts of DI particles. The biological properties of the persistently RVFV-infected mammalian cells are described and the molecular characteristics of the viral components analysed.

**Methods**

**Cells.** Clones VC10 and C1008 of Vero cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Vero cells from Institut Pasteur-Merieux (IPM) were maintained in Iscove's modified Dulbecco's medium supplemented with 10% FBS. Penicillin (200 U/ml) and streptomycin sulphate (200 μg/ml) were added to the media.

**Virus.** The MP12 strain of RVFV obtained after 12 passages and plaque-purifications in the presence of 5-fluoro-uracil (Caplen et al. 1985) was kindly provided by John Morrill (Applied Research Division, USAMRIID) and passed once in Vero cells at low m.o.i. prior to use. Its titre was 2 × 10^10 p.f.u./ml. The ZH548 strain was plaque-purified twice and grown in Vero cells. Its titre was 7 × 10^8 p.f.u./ml. The CI3 strain previously described (Muller et al., 1995) was a clone isolated by plaque purification from a natural strain and the viral stock used in this work was further plaque-purified twice and propagated at low m.o.i. (0-01) in Vero cells. Its titre was 9 × 10^6 p.f.u./ml. Punta Toro, Sindbis, and Toscana viruses were kindly provided by D. H. L. Bishop and C. Giorgi, respectively.

**Virus titration.** Infectious virus was titrated by plaque assay or immunoprecipitation performed by infecting VC10 cells. Cells were incubated for 6 days under an overlay consisting of DMEM, 2% FBS, antibiotics and 1% Indubiose (IBF Biotechnics) at 37 °C. The lytic plaques were counted after staining with a solution of crystal violet (0.2% in 10% formaldehyde and 20% ethanol).

Infected cell foci were revealed by immunostaining; after elimination of the agarose overlay, the cells were fixed with 3:7 Formaldehyde, permeabilized with 0.5% Triton-X100 then incubated successively with a 1:200 dilution of a mouse polyclonal serum directed against the N protein, a 1:1000 dilution of mouse monoclonal antibody (MAb) specific for the G1 or G2 glycoprotein (MAb 5G2 or 4D4) diluted 1:100 or with a 1:100 dilution of a mouse normal ascitic fluid, followed by fluorescein-labelled goat anti-mouse IgG (Biosys) diluted 1:100. Labelled cells were examined under a Zeiss microscope equipped for epifluorescence microscopy.

**Northern blot analysis.** Cytoplasmic extracts were prepared in a lysis buffer containing 0.6% Nonidet P40 and RNA was extracted with phenol/chloroform. Total RNAs were separated by electrophoresis in a 1% denaturing agarose gel containing either 6% formaldehyde after denaturation in the presence of 50% formamide (Lehrach et al., 1977) or 5 mM-methyl mercapto hydrroxyde (Bailey & Davidson, 1976), and transferred by blotting onto Hybond N membranes (Amersham). The prehybridization and hybridization with the riboprobes were performed at 42 °C in a buffer containing 50% formamide, 5 × Denhardt's solution, 0.5% SDS, 5 × SSPE (0.9% NaCl, 10 mM-NaH₂PO₄, 1 mM-EDTA, pH 7.4) and 20 μg/ml salmon sperm DNA. The membranes were hybridized overnight and washed successively with 2 × SSPE, 0.1% SDS at room temperature, 1 × SSPE, 0.1% SDS for 15 min at 65 °C, and 0.1 × SSPE, 0.1% SDS for 10 min at 65 °C.

Hybridization with the oligodeoxynucleotides was performed under the same conditions except that the membrane was incubated at 39 °C and washed twice for 10 min in 0.1 × SSPE, 0.1% SDS at 12 °C below the melting temperature of the oligodeoxynucleotide. The membrane was autoradiographed on Fuji RX film and washed twice for 15 min at 95 °C in 0.1% SDS for reprobing.

