Deleted Viral RNAs and Lymphocytic Choriomeningitis Virus Persistence in vitro

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SUMMARY

Lymphocytic choriomeningitis virus (LCMV) infection of most tissue culture cell lines results in a non-cytopathic persistent infection. Persistent infections in vitro share many characteristics with persistent LCMV infection of mice; both are associated with decreased titres of infectious virus, restricted accumulation of viral glycoproteins at the surface of infected cells and the generation of interfering particles. We have used gel electrophoresis and hybridization techniques to analyse LCMV gene expression during persistent infection of a number of tissue culture cell lines. Our study has demonstrated that, although deleted viral RNAs can be detected during persistent LCMV infection in vitro, there may not be an obligatory association between deleted RNAs and persistence. In addition, we have found that LCMV interfering activity can be produced in the apparent absence of deleted intracellular viral RNAs.

INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV) readily establishes persistent infections in tissue culture cell lines and mice (Buchmeier et al., 1980; Lehmann-Grube et al., 1980). Persistent infections are characterized by reduced viral replication, limited accumulation of viral glycoproteins at the surface of infected cells and the generation of LCMV-specific interfering particles that appear very rapidly after initiation of infection (Buchmeier et al., 1980; Lehmann-Grube et al., 1980; Oldstone & Buchmeier, 1982; Popescu & Lehmann-Grube, 1977; Welsh et al., 1972, 1977; Welsh & Pfau, 1972; Welsh & Oldstone, 1977; Welsh & Buchmeier, 1979). The net result is a significant reduction in the production of infectious particles.

Tissue culture cell lines infected with LCMV at either high or low m.o.i. exhibit minimal cytopathic effects during acute or persistent infection. Two cell lines, MDCK and P15, however can be lytically infected by LCMV (Dutko & Pfau, 1978; Jacobson et al., 1979). These cell lines do not generate interfering activity but can be protected from lysis, and persistent infections can be established, when standard virus is co-administered with preparations of interfering particles that have been generated in another cell line. These observations suggest that the non-cytopathic character of LCMV infection is dependent on the rapid generation of interfering particles and that interfering and/or defective interfering particles are involved with the establishment and maintenance of persistent infections (reviewed in Barrett & Dimmock, 1986; Huang, 1973; Perrault, 1984).

Viral proteins appear to be qualitatively unchanged in purified virion preparations and cells during persistent LCMV infection in vitro (Welsh & Buchmeier, 1979). To date, however, there is only limited information concerning the state of the viral genome. Martinez Peralta et al. (1981) examined LCMV RNA (strain WE) in preparations of purified interfering particles and were able to detect the viral L segment and host 28S and 18S ribosomal RNAs but not the viral S segment. Van der Zeijst et al. (1983) detected a number of novel intracellular subgenomic RNAs of presumed viral origin during persistent infection of BHK cells. Gimenez & Compans (1980)
and Dutko et al. (1976), who studied the related arenaviruses Tacaribe and Pichinde respectively, suggested that, during persistence, a number of novel subgenomic RNAs appear in conjunction with loss of the S segment. Therefore, the presence of novel subgenomic viral RNAs and/or the loss of a viral genomic RNA segment have been associated with arenavirus persistence.

The LCMV model is of particular interest because (i) it is one of the few systems in which interfering particles can be detected during a persistent infection in animals (Jacobson & Pfau, 1980; Lehmann-Grube et al., 1980; Popescu & Lehmann-Grube, 1977) and (ii) there are extensive similarities between the persistent infections of tissue culture cells and mice (Buchmeier et al., 1980; Oldstone & Buchmeier, 1982). The availability of a good _in vitro_ model system for persistent infection of animals creates the opportunity for more controlled biochemical manipulations than are possible _in vivo._

The LCMV genome has recently been cloned and sequenced (Romanowski & Bishop, 1985; Singh et al., 1987; Southern et al., 1987) and specific probes are now available to characterize viral RNA species and the transcription and replication strategy of the virus during acute (Fuller-Pace & Southern, 1988; Singh et al., 1987; Southern et al., 1987) and persistent infection. We were particularly interested in evaluating whether changes in intracellular viral RNA species could be demonstrated in the context of persistent LCMV infections.

