An interfering activity against lymphocytic choriomeningitis virus replication associated with enhanced mutagenesis

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Previous studies have documented that, in the presence of the mutagenic base analogue 5-fluorouracil (FU), lymphocytic choriomeningitis virus (LCMV) that persisted in BHK-21 cells decreased its infectivity to a larger extent than intracellular viral RNA levels, prior to virus extinction. This observation, together with in silico simulations, led to the proposal of the lethal defection model of virus extinction. This model suggests the participation of defective-interfering genomes in the loss of infectivity by increased mutagenesis. Since LCMV naturally produces defective-interfering particles, it was important to show that a capacity to interfere is produced in association with FU treatment. Here, we document that BHK-21 cells persistently infected with LCMV grown in the presence of FU, but not in its absence, generated an interfering activity that suppressed LCMV infectivity. Interference was specific for LCMV and was sensitive to UV irradiation and its activity was dose- and time-dependent. The interfering preparations produced positive LCMV immunofluorescence and viral particles seen by electron microscopy when used to infect cells, despite some preparations being devoid of detectable infectivity. Interference did not involve significant increases of mutant spectrum complexity, as predicted by the lethal defection model. The results provide support for a specific interference associated with LCMV when the virus replicates in the presence of FU. The excess of interference relative to that observed in the absence of FU is necessary for LCMV extinction.

INTRODUCTION

As a consequence of the low fidelity of viral RNA-dependent RNA and DNA polymerases, virus replication within a host generates mutant spectra consisting of dynamic distributions of viral genomes in competition that are ranked according to their relative fitness. They have been termed virus quasispecies by analogy with the concept of molecular quasispecies, developed by Eigen and Schuster as a theory of molecular evolution of primitive replicons (Eigen, 1971; Eigen et al., 1988; Eigen & Schuster, 1979). Viral quasispecies are endowed with great adaptability, and both the composition of the mutant spectrum and interactions among its components may influence the evolutionary dynamics of the virus (reviewed by Domingo, 2006; Domingo et al., 2008a, b). A manifestation of adaptability is the selection of drug-resistant mutants that may contribute to treatment failures (Coffin, 1995; Nájera et al., 1995; Nijhuis et al., 2009). There is a need to develop new antiviral strategies for variable viruses that prevent or minimize selection of drug-resistant viral mutants. One such strategy, termed lethal mutagenesis, is based on the theoretical concept of entry into error catastrophe (Eigen, 1971; Eigen et al., 1988; Eigen & Schuster, 1979). According to this concept, for any replicating entity, there is an upper value of the mutation rate, termed the error threshold, which must not be crossed if the wild-type genome distribution is to be kept stable (Biebricher & Eigen, 2005; Eigen, 2002). Applied to viruses, an increase of the mutation rate above some critical value might lead to drift of sequences, disorganization of the quasispecies and, consequently, to loss of infectivity (Eigen, 2002). This hypothesis has been amply supported experimentally (Crotty et al., 2001; Grande-Pérez et al., 2002; Holland et al., 1990; Martin & Domingo, 2008; Martin et al., 2008; Pariente et al., 2001, 2003; Ruiz-Jarabo et al., 2003; Sierra et al., 2000; reviewed by Anderson et al., 2004; Domingo, 2005; Graci & Cameron, 2004).
The mutant spectrum can influence the behaviour of individual viral variants, and can suppress variants of superior fitness as well as virulent, drug-resistant or pathogenic variants present at low frequency in the population (Crowder & Kirkegaard, 2005; Chumakov et al., 1991; de la Torre & Holland, 1990; González-López et al., 2004; Teng et al., 1996). In agreement with the suppressive effects of mutant spectra, the transition to extinction of lymphocytic choriomeningitis virus (LCMV) during persistent infections in cell culture subjected to mutagenesis by 5-fluorouracil (FU) was characterized by a progressive decrease in virus infectivity that was more pronounced than the decrease of viral RNA levels (Grande-Pérez et al., 2005b). These observations, together with in silico simulations of RNA genome replication under mutagenesis, led to the proposal of the lethal defection model of viral extinction, which has been supported by additional theoretical studies (Iranzo & Manrubia, 2009). According to this model, as a result of modest increases in the mutation rate, lethality is limited, but the mutant spectrum is enriched with defector RNA genomes that perturb viral functions and mediate decreases of infectivity and extinction (Grande-Pérez et al., 2005a). Parallel studies carried out with the picornavirus foot-and-mouth disease virus (FMDV) have provided independent evidence for the suppressive effects of mutant spectra (González-López et al., 2004). In particular, well-characterized capsid and polymerase mutants of FMDV exerted a specific interference on FMDV RNA replication, and contributed to extinction (Perales et al., 2007, 2009).