The riboprobes were synthesized from partial or full-length L, M and S cDNA cloned in pBSks+ (Stratagene) downstream from the T7 or T3 promoter. The plasmid was linearized with the appropriate restriction enzymes and the RNA synthesized with the T7 or T3 RNA polymerase in the presence of [α-35P]GTP (Amersham), under the conditions recommended by the supplier. The oligodeoxynucleotides were labelled in the presence of [γ-32P]ATP (Amersham) using T4 polynucleotide kinase. Oligo-L (ACAAAAAGGCGCCCAATC, complementary to nt 6387–6404 of the L genome) is specific for the 3' end and Oligo-F (GCTAACATTTGGGCGCTTTTGTG, complementary to nt 1–26 of the genome). Oligo-H (GGAATCATGTTGGTTTCTGAT, complementary to nt 35–57 of the genome), Oligo-J (TATGAGGAGACATGTGCGGAT, complementary to nt 122–145 of the genome) and Oligo-D₃ (CTAGAAAACGTCGCTGTC, complementary to nt 231–248 of the genome) were specific for the 5' end.

**Intracellular nucleocapsid purification.** Cells were disrupted in presence of 0.6% Nonidet P40 and the nucleocapsids were purified on a CsCl gradient (20–40%) as described by Leppert et al. (1979). The ribonucleoprotein (RNP) fraction was made 0.3% in Sarkosyl, the RNA phenol/chloroform extracted and ethanol precipitated.

**Results**

**Establishment of persistent infection of Vero cells by RVFV**

When cultures of Vero cells were infected with RVFV, the peak of virus production occurred after 2 or 3 days, depending on the m.o.i., and viral replication was associated with cells rounding up, detaching from the plate and dying. After a few days (usually 5–6 days), when the dead cells were eliminated and fresh medium was added to the plates, we observed the presence of scattered foci composed of surviving cells which could be visualized after staining with crystal violet (not shown). To determine whether the surviving cells had been infected, those which remained attached to the plastic were
Fig. 1. Detection of Vero cells surviving after the acute phase of RVFV infection. Vero VCI 0 cells were infected with the MP12 or C13 strain at an m.o.i. of 0.001. After 3 days, dead cells were eliminated and fresh medium was added. The remaining cells were incubated for an additional 3 days at 37 °C, formaldehyde-fixed and Triton-X100-permeabilized then incubated with antibodies directed against RVFV N protein and the foci revealed by immunoperoxidase staining.

fixed and treated with antibodies against the N protein. Fig. 1 shows that the foci were stained by immunoperoxidase and, at the microscopic level, almost all the cells exhibited a positive reaction in the cytoplasm (not shown).

Since the first experiments were performed with the attenuated MP12 strain and clone VC10 of Vero cells, it was important to know whether the persistent infection was due to this particular clone of Vero cells or could be achieved with other strains. Therefore, other strains, C13 and ZH548 and other clones of Vero cells, C1008 and IP-M were tested. Persistently infected cells were observed in VC10 cells with the attenuated C13 and virulent ZH548 strains as well as after infection of the two clones of Vero cells 1008 and IP-M with the C13 strain. The number of the surviving cell foci varied with the RVFV strains and the clone of Vero cell. This is illustrated in Fig. 1 in which the VC10 cells were stained 6 days post-infection (p.i.) at an m.o.i. of 0.001. Foci were more numerous after infection with C13 than MP12 strain (Fig. 1) and in the case of the virulent strain, the overall CPE was more pronounced and the number of foci was reduced when compared to the attenuated strains MP12 and C13 (not shown). The foci observed after infection of the three subclones of Vero cells with C13 strain exhibited a slightly different aspect: they appeared larger and more numerous with IP-M and tiny and less abundant with C1008 (not shown). These variations might be related to the intrinsic properties of the cells.

Once the cells had survived the primary acute infection, foci developed and maintenance medium was changed weekly until the monolayer became confluent (after approximately 3 weeks). The persistently infected cultures displayed a slightly lower multiplication rate than uninfected cells but the morphological aspect of the cells was unmodified. Several cultures of persistently infected cells were established after primary infections with different virus strains and up to this time, these cell lines have been passaged weekly at a 1 to 5 dilution. Some of the cultures have been studied further for the expression of viral genomic RNA and structural proteins as well as for the production of infectious virions.