**METHODS**

*Virus stocks and infection of tissue culture cell lines.* Persistent infections were initiated either with uncloned preparations of LCMV Armstrong CA1371 or with a triply plaque-purified stock (clone 53B) derived from the uncloned stock. Semi-confluent monolayers of cells were infected with virus at multiplicities of 0.01 to 0.1 p.f.u. per cell and the cultures were trypsinized and replated when confluent. In all cases there was no reduction in the rate of cell division as a consequence of LCMV infection and the persistent infections became stabilized, as judged by reduced production of infectious virus and cell surface glycoprotein (GP) accumulation, within a few weeks. Infectious LCMV was quantified by plaque assay on Vero cell monolayers (Welsh & Buchmeier, 1979). LCMV interfering activity was determined by inhibition of plaque formation (Welsh & Buchmeier, 1979).

*Virion concentration.* A simplified scheme for concentration of virions was used to avoid the loss or inactivation of virus commonly observed during purification on Renografin gradients. Tissue culture cell supernatants were centrifuged in 30 ml Corex tubes at 8000 r.p.m. for 30 min in an HB-4 rotor in an RC28 Sorvall high speed centrifuge to remove cells and clarify the supernatant. The supernatant was decanted and centrifuged for 1 h at 30000 r.p.m. in a TY35 rotor in a Beckman ultracentrifuge (L8-M). The supernatant was decanted, discarded, and the pelleted material resuspended in Eagle's minimum essential medium (concentrated virion preparation). Using this concentration scheme a 12-fold, and in a later experiment a 120-fold, concentration of supernatant virus was obtained. The total titre of infectious virus calculated for the concentrated and stock supernatants was approximately the same.

*Vesicular stomatitis virus (VSV) plaque assay.* VSV stocks were titrated on L929 cell monolayers. A standard dilution that gave approximately 50 plaques/well was used for interference assays. Interference mediated by concentrated virion preparations (from GH3 cells) and by interferon gamma was determined by reduction in VSV plaque formation.

*RNA purification from tissue culture cells.* Total intracellular RNA was isolated by a modification of the method of Chirgwin et al. (1979). Cell monolayers were washed with sterile phosphate-buffered saline pH 7-4. and then 10 ml of GTC (4 M-guanidinium thiocyanate, 25 mM-sodium citrate, 0.5% Sarkosyl, 0.1 M-2-mercaptoethanol) was added. The resultant viscous solution was vortexed for approximately 45 to 60 s, layered over 2 ml of diethyl pyrocarbonate-treated 5.7 M-caesium chloride (Sigma), 100 mM-EDTA, and centrifuged in an SW41 rotor for 16 h at 35000 r.p.m. at 18 °C in a Beckman ultracentrifuge. The RNA pellet was washed with 70% ethanol and then repelleted.

*Nucleic acid concentration.* Purified RNA pellets were resuspended in sterile water and the concentration and purity were determined by absorbance measurements at 260, 280 and 320 nm. In addition, the amount and integrity of ribosomal RNA was assessed by electrophoresis of total intracellular RNA on non-denaturing 1% agarose mini gels.

*RNA electrophoresis.* RNA was denatured with glyoxal (1 M) and DMSO (50%) as previously described (Francis & Southern, 1988; McMaster & Carmichael, 1977). A standardized amount of total intracellular RNA (5 or 10 μg) was electrophoresed on a 1.5% agarose gel in 10 mm-NaH₂PO₄/Na₂HPO₄ pH 6-5 (Francis & Southern, 1988; Maniatis et al., 1982). Electrophoresis was performed at 14 mA constant current (approx. 2.5 V/cm) for 16 h with continual recirculation of the phosphate buffer.
RNA transfer and hybridization conditions. Transfer of RNA from agarose gels to nitrocellulose or Nytran filters was performed under standard conditions (Thomas, 1983). Filters were air-dried and baked for 2 h at 80 °C. Prehybridization and hybridization with nick-translated probes were performed in 50% formamide (deionized by treatment with Bio-Rad AG 501-X8), 5 × SSC, 2.5 × Denhardt's solution, 50 mM-phosphate buffer pH 6.5, 0.1% SDS, and 100 µg/ml of sonicated salmon sperm DNA for 24 and 48 h, respectively. Hybridization with radiolabelled RNA probes was for 24 h at 55 °C.