Arenaviruses are attractive pathogens to investigate the mechanisms and possible application of lethal mutagenesis because of the very limited number of effective antiviral inhibitors available and the impact of arenavirus diseases on human health (Buchmeier et al., 2007; Macher & Wolfe, 2006; Peters, 2002). Administration of FU to mice impeded the establishment of a persistent infection with LCMV, an observation that constituted a proof-of-principle of the feasibility of a lethal mutagenesis approach in vivo (Ruiz-Jarabo et al., 2003). LCMV is the prototype arenavirus; its genome includes two RNA segments, L and S, that use an ambisense strategy for gene expression. The L segment encodes the 90 amino acid Z protein, a zinc-finger protein implicated in budding (Pérez et al., 2003; Salvato et al., 1992), and the L protein, the RNA-dependent RNA polymerase, of 2210 amino acids (Fuller-Pace & Southern, 1989; Lee et al., 2000; Lopez et al., 2001; Singh et al., 1987). The S segment encodes the glycoprotein precursor (GP-C) and the nucleoprotein (NP) (Buchmeier et al., 2007).

In the present study, we have used LCMV to demonstrate that virus extinction by enhanced mutagenesis is achieved through the specific interference generated during LCMV replication in the presence of a mutagenic agent. The interference required FU mutagenesis, since it was not observed in infected cultures maintained under the same conditions but in the absence of FU. Under basal mutation rates, the accumulation of interfering activity was insufficient to achieve LCMV extinction. The results provide further support for the lethal defection model of virus extinction and reinforce the concept that limited mutagenesis may suffice to drive a virus towards extinction.

**RESULTS**

**BHK-21 cell cultures persistently infected with LCMV in the presence of FU accumulate an interfering component**

The previously proposed lethal defection model of LCMV extinction (Grande-Pérez et al., 2005b) has been further tested by monitoring LCMV infectivity and genomic RNA levels in the culture medium of BHK-21 cells persistently infected with LCMV, grown in either the absence or presence of FU. Infectivity was determined by plaque assay on Vero cells, in which several rounds of replication and cell-to-cell propagation of viruses occur (see Methods for a detailed procedure). Parallel, persistently infected cultures were maintained either in periodically renewed medium (Fig. 1a; first design) or in the same culture medium (Fig. 1b; second design). In the first design, at fixed time intervals, all the medium on a monolayer was removed, and fresh medium with FU was added. In the second design, the medium removed for analysis (300 μl; 6% of the total) was replaced at fixed time intervals by the equivalent volume from a culture medium of BHK-21 cells grown in parallel in the presence of the same concentration of FU. The results (Fig. 1a, b) show that, despite a decrease of infectivity of several logarithms in the presence of FU, extinction (loss of infectivity) was only achieved in the cultures in which the medium was not changed (second design) (Fig. 1b). In this case, the infected cultures maintained LCMV RNA levels that were 101- to 102-fold higher than in cultures in which the medium was periodically renewed (Fig. 1c, d). Increased viral RNA levels relative to infectivity led to statistically significant decreases of specific infectivity (Fig. 1f). The difference of specific infectivity between the LCMV populations produced in the presence and absence of FU was significant (P<0.05, Student’s t-test; Fig. 1f) throughout the duration of the infection (Fig. 1b). In contrast, this difference was not significant when extinction did not occur (first design) (P=0.15, Student’s t-test; Fig. 1e). The results were similar in duplicate cultures, and indicate that the interfering activity generated during persistent LCMV infections, and the ensuing LCMV extinction, are dependent on the presence of FU and on another component that accumulates in the culture medium; FU per se is not sufficient to promote extinction. The results also suggest that supernatants of infections carried out in the presence of FU produce a significant decrease of LCMV infectivity, with a modest decrease of LCMV RNA levels, in agreement with previous results (Grande-Pérez et al., 2005b).
Deprivation of standard ribonucleotides can limit RNA genome replication, and nucleotide pool imbalances can be mutagenic (Anglana et al., 2003; Kunz et al., 1994; Mathews, 2006). The concentration of standard nucleoside triphosphates was 2- to 19-fold lower in the presence of FU triphosphate (FUTP), but did not differ significantly between the persistently infected cell cultures in which extinction did not occur (first design) and those in which it occurred (second design) (Fig. 2). Furthermore, intracel-
Fig. 2. Levels of NTPs in persistent infections of LCMV in BHK-21 cells. Values have been normalized to the value for NAD in the same cell extract. (a) FPLC analysis of NTP levels in cell-culture supernatants, corresponding to the experiment shown in Fig. 1(a) (resistance to extinction) in the absence (−FU) or presence (+FU) of FU, at the indicated times post-infection. (b) FPLC analysis of NTP levels in cell-culture supernatants, corresponding to the experiment shown in Fig. 1(b) (viral extinction) in the absence (−FU) or presence (+FU) of FU, at the indicated times post-infection.
lular FUTP levels were higher in those cultures in which extinction was not observed. This result argues against an imbalance of nucleotide pools or a decrease in the FUTP level as the mechanism of the observed resistance of LCMV to extinction.