Biological properties of persistently infected cells

First, we plaque assayed the infectious particles present in the culture medium harvested from various passages. One of the VC10 cultures persistently infected with the strains C13 or MP12 was analysed (Fig. 2). The titres of extracellular virus decreased progressively and, at passage 4 of the MP12 virus-infected cells or passage 19 of the C13 virus-infected cells, infectious virus was no longer detectable. Similar patterns of virus production were observed with two other persistently infected cell lines obtained after infection with these strains. As the production of infectious virus decreased, the plaque size diminished, suggesting that the particles produced were less and less cytopathic. This was confirmed by titrating the viral product by an assay which detects foci expressing the viral antigens (focus forming units; f.f.u.). Various dilutions of viral suspension were inoculated into cells which were incubated under an agarose layer. The presence of infected cells was revealed by antibodies against the N protein and immunoperoxidase staining. It is of note that, when the standard stock was assayed by the two methods, the titres were similar but the foci were stained only at the periphery, the centre being lysed. Comparison of the titres of virus produced by the persistently infected cells determined by the two methods indicated that detection of foci was 10- to 100-fold more sensitive than plaque formation. For instance, at passage 8 of the C13 virus persistently infected cells, the viral titre was $8 \times 10^4$ p.f.u. and $4 \times 10^5$ f.f.u./ml. The foci observed in these assays were non-lytic and their size diminished with the increasing number of passages. In this particular cell line the presence of virus was detected until passage 18 when assayed by plaque formation and passage 27 when assayed by formation of immune foci.
Fig. 2. Yield of cytopathic infectious virus during the passages of C13 and MP12 persistently infected Vero cells. Vero VC10 cells were infected with the C13 or MP12 strain at an m.o.i. of 0.001. After the acute phase, the surviving cells were maintained in the culture for 3 weeks before the first passage. Then they were passaged weekly. The medium was collected prior each passage and the virus titre determined by plaque assay on Vero cells. Virus produced in the acute infection (0) is given as a reference. Passage 1 of the C13 persistently infected cell line was not tested.

Table 1. Interference of particles produced by Vero cells persistently infected with MP12 at passage 2

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Normal infection</th>
<th>Coinfection</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1·0 x 10⁴</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RVFV-MP12</td>
<td>8·5 x 10⁷</td>
<td>0·8 x 10⁴</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>RVFV-C13</td>
<td>3·0 x 10⁷</td>
<td>3·2 x 10⁴</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>Punta Toro</td>
<td>1·1 x 10⁷</td>
<td>2·2 x 10⁶</td>
<td>20%</td>
</tr>
<tr>
<td>Toscana</td>
<td>1·25 x 10⁷</td>
<td>1·6 x 10⁵</td>
<td>&gt; 100%</td>
</tr>
<tr>
<td>Germiston</td>
<td>3·9 x 10⁷</td>
<td>5·5 x 10⁶</td>
<td>&gt; 100%</td>
</tr>
<tr>
<td>Sindbis</td>
<td>1·8 x 10⁸</td>
<td>2·9 x 10⁸</td>
<td>&gt; 100%</td>
</tr>
</tbody>
</table>

* Fresh Vero cells were coinfected with the virus and medium from persistently infected cells.
† Virus present in the medium was harvested 5 days after superinfection (or 2 days for Sindbis virus).

Altogether, this indicates that the production of particles, lytic or non-lytic, finally stopped after a limited number of passages.

To characterize the persistently infected cells further, we checked whether interfering particles were formed and released in the medium. The presence of interfering particles in cells coinfected with a standard virus would block the multiplication of the latter and protect the cells against the CPE. Thus, the assay consists of coinfecting fresh Vero cells with the standard virus, (homologous or heterologous) and undiluted culture medium from the persistently infected cells. The results summarized in Table 1 indicated that production of homologous viruses (the same or a different strain) was drastically reduced in cells coinfected with the medium harvested from persistently infected cells, while the growth of the heterologous viruses, Toscana, Germiston and Sindbis was unaffected. Among the two phleboviruses tested, the multiplication of Punta Toro, but not Toscana, was partially inhibited. This may indicate that Punta Toro virus is more closely related to RVFV than Toscana virus.

Table 2. Susceptibility of Vero cells persistently infected with MP12 to superinfection

<table>
<thead>
<tr>
<th>Superinfecting virus</th>
<th>Vero cells at passage 5</th>
<th>at passage 34</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RVFV-MP12</td>
<td>1·85 x 10⁷</td>
<td>&lt; 4·0 x 10⁴</td>
</tr>
<tr>
<td>RVFV-C13</td>
<td>3·0 x 10⁷</td>
<td>&lt; 4·0 x 10⁴</td>
</tr>
<tr>
<td>Punta Toro</td>
<td>1·25 x 10⁷</td>
<td>2·2 x 10⁴</td>
</tr>
<tr>
<td>Toscana</td>
<td>3·9 x 10⁶</td>
<td>6·4 x 10⁷</td>
</tr>
<tr>
<td>Germiston</td>
<td>1·8 x 10⁸</td>
<td>6·0 x 10⁷</td>
</tr>
</tbody>
</table>