After hybridization, the filters were washed three times in 2 × SSC, 0.1% SDS for 30 min at 37 °C and once in 0.1 × SSC, 0.1% SDS for 30 min at 55 °C. The wash in 0.1 × SSC, 0.1% SDS was repeated for 30 min at a temperature higher than 55 °C if necessary as indicated by an excessive background.

Synthesis of hybridization probes. Purified LCMV cDNA sequences were labelled with [α-32P]dATP and [α-32P]dCTP to a specific activity of >2 × 10^8 c.p.m./µg by the procedure of Rigby et al. (1977). Single-stranded RNA probes were synthesized in the presence of [α-32P]UTP using the SP6 promoter and polymerase system (Melton et al., 1984). All LCMV hybridization probes contained LCMV sequences exclusively.

RESULTS

Novel subgenomic viral RNAs are detectable during persistent infection of tissue culture cell lines

Total intracellular RNA was analysed from four cell lines [GH3, rat pituitary cells (Tashjian et al., 1968); RIN, rat insulinoma cells (Gazdar et al., 1980); L, mouse fibroblasts; B-113, a B cell hybridoma (M. B. A. Oldstone et al., unpublished results)] that were persistently infected with LCMV Armstrong CA1371. After size-fractionation on denaturing agarose gels and transfer to nitrocellulose filters, the RNAs were hybridized with radiolabelled virus-specific nick-translated probes from the nucleoprotein (NP) and GP coding regions of the S segment (Fig. 1a, b, respectively). Uninfected cells did not show any virus-specific hybridization but the persistently infected cell lines demonstrated a signal from the S segment and from subgenomic viral mRNAs transcribed from the NP- and GP-coding regions (Southern et al., 1987). In addition, multiple new subgenomic viral RNAs were evident in the GH3, RIN and B-113 cells (using NP and GP segment probes) but no such subgenomic RNAs could be detected in the L cell RNA.

Detection of viral L segment RNAs during persistent infection

We analysed the persistently infected cells for the presence of the full length L segment and any subgenomic L-derived RNAs. Fig. 2 compares the results obtained with persistently infected GH3 cells with those from acutely infected BHK cells. The filter was sequentially hybridized with S segment (Fig. 2a) and L segment (Fig. 2b) nick-translated probes. Two faint subgenomic L bands, present in both the acutely and persistently infected cell RNA samples, have not been associated with expression of the L RNA segment and may be minor degradation products or representative of low-level cross-hybridization to viral S sequences (see legend to Fig. 2).

The L segment was readily detected during both acute and persistent infection but without any indication of unique, L segment, subgenomic RNAs (Singh et al., 1987). The acute and persistent infections were not identical because the relative levels of genomic S and L RNAs changed and the latter was under-represented in persistent infections (compare the signal ratios of L and S in lanes 2 and 4, Fig. 2a, b).

Systematic analysis of novel subgenomic RNAs found during persistent infection of GH3 cells

We extended the characterization of novel subgenomic S RNAs in GH3 cells by examining the sequence content and polarities of the intracellular viral RNAs. Samples containing equivalent amounts of persistently infected GH3 cell RNA or BHK cell RNA (48 h after acute infection with LCMV) were separated on a denaturing agarose gel (see Methods). After transfer of the RNA to a nitrocellulose filter, the filter was cut into strips and incubated with single-stranded RNA probes that detected genomic sense or genomic complementary sense sequences from either the 3' terminal NP region [approx. nucleotides (nt) 35 to 450], the internal NP region (approx. nt 1200 to 1600), or the 5'-terminal GP region (approx. nt 2300 to 3400) of the S segment (Fig. 3a).
Fig. 1. Analysis of intracellular S RNAs from cell lines persistently infected with LCMV. Total intracellular RNA from four cell lines, persistently infected with LCMV for a minimum of 1 year, was size-fractionated by agarose gel electrophoresis under denaturing conditions. After transfer of the RNAs to a nitrocellulose filter, hybridization reactions were performed using radiolabelled nick-translated probes from the viral S segment. The filter was hybridized with an NP region probe (a), completely stripped of signal, and rehybridized with a GP region probe (b). RNAs from uninfected cell lines were used as negative controls (lanes 2, 4, 6 and 8). Lanes 1 and 2, GH3 cells; lanes 3 and 4, RIN cells; lanes 5 and 6, L cells; lanes 7 and 8, B-113 cells. The NP and GP mRNAs are indicated and prominent subgenomic RNAs have been marked with asterisks.