Detection of virus-specific antigens and virus particles in cells infected with FU-treated viral populations

In other virus systems, specific interference by viral mutants required that the interfering genomes be replication-competent (Crowder & Kirkegaard, 2005; Perales et al., 2007). To test whether interfering preparations expressed viral antigens, the supernatants of cultures persistently infected with LCMV in the presence of FU, either with or without detectable infectivity [culture media obtained at 96 h post-infection (p.i.) in the experiment of Fig. 1a, b] were applied to BHK-21 cell monolayers, using the standard infection protocol. In all cases, positive NP-specific immunofluorescence and viral particles were observed (Fig. 3). Thus, the interfering supernatants contain replication-competent viral particles.

Stochastic LCMV extinction in BHK-21 cells. Interference exerted by cell-culture supernatants displaying different infectivity levels

Previous studies have established that the decrease of LCMV infectivity prompted by FU treatment occurs through sharp fluctuations of infectivity that eventually result in virus extinction (Grande-Pérez et al., 2005b). The stochastic nature (chance occurrence) of the fluctuations was investigated by determining the infectivity of several replicate BHK-21 cultures persistently infected with LCMV in the presence of FU. The results document wide variations of infectivity among different replicates (3–6), prior to extinction (Fig. 4a). Two replicates that differed by about 100-fold in infectivity in the cell-culture supernatants at 88 h p.i. (asterisks in Fig. 4a) showed minor differences in interference activity (Fig. 4b), suggesting that the presence of infectivity is not a requirement for interference. Similar results were obtained with other samples (Fig. 4b–d).

Interference is LCMV-specific and UV radiation-sensitive

 Supernatants with interfering activity obtained at 102 h p.i. (Fig. 1a) were used to perform additional interference assays, aimed at determining the nature of the interfering activity. First, interference was consistently exerted on cultures of BHK-21 cells infected with LCMV, but not on parallel BHK-21 cultures infected with either encephalomyocarditis virus (EMCV) or vesicular stomatitis virus (VSV) (Fig. 5a). This result rules out induction of unspecific antiviral components (such as interferon) as mediators of interference. This is in line with the fact that BHK-21 cells (like Vero cells) are unable to induce interferon upon viral infection.

Interference was not due to FU that remained in the inoculum, since intracellular FUTP was not detected in cells in which interference occurred or in parallel control monolayers (Fig. 5b; arrows). However, FUTP was detected in FU-treated LCMV-infected monolayers at the same times post-infection (Fig. 5c). Interference was not observed when LCMV was cooinculated with mock-infected supernatant in the presence of FU (Fig. 5b; red dashed line). FU has an effect on the infectivity of EMCV and VSV (data available upon request), although the interference experiment was negative for EMCV and VSV (Fig. 5a).

Nucleic acids are much more sensitive to inactivation by UV irradiation than proteins, through formation of cross-links by UV in the 200–300 nm wavelength range. The interference decreased 100-fold when the interfering supernatants were irradiated with UV light (Fig. 5b; green lines). As expected, neither DMEM with FCS nor the neutralizing activity of mAb SD6 (specific for FMDV; described in Mateu et al., 1987) was affected by the same doses of UV irradiation. These results exclude proteins or small molecules as the interfering component. The interfering activity was not lost upon filtration through a 0.2 μm filter or by RNase A treatment (Fig. 5d). The interfering activity manifested a dose effect, and it increased slightly with the time allowed for LCMV to replicate in the presence of FU (Fig. 6). RNA purified from culture supernatants in which extinction was achieved did not interfere (Fig. 5d). These results strongly suggest that the interfering component was associated with LCMV particles, as proposed in the lethal defection model.