To determine whether the persistently infected cells could support the growth of homologous or heterologous viruses, early and late passages of cells persistently infected with MP12 virus were superinfected (Table 2). Superinfection with the same or a different strain of RVFV did not lead to any CPE and infectious virus was not produced. However, these cells were still susceptible to superinfection with other viruses of the same genus, (Punta Toro and Toscana), the same family (Germiston bunyavirus), or a different family (Sindbis).
Synthesis of the viral genomic RNA in Vero cells persistently infected with RVFV

Genomic RNAs synthesized in several persistently infected cell lines were analysed by Northern blot using viral complementary-sense riboprobes specific for the three segments. Fig. 3 shows the viral RNAs in various passages of one VC10 cell line established after infection with C13 virus. Because the transfer of high molecular mass RNAs was very inefficient after migration in the presence of methyl mercuric hydroxide (Fig. 3 d), analysis of the L segment was performed after migration in the presence of formaldehyde (Fig. 3 a). However, the same results were obtained in both types of gels. The three RNA segments or their derivative products were detected at each passage tested. Except for the very first passages, the amount of each of the three segments (standard plus derivative products) was reduced when compared to the acute infection (lane 28 h) and varied independently from one passage to another.

After hybridization with the L-specific probe, the standard size L genomic RNA (6.4 kb) was detected and, as early as passage 3 or 4, a slowly-migrating RNA of approximately 11 kb was generated. It appeared to be the dominant species expressed from passages 4 to 7, decreased afterwards but was still detectable in passage 31 (Fig. 3 a). Starting from passage 7 or 8, additional discrete bands migrating faster than the standard molecule and estimated to be approximately 3.2 and 2.9 kb were visible. Other defective molecules particularly abundant in passage 31 were detected. Their sizes are approximately 5.3, 4.8 and 2.1 kb. The ratio of the full-length
Detection of the L genome with:

<table>
<thead>
<tr>
<th>Probe Type</th>
<th>3' End</th>
<th>Internal Region</th>
<th>5' End</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboprobe</td>
<td>5'</td>
<td>3'</td>
<td>5'</td>
</tr>
<tr>
<td>Oligonucleotide</td>
<td>5'</td>
<td>3'</td>
<td>5'</td>
</tr>
</tbody>
</table>

(a) Total cytoplasmic RNAs extracted from passages 2, 4, 15, and 31 of the cell line persistently infected with CI3 virus reported in Fig. 3 were electrophoresed in a formaldehyde/1% agarose gel and transferred onto a nylon membrane for hybridization. The locations of the three different L-specific probes (positions 5926-6404, 3059-4084 and 1-492 of the antigenome) are indicated. The migrations of the standard L genomic RNA and the 28S and 18S ribosomal RNAs are marked. RNAs from mock (mock) and acutely infected cells (28 h) were analysed in parallel. (b) Hybridization of the viral RNAs from passage 31 with 32P-labelled oligodeoxynucleotides specific for the 5' end of the genome: Oligo-F (complementary to position 1-26 of the genome sense) and Oligo-H (complementary to position 37-57). Oligo-L (complementary to position 6387-6404) which anneals to the 3' end was used as a control.
L RNA to the L derivatives decreased rapidly, the standard L molecule being only faintly detected in passage 3 or 4.

Hybridization with the M-specific probe (Fig. 3b) revealed the standard M genomic RNA (3.84 kb) and a faster-migrating band (approximately 3.5 kb), the relative ratio of the two bands varying with passages. Fig. 3(c) shows the hybridization with the S-specific probe. The standard genomic RNA (1.15 kb) was detected in the first passages (until 7 or 8) and a slower-migrating band of approximately 2.3 kb appeared in the following passages. No S RNA migrating faster than the standard RNA was detected.

