Influenza C virus RNA is uniquely stabilized in a steady state during primary and secondary persistent infections

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The ability to establish persistent infections in vitro and in vivo has been illustrated for different human RNA viruses. However, little insight has been gained regarding the intracellular state of viral RNA species and the regulatory processes governing their long-term continuance. In this report, primary persistence of a variant of influenza C/Ann Arbor/1/50 virus in infected MDCK cells and secondary infections in human cell lines were investigated. Different PCR and staining techniques were applied for the description of low viral loads. The RNA pattern in primary persistence indicates that viral RNA synthesis is quantitatively linked to productive and non-productive phases, with negative-strand RNA being present continuously. In single cell cultures, derived from the primary line, all clones tested were positive by nested PCR and Southern blot screening. This suggests that a true steady-state persistence of influenza C virus is established in each individual cell of the infected population. Secondary infection experiments, in terms of transfer of the persistent virus variant to different cell types, showed that a re-establishment of persistence can be accomplished in vitro. The stable persistent status remained reserved for distinct host cell lines. Hereby, vRNA is stably maintained in a cell-type specific manner, whereas gene expression (e.g. HEF glycoprotein production) occurs in a variable fashion. These data point out novel characteristics in the understanding of influenza virus persistence.

Introduction

Persistent infections with influenza viruses are poorly understood, since their occurrence in cell culture is rare and often transient (De Barun & Nayak, 1980; Frielle et al., 1984). Several virus mutants, however, with the ability to persist in vitro for longer periods have been recently described, including influenza virus types A (Urabe et al., 1992, 1993), B (Clavo et al., 1993) and C (Camilleri & Maassab, 1988). The latter report describes a persistent variant of influenza C/Ann Arbor/1/50 virus which was established in MDCK cells by rescuing cells which had survived experimental infection (H. J. Maassab, personal communication). This cell culture has been passaged continuously since 1984 with no signs of loss of virus persistence. As an ideal and unique model for persistent influenza virus, two fundamental issues should be illustrated: firstly, the proportion of infected cells performing virus spread and secondly, the re-establishment of persistence after secondary infection in different cell lines.

Regarding the first point, persistent infections in vitro have been categorized into two types: 'carrier cultures' and 'steady-state' infections. In carrier cultures a small number of short-lived infected cells allows virus dissemination leading to horizontal infection of new subpopulations (Walker, 1964). In a steady-state type of persistence all cells are infected and continuous cell multiplication is accompanied by vertical virus transfer (Joklik, 1977). In both cases, however, the regulatory basis is unknown.

As far as the second issue is concerned, poliovirus (Calvez et al., 1993) and rubella virus variants (Williams et al., 1994) were shown to possess the potential for developing a secondary persistence in cell types different from the original ones. This property points clearly to viral determinants for persistence, possibly influenced by cell–virus interactions.

The data presented here indicate that persistent infection with influenza C virus represents the ‘steady-state’ type with genomic RNA detectable at stable levels. Furthermore, a re-established form of persistence is supported by the original MDCK culture and in secondary terms by human embryonic lung cells. This suggests a virus-determined mechanism of persistence which is subordinated to cell-type specificity.
Methods

Virus and cell culture. Influenza C virus, strain Ann Arbor/1/50 — wild-type (C/AA-wt) or persistent variant (C/AA-pi), were grown in MDCK cells or embryonated hens’ eggs. MDCK, WI-38, HEK-293 (all distributed by ATCC), MeluSo (established and placed at our disposal by E. P. Rieber, Institute for Immunology, LMU Munich; Johnson et al., 1982) and persistently infected MDCK-pi cells (kindly provided by H. F. Maassab) were cultivated in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum. The MDCK-pi culture has been maintained at 33 °C with twice weekly feedings by replacing 50% of the growth media, as reported earlier (Camilleri & Maassab, 1988). Raji cells (ATCC) were grown in suspension using 10% serum-complemented RPMI 1640 medium.