Mutant spectrum complexity of LCMV in FU-treated cultures

LCMV displays error-prone replication, and naturally produces defective-interfering (DI) particles (Lazzarini et al., 1981; Meyer & Southern, 1997; Popescu et al., 1976; Sevilla et al., 2002; Welsh & Oldstone, 1977). To compare the mutant spectrum complexity of LCMV in persistently infected BHK-21 cells, grown in either the absence or presence of FU, regions of the Z, L, GP and NP genes were subjected to a clonal analysis. The populations passed in the presence of FU showed increased mutation frequency compared with populations passed in the absence of FU, as expected (Grande-Pérez et al., 2002; Ojosnegros et al., 2008). The only exception was at 48 h p.i. in populations subjected to design 1, which had a mutation frequency similar to the control populations and significantly lower than the population at 48 h p.i. treated with FU under design 2 (P<0.05) (Table 1). Possible reasons for this difference were not investigated. A comparison of the mutation types recorded in all mutant spectra analysed (Table 2) indicated an abundance of U→C and A→G transitions in virus from population subjected to FU
Fig. 3. Detection of LCMV NP and LCMV particles in BHK-21 cells treated with interfering cell-culture supernatants. (a) Detection of NP by immunofluorescence (panels 2, 5 and 8). Immunofluorescence of BHK-21 cells infected with LCMV at an m.o.i. of 0.01 p.f.u. per cell at 24 h p.i. (panels 1, 2, 3), BHK-21 cells overlaid with culture supernatant of BHK-21 cells infected with LCMV in the presence of FU, under conditions that eventually led to LCMV extinction (panels 4, 5, 6) (Fig. 1b, 96 h p.i.) and BHK-21 cells overlaid with culture supernatant of BHK-21 cells infected with LCMV in the presence of FU, under conditions that did not lead to LCMV extinction (panels 7, 8, 9) (Fig. 1a, 96 h p.i.). Panels 3, 6 and 9 show the corresponding samples with nuclei labelled with DAPI, and panels 1, 4 and 7 show merged images. Original magnification was ×63. (b) LCMV particles (indicated by arrows) in sections of BHK-21 cells negatively stained with OsO₄ and K₃Fe(CN)₆. Panel 1: BHK-21 cells infected with LCMV at an m.o.i. of 1 p.f.u. per cell, at 24 h p.i. Panel 2: as panel 1, in the presence of 100 µg FU ml⁻¹. Panel 3: BHK-21 cells overlaid with culture supernatant of BHK-21 cells infected with LCMV under conditions that led to LCMV extinction (Fig. 1b; 96 h p.i.). Panel 4: BHK-21 cells overlaid with culture supernatant of BHK-21 cells infected with LCMV under conditions such that LCMV was not extinct (Fig. 1a, 96 h p.i.).
Fig. 4. Stochastic extinction of LCMV in BHK-21 cells and interference produced by FU-treated populations. (a) Monolayers of 3 \times 10^6 BHK-21 cells were infected with LCMV at an m.o.i. of 0.01 p.f.u. per cell in DMEM in the absence (−FU) or presence (other bars) of 100 μg FU ml⁻¹ (several dishes per time point). After the adsorption period, the monolayers were overlaid with medium. At the indicated times post-infection, the supernatant of each plate was collected and assayed for infectivity in Vero cell monolayers. Supernatants used in experiments detailed in (b) are labelled as 1* and 2*. (b–d) Replica monolayers of 3 \times 10^6 BHK-21 cells were infected with 0.01 p.f.u. per cell in the absence (LCMVA, LCMVB) or the presence of supernatants labelled 1* or 2* (b) or of the supernatants LCMV + FU96A, LCMV + FU96B (described in Fig. 1b) (c) or LCMV + FU102A, LCMV + FU102B (described in Fig. 1a) (d). At the indicated times post-infection, 300 μl supernatant was collected and virus infectivity was determined.