These observations were not restricted to this particular persistently infected cell line. Similar molecules were observed in other cell lines established after infection with the same strain but at different m.o.i. or with other strains such as the attenuated MP12 or the virulent ZH548. These cell lines synthesized similar abnormal molecules migrating faster or slower than the standard genome RNAs, especially the 11 kb long L RNA and a slow-migrating S band. In the cells persistently infected with MP12 or ZH548 virus, the size of the abnormal S segment was estimated to be approximately 3.3 kb. The S segment of most of the strains is 1690 nt long (Giorgi et al., 1991) except for C13 which has a 570 nt long deletion in the NSs gene (Müller et al., 1995). This strongly suggests that the slow-migrating S and L bands represent a duplicate form and are generated in cells persistently infected with the three strains.

The abnormal L RNAs were characterized further by hybridization with viral complementary sense riboprobes specific for the genomic 3' or 5' ends or the internal region (Fig. 4a). The probe specific for the 3' end detected the same molecules as did the full-length probe whereas the probe specific for the 5' end hybridized only with the 4.8 and 11 kb long RNAs and the internal probe did not reveal the fast-migrating molecules, or if any, very weakly. This suggests that all the fast-migrating L RNAs possess the standard 3' end, the 4.8 kb long RNA is internally deleted and the others deleted either internally or from the 5' end. To determine the 5' end of these defective RNAs, hybridization was carried out with several oligodeoxynucleotides mapping in the region corresponding to the first 250 nucleotides (Fig. 4b). The probe hybridizing with the first 26 nucleotides of the 5' genomic end (Oligo-F) detected all the molecules whereas the probes located at positions 37–57 (Oligo-H) or downstream, such as Oligo-J and D2 (not shown) detected only the 4.8 kb species already revealed by the riboprobe specific for the 5' end. These results indicated that all the defective L molecules were generated by internal deletions, and some of them conserved only a short region (less than 57 bases) of the 5' end.

To determine whether the defective RNAs were encapsidated, the cytoplasmic extract from passage 19 of the persistently infected cells was centrifuged in a CsCl gradient and the nucleocapsid fraction was analysed by Northern blot (Fig. 5). The two fast-migrating L molecules (3.2 and 2.9 kb long species) and the two M molecules (the standard and the 3.5 kb subgenomic) sedimented at the density of the RNP's. This passage contained very low amounts of S and L (standard and duplicate) forms. Probing of the Northern blot with the S riboprobe did not detect any S RNA (not shown), probably because of the low level of synthesis and loss during the purification. In the pellet fraction no genomic segment (standard or abnormal) was detected.

Expression of the viral structural proteins

Expression of the viral structural proteins in various RVFV persistently infected cells was analysed by immunofluorescence, using a polyclonal serum specific for the N nucleoprotein or MAbs specific for the two glycoproteins, G1 and G2. We observed that, at all the passages tested and still after more than 1 year, all the cells were stained with the antibodies against N, G1 and G2 proteins, although fluorescence was less intense than in acute infection and varied to some extent from cell to cell (Fig. 6b). Fluorescence associated with the N protein showed a fine granulation localized in the cytoplasm and the G1 and G2 glycoprotein staining appeared as large granules in close proximity to the nucleus, evoking the classical pattern of the Golgi apparatus. These localizations were similar to those observed in an acute infection (Fig. 6a) (Wasmoen et al., 1988). Altogether, these experiments demonstrate that persistently infected cells express the viral structural antigens long after they have stopped producing infectious virions.
Fig. 6. Immunofluorescence of the viral proteins in Vero cells persistently infected by RVFV. Cells infected with the C13 strain and harvested at 28 h p.i. (a-c) or at passage 42 (d-f) were formaldehyde-fixed, acetone-permeabilized and treated with either a mouse anti-N protein polyclonal antibody (a, d), an anti-G1 glycoprotein MAb (b, e), or normal mouse ascites (c, f) then with goat fluorescein-labelled anti-mouse immunoglobulins. The exposure times for the persistently infected cells were two- to fourfold longer than for the acutely infected cells. Scale bar represents 60 μm.

Discussion

Like most of the viruses of the *Bunyaviridae* family, RVFV gives rise to CPE in mammalian cells. However, during infection of Vero cells, a small proportion of the cells, although being infected, escaped from death and kept proliferating. In all the cell lines which have been tested with the three different strains, infectious virus was produced during a limited number of passages, but the structural proteins N, G1 and G2, and the three genomic segments or related products were continuously
expressed for more than 1 year. This implies that the L polymerase remained functional during all these passages. Long-lasting expression of the viral proteins explains also why the persistently infected cells were resistant to RVFV super-infection.