Single cell cloning. MDCK-pi cells were grown to confluence and harvested by trypsinization. For cloning, cells were counted and seeded to single cell densities in 96-well plates (serial dilutions containing 0, 0.5, 1, 2 and 5 cells per well). Cell growth was monitored daily under the microscope and microcolonies growing out from individual foci were marked and passaged to cloned cell lines at 37 °C for optimal division rates.

Infection tests and virus re-isolation. In infection tests, egg-grown virus with titres between 60 and 130 HAU/ml was utilized as inoculum. Infectious particles ratios were determined by an infectivity assay, as described earlier (Marschall et al., 1994) and viruses which lay within a twofold range were chosen (i.e. progeny production in standard MDCK monolayers between 8 and 16 HAU/ml 2 days post-infection). Virus incubation was carried out in 1 ml volume in 50 ml culture flasks for 1 h at 33 °C. Cultivation in fresh medium was performed at 33 °C. Re-isolation was achieved by direct inoculation of culture medium samples in 10-day-old embryonated hens’ eggs. Each sample of 50 μl was injected into the amnion cavity, incubated for 3 days at 33 °C and harvested for titration of haemagglutination activity.

Amplification of viral sequences by RT-PCR. Sequences from virus genomic segments were amplified from total cell-derived RNAs (Chomczynski & Sacchi, 1987) in a reverse transcription-polymerase chain reaction (RT-PCR). Five μg of RNA was set in 10 μl of 100 mM-Tris—HCl pH 8.3, 10 mM-MgCl2, 140 mM-KCl, 10 mM-DTT, 400 μM-dNTPs, 3 pmol of influenza virus universal primer (Unil) and 12.5 U of AMV reverse transcriptase (Boehringer Mannheim). The mixture was incubated at 42 °C for 60 rain and the reaction terminated by either the positive- or the negative-sense primer for reverse transcription. The minus-stranded cDNA of the respective segment was subsequently amplified in the PCR by the addition of the second primer of the pair. Positive reactions gave information about the initial presence of the distinct strand of RNA template. All primer compositions were controlled for reliability and for their suitability in semi-quantitative evaluations by the use of standard cDNA templates.

Nested PCR. A second round of PCR, including nested primers at staggered locations, was performed for improving sensitivity. Five μl of each PCR sample was subjected to a new 50 μl volume setup and reacted under the same cycling conditions.

PCR Southern blotting. Agarose gels (1–2%) were blotted onto nylon membranes by capillary transfer in 20 × SSC buffer (3 M-NaCl, 0.3 M-sodium citrate pH 7.0) in order to hybridize with a PCR digoxigenin (DIG)-labelled probe, at a stringency of 68 °C [5 × SSC, 1% w/v blocking reagent (Boehringer Mannheim), 0.1% w/v NaCl, 0.1% w/v N-lauroyl sarcosine, 0.02% w/v SDS]. Staining was performed with anti-DIG alkaline phosphatase-conjugated antibodies and NBT/BCIP colour substrate (Boehringer Mannheim) at room temperature in the dark.

In situ esterase assay. Direct staining of the viral enzymatic activity in infected cells was attained as published by Wagaman et al. (1989). Briefly, fixation occurred for 30 sec on ice (45% acetone, 19% formalin, 1-4 mM-Na2HPO4, 7-3 mM-KH2PO4). The substrate analogue for viral receptor-destroying enzyme (RDE) activity, α-naphthyl acetate-pararosanilin (0.5 ml 2% α-naphthyl acetate in ethylene glycol monomethyl ether, 8.9 ml 0.067 M-phosphate buffer pH 6.3, 0.3 ml 4% pararosanilin in 2 M-HCl, 0.3 ml 4% NaNO3, adjusted to pH 6.1 with NaOH for immediate use) was incubated on the cell layer for 15 min at room temperature. After rinsing repeatedly, cells carrying enzymatically active viral glycoprotein (HEF) were stained red.