Fig. 5. Specificity of the interference exerted by culture medium from BHK-21 cells persistently infected with LCMV in the presence of FU. (a) Duplicate monolayers of 3 \times 10^6 BHK-21 cells were infected with either EMCV, VSV or LCMV alone (Virus A, virus B) or with EMCV, VSV or LCMV together with supernatant from BHK-21 cells persistently infected with LCMV in the presence of FU (at 102 h p.i.; from the assay shown in Fig. 1b, which led to LCMV extinction) (Virus A + FU102, virus B + FU102). Infections were carried out at an m.o.i. of 0.01 p.f.u. per cell. Samples were titrated in triplicate; standard deviations are given. (b) Same as (a) in an infection with LCMV (blue lines) or either coinfected with the 102 h p.i. supernatant (Fig. 1a) (purple lines) or irradiated with UV light (green lines) or coinfected with supernatant of a mock-infected BHK-21 cell culture in the presence of FU (dashed red line). Samples were titrated in triplicate; standard deviations are given. (c) Confluent monolayers were extracted as described in Airaksinen et al. (2003) and the NTPs were separated by HPLC (black arrows). A_{254} is expressed in mAU. Elution profiles: 1, LCMV-infected BHK-21 cell extracts at 0 and 48 h p.i.; 2, LCMV-infected BHK-21 cell extracts at 0 and 48 h p.i. in the presence of 100 μg FU ml⁻¹; 3, BHK-21 cell extracts at 0 and 48 h p.i. coinfected with LCMV and medium from infections from which the supernatant was removed and virus was not eliminated at 102 h p.i. (LCMV + FU102) in the absence of FU. (d) Duplicate monolayers of 3 \times 10^6 BHK-21 cells were infected with either LCMV or LCMV together with RNase A (LCMV + RNase A) or with supernatant from BHK-21 cells persistently infected with LCMV in the presence of FU (96 h p.i. from assay Fig. 1b that led to LCMV extinction) (LCMV + FU96), or both (LCMV + FU96 + RNase A) or this supernatant filtered through a 0.2 μm filter (LCMV + FU96 filtered) or, finally, with viral RNA extracted from this supernatant (LCMV + RNA from FU96) as indicated. The two groups of bars represent duplicate experiments. At 48 h p.i. samples were titrated in triplicate.
Interference by LCMV

(a) 

(b) 

(c) 

(d)
mutagenesis, as expected (Agudo et al., 2008). For unknown reasons, a relatively high frequency of G→U and C→A transitions (27%) was observed in Z, but not in other genes. This bias was not investigated further. Examination of the presence of A, C, G or U at positions -1, -2, +1 and +2, relative to the site in which an A→G mutation occurred, suggests that the FU-mediated increase in mutation frequency was not due to ADAR-like activities (Polson & Bass, 1994; Zahn et al., 2007) (Table 2).

In conclusion, extinction of LCMV that replicates in BHK-21 cells in the presence of the mutagenic base analogue FU can be avoided by renewing periodically the cell-culture medium with fresh medium even while maintaining the same FU concentration. FU-treated populations produce an interfering activity that does not require the presence of detectable infectivity and which is specific for LCMV, UV radiation-sensitive and displays a dose- and time-dependent behaviour. Production of the interfering activity requires viral replication in the presence of FU. The results suggest that the interfering activity is associated with LCMV as a result of FU-mediated mutagenesis.

**DISCUSSION**

Negative-strand RNA viruses produce DI genomes, which include point mutations and deletions, and particles that can modulate replication of the corresponding standard virus (Holland, 1990; Palma & Huang, 1974; Roux et al., 1991). LCMV gene expression can be regulated by different types of DI genomes (Meyer & Southern, 1997; Popescu et al., 1976; Welsh & Buchmeier, 1979; Welsh et al., 1972; Welsh & Oldstone, 1977; Welsh & Pfau, 1972). DI RNAs can be viewed as one class of defective genomes, among many types of mutants generated as a result of high mutation rates during RNA genome replication, when complementation by the corresponding standard virus is allowed (Holland et al., 1982; Sevilla et al., 2002).

The lethal defection model of virus extinction (Grande-Pérez et al., 2005b) emphasizes the role of a class of defective genomes, termed defectors, in extinction. To substantiate this model for LCMV, it was essential to demonstrate that the interfering activity generated by the FU treatment was superior to the interfering activity naturally associated with stable LCMV replication. This point has been proven in the present study by documenting FU-dependent generation of interfering activity in sufficient levels so as to drive LCMV towards extinction. No extinction was mediated by supernatants of LCMV-infected cultures maintained in the absence of FU. The interfering activity was specific for LCMV and, therefore, could not be the result of any general antiviral response or other FU-induced unspecific perturbation of the cultured cells.