The results of the hybridizations to detect genomic sense sequences are shown in Fig. 3(b). The acutely infected BHK cell RNA (lane 1) displayed the expected signal from the S segment and the GP mRNA with the GP region probe. The probes from the NP and GP regions detected different virus-specific RNAs in the GH3 RNA samples (see also Fig. 1) and, by extending this analysis, we could begin to assemble maps of the genomic S RNA sequences that were present or absent from individual subgenomic RNAs. In most cases, a given subgenomic RNA appeared to hybridize with probes of each polarity, providing evidence for intracellular replication of the subgenomic species.

Interference during persistent LCMV infection of GH3 cells

The GH3 intracellular RNA used in Fig. 1 and 2 had been extracted from an LCMV Armstrong CA1371 persistently infected cell line but, after storage in liquid nitrogen, viable cells could not be recovered. We therefore examined other GH3 cells and expanded a cloned GH3 cell line, GH3-21, that had been persistently infected with LCMV Armstrong CA1371 (clone 53B) to test whether these cells produced any interfering activity. Multiple experiments were performed in which stock supernatant from GH3-21 cells was stored (at -70 °C) and the remaining supernatant (approx. 300 ml, equivalent to culture medium from 10 T175 culture vessels) was concentrated (see Methods). The stock supernatant and the concentrated virion preparations were titrated for infectious virus, and the virus preparations were tested for interference with standard LCMV (SV) replication and for the presence of interferon (see Methods). The stock supernatant showed a sequential reduction in virus titres at all dilutions (approximate titre 1.8 x 10^5 p.f.u./ml) whereas infectious virus could be detected only in the concentrated virus preparations at a 1:2000 dilution, but not at a 1:2 or 1:20 dilution. The titre of infectious virus in the concentrated virion preparation was approximately 4 x 10^6 p.f.u./ml (as shown in Fig. 4a) and, since the virion preparation was originally concentrated 12-fold, this was in reasonable agreement with the titre of virus in the stock supernatant. The concentrated virion preparation interfered with plaque formation by SV (Fig. 4b) and there was no role for interferon in this process (Fig. 4c). The interfering activity in the concentrated GH3 stock was subsequently shown to be virus-specific (Table 1). A 10^-5 dilution of SV produced 200 p.f.u./ml,
Persistent LCMV infection

in vitro

Fig. 2. Detection of L-derived RNAs during acute and persistent LCMV infection. Total intracellular RNAs extracted from uninfected GH3 cells (lane 1), persistently infected GH3 cells (lane 2) and two independent acute infections of BHK cells (lanes 3 and 4) were hybridized with (a) S segment (NP region) and (b) L segment nick-translated probes. The filter was first hybridized with the S segment probe, completely stripped, and then rehybridized with the L segment probe. The S segment and NP mRNAs are indicated in (a) and the L segment RNA in (b). Faint low Mr bands detected with the L probe may represent breakdown products and/or demonstrate low-level cross-hybridization to the genomic S RNA segment. The appearance of one band (X) may also be due to a high concentration of 28S ribosomal RNA that produces a local distortion in the gel.