Results and Discussion

Productive and non-productive transcript levels

It is important for our understanding that virus titres in the supernatant of persistently infected MDCK cells (MDCK-pi) were shown to be variable (Marschall et al., 1993). In consequence, cells were harvested for transcript analysis both during periods of virus production and during non-productive phases in the complete absence of virus shedding. Early (NS) and late cycle genes (HEF, M) were amplified from total RNAs in a strand-specific RT—PCR approach, using either the positive- or the negative-sense primer for reverse transcription. The choice of primer pairs, spanning genomic introns, allowed us to differentiate between full-length and spliced RNA species. Minus strand-specific amplification (Fig. 1a) was successful in all cases, with high yields in producer samples indicating ongoing genome replication. In comparison reactions run with samples from the non-productive phase yielded lower amounts (particularly HEF), reflecting limited amounts of minus-strand templates. But, importantly, detection was stable and reproducible in several experiments (not shown). Using the plus strand-specific reaction (Fig. 1b) however, strong signals were observed only during productive phases, while PCR performed on templates from the
non-productive phases was near to the detection limit (NS, M) or below this (HEF). Since it cannot be precluded that quantitative product differences between the single segments partly depend on variable PCR primer efficiencies, wider statements appear problematic. Nevertheless, from the view of several independent experiments and previous data (Marschall et al., 1993) one general statement can be made: positive-sense RNA (antigenomes, spliced and unspliced messengers) is variably expressed and can be silenced during long-term...
Secondary transfer of persistent infection

The fundamental question of whether persistence is governed by determinants of the virus or of the host cell was addressed in infection assays. For this purpose virus stocks, i.e. early passages of influenza C/Ann Arbor/1/50 wild-type (C/AA-wt) and its persistent variant (C/AA-pi), comprising equivalent m.o.i. (see Methods), were assayed in parallel. As target cells, MDCK and different human lines were grown to confluency in 25 cm² flasks, freshly infected and continuously cultivated. At the indicated times of cell culture splitting (Fig. 3), aliquots were taken for total RNA extraction and RT-PCR analysis. RT was performed with the universal primer whereas PCR analysis. RT was performed with the universal primer whereas PCR was specifically directed to segment 7 (NS). The time course of the detected reaction product (898 bp) illustrates the presence and stability of intracellular viral RNA. All kinetics shown were reproduced with C/AA-wt and C/AA-pi virus by duplicate experiments. As a striking result, continued RNA signals of C/AA-pi virus were demonstrated in both the original MDCK line (kidney cells) and in human embryonic lung cells (Wi-38). To the contrary, C/AA-wt virus was cleared in the period from day 3 to 17 in the different cells. Thus, persistent infection by C/AA-pi virus can be transferred to its primary adapted host cell and also to a secondary cell type of unrelated origin. This behaviour reflects the genetic potential of C/AA-pi virus to determine persistence.

Cell-type specific support of persistence

The infection experiment described in Fig. 3 was further analysed to study different host-dependent parameters, such as virus uptake, initial productivity, intracellular
RNA persistence and continued particle release. The central finding of this experiment is that only two out of five cell lines support persistence for periods longer than 2 months. In the other three cell types, however, C/AA-pi virus infection is abortive and shows patterns in PCR kinetics which are indistinguishable from C/AA-wt (Fig. 3). This phenomenon is not directly correlated to the rate of virus production as measured by haemagglutination (HA) titres in the culture medium at early times post-infection (p.i.) (Table 1). Of note, Wi-38 cells, which were successfully infected for long periods at the RNA level, only released virus to very low titres, which was in contrast to the case with MDCK cells. On the other hand, cells with the capacity to produce higher viral yields in comparison to MDCK, e.g. HEK-293, failed to maintain persistent virus under the conditions tested. To confirm these data, virus was re-isolated from medium samples, taken from all assayed cultures 2 months p.i. Infectious virus was detected exclusively in supernatants of MDCK-pi and Wi38-pi cells, which underlined their suitability as hosts for a continuous form of persistence. Given this information, Wi38-pi cells were reacted in the in situ esterase assay for influenza C virus. HEF expression in Wi38-pi cells was shown to be limited to a small number of positive cells. Surface and cytoplasmic staining were both detectable. It is worth noting that in Wi38-pi cells viral gene expression is obviously down-regulated to very low levels (see virus titres in Table 1), comparable to the described phases of non-productivity in MDCK-pi cells.