Two types of abnormal genomic forms were generated during the cell passages: slow- and fast-migrating RNAs. Slow-migrating L and S RNAs were estimated to be approximately twice the size of the standard segments. RNAs migrating more slowly than the standard genome have also been reported in mosquito C6/36 cells persistently infected with Bunyamwera virus (Scallan & Elliott, 1992) and in the arenaviruses Pichinde (Shivaprakash et al., 1988) and Tacaribe (Iapalucci et al., 1994). It is noteworthy that all these viruses possess circular RNPs due to complementary 5' and 3' terminal sequences. This partially double-stranded RNA structure of the genome may favour the generation of duplicate forms, as it occurs more frequently for rota- and orbiviruses (For a review see Estes, 1996; Eaton & Gould, 1987). Fast-migrating RNAs representing deleted forms of the L and M segments were also generated. As revealed in Fig. 3, the 3-2 and 2-9 kb L RNAs were generated in the early passages and the pattern of hybridization with the probes (Fig. 4) suggests that the deletion occurred between position 26 and 57 at the 5' genomic end. Except for the 4-8 kb long L RNA which hybridized with the 5' terminal riboprobe, most of the deletions might have occurred at the same site. We do not know whether the deleted sequences are collinear or not. Internal deletions appear to be a common default described for several members of the family (Patel & Elliott, 1992; Resende et al., 1991; C. Giorgi, personal communication). It would be interesting to sequence the defective RNAs and determine where the deletions occurred in the sequence and if the sequences at the junctions were repeated, as has been reported for tomato spotted wilt virus (TSWV) DI RNAs (Resende et al., 1992) and Toscana virus DI RNAs (C. Giorgi, personal communication).

Among the mechanisms responsible for the establishment of persistent infections the putative role of interferon could be eliminated, since Vero cells are deficient for this component (Desmyter et al., 1968). Infections of mosquito cells with arboviruses normally lead to persistence. In these cells, the N protein is overexpressed and viral mRNAs are encapsidated (Rossier et al., 1988; Hacker et al., 1989). It could well be that a similar situation occurred in some of the Vero cells infected with RVFV. Persistence has been described in vertebrate cells infected with many viruses. This has been documented for DI particles, temperature-sensitive and other mutations (For reviews see Holland et al., 1980; Youngner & Preble, 1980; Roux et al., 1991). DI particles were probably the cause of the persistent infection of Vero cells with the phlebovirus Toscana (Verani et al., 1984) and nairovirus Dugbe (David-West & Porterfield, 1974). In the experiments reported in this paper, we used plaque-purified viruses grown at low m.o.i but we cannot rule out the possibility that DI particles were present, even if only in minor amounts. The persistently infected cells became resistant to superinfection with the same virus and the supernatants produced DI particles. We do not know whether these particles were present in the stock used for the primary infection in trace amounts and were responsible for the persistence or whether they were generated after persistence has been established.

At this point, we observed that the three viral strains were able to induce persistence, however production of persistently infected cultures was easier with the attenuated strain C13 than with MP12 and more difficult with the virulent strain ZH548. The major difference between C13 and MP12 (or ZH548) strains resides in the S segment which contains a large in-frame deletion in the NSs gene in C13 virus (Müller et al., 1995). Experiments are in progress to determine whether the NSs protein plays a role in persistence.

Persistence of RVFV in cell culture was not restricted to Vero cells but was also established in a murine neuroblastoma cell line, the Neuro 2A cells and to a lesser extent in the human and simian fibroblasts MRC5 and CV1. This raises the question of whether persistence occurs also in infected animals. Several reports indicating that infectious viruses were recovered in various animal organs relatively late after inoculation with RVFV (Kasahara & Koyama, 1973; Yedloutschnig et al., 1981; Anderson et al., 1987) and the fact that the C13 strain was found to be highly immunogenic for mice, support this idea. Although further experiments using sensitive methods will be necessary to look for the presence of virus in the organs of infected animals, this aspect of RVFV biology must be taken into account when using attenuated strains as vaccines.

We would like to thank J. F. Saluzzo, C. Prehaud, N. Lopez and R. Müller for fruitful discussions and J. Smith for kindly providing MABs. This work was supported in part by la Direction Centrale du Service de Santé des Armées (DGA-DRET).

References


Received 29 March 1996; Accepted 7 August 1996