Table 1. Detection of LCMV interfering activity

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<tr>
<th>Dilution</th>
<th>SV stock (p.f.u./ml)</th>
<th>Treatment</th>
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<tr>
<td>10⁻⁵</td>
<td>10⁻⁶</td>
<td>10⁻⁷</td>
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<tr>
<td>200</td>
<td>20</td>
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<tr>
<td>50</td>
<td>ND*</td>
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<td>0</td>
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<td>125</td>
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* ND, Not determined.
† Virus incubated for 30 min at 37 °C as a control for treatments below.
‡ Guinea-pig anti-LCMV, 1:100 dilution.
§ Concentrated virion preparation from persistently infected GH3-21 cells, 1:2 dilution.
|| Concentrated virion preparation, 1:2 dilution, neutralized with anti-LCMV, 1:100 dilution, for 30 min at 37 °C before being added to the Vero cells with the SV stock (which was not heated).
whereas inclusion of either antiserum to LCMV or the preparation of GH3 particles (1:2 dilution) completely prevented the formation of virus plaques. Pretreatment of the particle preparation with anti-LCMV antibody eliminated most or all of the interfering activity (Table 1).

**Evidence for subgenomic viral RNAs in persistently infected cell lines exhibiting interference**

Total intracellular RNA from persistently infected GH3-21 cells (this study) and long term persistently infected BHK and L cells (cells kindly provided by Ray Welsh, University of Massachusetts Medical School, Worcester, Mass., U.S.A.) were analysed by hybridization with virus-specific single-stranded RNA probes. The persistently infected BHK and L cell lines (initiated with an uncloned LCMV Armstrong CA1371 stock) had previously been shown to exhibit interference and decrease expression of surface GP (R. Welsh, personal communication). The L cells did not contain any subgenomic viral RNAs, except GP mRNA, but the BHK cells exhibited four viral RNA molecules, a GP mRNA, two non-identical but approximately S segment size RNAs and a distinct subgenomic RNA of approximately 2300 nt (Fig. 5). The persistently infected GH3-21 cells showed weak, diffuse subgenomic RNA bands at the time samples were removed for biological assays (Fig. 4 and Table 1) but these RNA species
Fig. 4. Detection of interfering activity in supernatant medium collected from cultures of GH3-21 cells persistently infected with LCMV. (a) Virus titration with the supernatant (○) and a 12-fold concentrated supernatant (see Methods) of persistently infected GH3 cells (●) at various 10-fold dilutions (unconcentrated GH3 cell supernatant dilutions 10^{-1} to 10^{-6}; GH3 concentrated cell supernatant dilutions 1:2, 1:20, 1:2000, 1:200000, 1:2000000). Titres (p.f.u.) are the number of virus plaques per well (six-well plates) per ml of stock. Titres of 1.8 × 10^5 p.f.u./ml for the GH3 SN and 4 × 10^6 p.f.u./ml for the GH3 conc. preparations were observed in this experiment. (b) Titration (p.f.u.) of a stock of LCMV clone 53B (SV) alone (upper line) and after co-incubation with a 1:2 dilution of the GH3 conc. (SV + GH3 conc. 1:2; ●). A titre of 1.8 × 10^8 p.f.u./ml was measured for SV in this experiment and there was no detectable p.f.u. for SV + GH3 conc. (c) Assays for interferon activity. Titration (p.f.u.) of a VSV stock alone (○), VSV treated with 20 units of interferon gamma (□), VSV treated with GH3 conc. (●) and a control without any added virus (■).

appeared to be more heterogeneous than in the analysis performed several months earlier (compare Fig. 3 and 5).

**DISCUSSION**

We have demonstrated that a number of cell lines (GH3, RIN, B-113 and BHK) may exhibit one or more novel subgenomic viral RNAs when persistently infected with LCMV. We did not detect the elimination of either the genomic L or S RNA segments or viral mRNA species
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Fig. 5. Analysis of intracellular viral RNAs from persistently infected cell lines that produce LCMV interfering particles. Total intracellular RNA from acutely infected BHK (lane 1), uninfected BHK (lane 2) and persistently infected cells: BHK (lane 3), L (lane 4) and GH3-21 (lane 5, purified in April 1987 at the time of the biological interference assays) was analysed using a single-stranded RNA probe to detect genomic sense sequences from the GP coding region of the S segment. The genomic S segment and GP mRNA are indicated.