In conclusion, this study contributes to the molecular understanding of RNA virus persistence, which has become a recent focus of interest (reviewed by Oldstone, 1989, 1991). (i) Genomic RNA of persistent influenza C virus has been demonstrated to be uniquely stable, even in the absence of continuous gene expression. The lack of a regulated cycle with early and late stages of infection, typical for productive influenza C virus strains (Meier-Ewert & Compans, 1974), appears significant. Reports on the remarkable intracellular stability of inactivated influenza A virus segments are indicative of a general potential to preserve quiescent RNA (Cane & Dimmock, 1990). In this context the important role of the influenza A virus NS1 protein, controlling processing and transport of mRNA, has recently been discovered (Fortes et al., 1994; Qian et al., 1994; Qiu & Krug, 1994). NS1 deregulation during persistence was suggested previously (Lucas et al., 1988). (ii) The established form of persistence is classified as a true steady state, in which all cells are infected. This finding is paralleled by the characterization of long-term persistent measles virus in culture (Fernandez-Munoz & Celma, 1992). As typical in our study, genomic RNA remained constantly detectable in each single cell clone, even when grown at low-permissivity temperatures (37 °C) in the absence of virus shedding. (iii) Besides MDCK, the original cell line, a human lung cell culture was identified supporting secondary viral persistence. This kind of in vitro transfer of persistent infection was also performed successfully with a poliovirus variant. Here the viral origin of determinants for the persistent infection phenotype were mapped in the virion protein sequences, VP1 and VP2, by gene recombination (Calvez et al., 1993). Sequence analysis of the surface glycoprotein HEF of C/AA-pi virus revealed a distinct variation in the putative receptor binding domain, associated with the capacity to infect cells carrying low receptor levels (Marshall et al., 1994). (iv) Cell-type specificity for persistent infection, as outlined by our results, has been investigated intensively for latent DNA viruses, for instance Epstein–Barr virus (Bogedain et al., 1994; Marshall et al., 1991; Schwarzmann et al., 1994). In this case latency is characterized by complex down-regulation of gene expression, as accomplished by virus–cell interregulation in privileged tissues. The data presented here are comparable to these critical requirements, in terms of a cellular background allowing the establishment of persistence. The molecular basis of influenza A virus tropism has recently been attributed to multiple intracellular steps in both entry and release (Gujuluva et al., 1994). A strategy for RNA viruses to reside in distinct, specialized

### Table 1. Tropism and stability of persistent influenza C virus in culture

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Species and tissue type</th>
<th>Virus uptake*</th>
<th>Permissiveness for productive infection†</th>
<th>Establishment of long-term persistence‡</th>
<th>Infectious progeny virus§</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDCK</td>
<td>Canine kidney</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HEK-293</td>
<td>Human embryonic kidney</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MeJuSo</td>
<td>Human melanoma</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wi-38</td>
<td>Human embryonic lung</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Raji</td>
<td>Human B lymphocytes</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</tr>
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</table>

* Virus uptake/attachment was shown by RT-PCR from total RNA 2 days p.i.
† Virus yield ≥ 4 HAU/ml during the first week.
‡ Stability of intracellular viral genome > 2 months.
§ Culture medium samples were tested for infectivity in embryonated hens' eggs 2 months p.i.
cells for long periods without continuous replication was proposed by Fears et al. (1994): persistent paramyxovirus SV5 was found to remain inactive in cytoplasmic inclusion bodies of cellular subpopulations. In analogy, expression of persistent influenza C virus in MDCK cells is strictly silenced during non-productive intervals. In this context it is important to note that a variant-specific point mutation of amino acid 3 in the viral RNA polymerase I has been identified (Lapatschek et al., 1995). Future studies will elucidate the role of quiescent viral RNA.

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References


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