during the course of persistence, although the L segment does appear to be selectively under-represented in comparisons of persistent and acute infections. The viral NP and GP mRNAs were both readily detected during persistent infection of all cell lines we examined. In other studies, where intracellular viral RNAs have been analysed during persistent arenavirus infection, van der Zeijst et al. (1983) purified viral nucleocapsids and found a number of viral RNA species present that were different from those normally found in LCMV. None of these RNAs could be translated in vitro. Dutko et al. (1976) were able to detect a 15S RNA, which is approximately the size of the viral mRNAs, in Pichinde virions from acutely but not persistently infected BHK cells but it is unclear what this 15S RNA represents. Immunofluorescence and immunoblotting analyses of viral GP expression in persistently infected cells reveal different levels of GP expression that are dependent on the particular cell line examined (S. J. Francis, unpublished results). Our detection of GP mRNA both in vitro (this study) and in vivo (Francis & Southern, 1988) suggests that post-transcriptional regulation of GP must account for at least part of the decreased expression of GP. This is consistent with the observation of Welsh & Buchmeier (1979) that a large percentage of persistently infected cells contain cytoplasmic GP but that surface accumulation of GP is decreased relative to acute infections.

The generation of new subgenomic viral RNAs appears to depend on both the particular cell line and the viral stock used to initiate infection. Persistent infection of BHK cells initiated by
cloned isolates of LCMV Armstrong CA1371 may or may not give rise to subgenomic RNAs (Fig. 5). Differences in the virus stocks and/or the BHK cells may be responsible for this variability. The use of the uncloned stock, LCMV Armstrong CA1371, often appears to result in the accumulation of subgenomic S segment RNAs. The RNAs which we studied were extracted from cell lines that had been persistently infected for various but extensive times (> 1 year) prior to analysis. Therefore we cannot comment on whether the initial pattern of subgenomic RNAs found in any particular cell line was similar to that in other cell lines or was related to those, if any were present, contained in the LCMV Armstrong CA1371 virion preparations. Since each cell line appears to contain unique viral subgenomic RNAs there must have been either a selection and/or evolution of the original RNA species of the LCMV Armstrong CA1371 stock. Interestingly, not all persistent infections (i.e. in L cells) initiated with LCMV Armstrong CA1371 contained subgenomic viral RNAs yet they still exhibited interfering activity. This could be explained either by an inability of our routine analytical methods to detect subgenomic RNAs of less than 400 to 500 bases or by the presence of viral segments with minor deletions or sequence aberrations that were not large enough to produce a shift in size that was detectable by denaturing agarose gel electrophoresis. Although most defective interfering RNAs described in other viral systems (Barrett et al., 1984; Huang, 1973; Perrault, 1984; Rao & Huang, 1982; Weiss et al., 1983) have contained readily detectable deletions, copy-backs, and/or rearrangements, there may not be an absolute requirement for such major defects (for example see Schubert et al., 1984).

Our description of novel LCMV intracellular subgenomic RNAs during persistent infections agrees with prior observations made with closely related arenaviruses. Dutko & Pfau (1978) and Gimenez & Compans (1980) studying Pichinde and Tacaribe viruses, respectively, reported the appearance of new RNAs in virions purified from persistently infected BHK cells. We have been unable to detect any subgenomic viral RNAs in virions from persistently infected BHK cells. A newly initiated persistent infection of BHK cells has not yet generated any novel subgenomic RNAs. Virion particles are released by these cells (F. Fuller-Pace, unpublished observations) but there is no detectable infectious virus, even when titrated out to a 10^-9 dilution. We have found that the viral genomic S and L segments are present intracellularly and in virions that are formed during the persistent infection of these BHK cells.

Persistent infection with arenaviruses appears to be a highly complex phenomenon and is associated with different alterations in viral gene expression which depend on the cell type and the virus stock used to establish persistence. Interfering viruses that arise during persistent infections may or may not be correlated with intracellular subgenomic viral RNAs. The significance of variation in the relative levels of the genomic L and S segments during acute and persistent infection remains uncertain. At this time it is unclear whether the changes we have described are causal or consequential to the state of LCMV persistence.

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