All evidence points to a subset of LCMV particles as the component where the interfering activity resides, as

![Fig. 6. Dose and time effects on interference.](image)
Table 1. Genetic complexity in the mutant spectra of LCMV replicated in BHK-21 cells, in the absence and presence of FU, at different times post-infection

Viral RNA was sampled from the culture supernatant of BHK-21 cells persistently infected with LCMV, subjected to either experimental design 1 or 2, at the indicated times post-infection. The mutation frequency (substitutions per nucleotide) and Shannon entropy in the mutant spectra were determined for the indicated genes, as detailed in Methods. Values showing a significant difference (P<0.05) between designs 1 and 2 in the presence of FU are highlighted in bold. ND, Not determined.

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expected from prior studies on LCMV interference and as proposed in the lethal defection model. In particular, analysis of the LCMV genomes present in the interfering populations suggests that FU mutagenesis was involved in generating viral populations with a modest increase of mutant spectrum complexity. The types and location of the mutations indicate that ADAR 1-L or related activities were not involved in the production of mutated LCMV genomes (Polson & Bass, 1994; Zahn et al., 2007). The mutational bias found, an abundance of A→G and U→C transitions, coincides with that observed previously in FMDV and LCMV populations treated with FU (Grande-Pérez et al., 2002, 2005b; Pariente et al., 2001; Sierra et al., 2000). Although the interfering activity was not dependent on the infectivity present in the interfering populations, it was dependent on the dose and age of the supernatant. It would be interesting to test whether the FU-generated interfering LCMV genomes can also exert an interference on other Old World and New World arenaviruses. The results support the lethal defection model of virus extinction and predict that low mutagen doses, capable of increasing interference levels, may be used in lethal mutagenesis protocols without the need to reach high virus lethality as a result of mutagenesis.

METHODS

Cells and virus. Growth of BHK-21 and Vero cells and virus infections were carried out as described previously (Grande-Pérez et al., 2002; Meyer et al., 2002; Sierra et al., 2000). LCMV ARM 53b is a triple plaque-purified clone from LCMV ARM CA 1371, passaged four times in BHK-21 cells. Rueckert EMCV strain and the Mudd–Summers strain of VSV Indiana serotype were used in this study.

Virus infections. Semiconfluent monolayers of BHK-21 cells (about 2.8 x 10^6 cells in 100 mm-diameter dishes) were infected with LCMV ARM 53b at an m.o.i. of 0.01 p.f.u. per cell; the infected cultures were maintained in 5 ml DMEM supplemented with 10% FCS, 2% L-glutamine, 0.52% glucose and 50 μg gentamicin ml^-1, in either the absence or the presence of FU (100 μg ml^-1), at 37°C and 7% CO2. Supernatants from infected cells (and from mock-infected cells) were harvested at the times post-infection indicated in the corresponding experiments, clarified by centrifugation at 2400 r.p.m. for 30 min at 4°C and stored at -80°C. Virus titres were determined on Vero cell
with 0.2 J cm\(^{-2}\) using a UV 254 nm Energy Cross-linker CL-508 (UVtec Ltd). As a control, 10-fold dilutions of LCMV were treated in the same manner, and no infectivity remained at any dilution (0 to 10\(^{-10}\)). As an additional control, DMEM supplemented with 10% FCS, 2% L-glutamine, 0.52% glucose and 50 μg gentamicin ml\(^{-1}\) was irradiated under the same conditions, and no effect on the growth of BHK-21 cells was observed. Also, the FMDV-specific mAb SD6 was irradiated under the same conditions, without any effect on its FMDV neutralization activity.

**Drug treatment.** Preparation of FU stock solutions, determination of FU toxicity for BHK-21 cells and procedures for infections in the presence of FU have been described previously (Grande-Pérez et al., 2002; Ruiz-Jarabo et al., 2003). At least 80% cell survival was scored after the FU treatments performed in the present study, in agreement with previous determinations (Sierra et al., 2000). LCMV was considered extinguished when no RT-PCR-amplifiable material and no infectivity (limit of detection ≤33 p.f.u. ml\(^{-1}\)) could be observed in the cell-culture supernatant after three blind passages of the undiluted viral population in BHK-21 cells in standard culture medium, in the absence of mutagens (Pariente et al., 2001; Sierra et al., 2000). In addition, extinction was also ascertained in a cellular extract of the BHK-21 cells after the first blind passage. Supernatants used to interfere were preincubated with 10 μg RNase A ml\(^{-1}\). A sample of LCMV with DMEM was pretreated in the same way before the infection.

**RNA extraction, RT-PCR, nucleotide sequencing and molecular cloning.** Total RNA from the supernatant of cell cultures was extracted of the BHK-21 cells after the first blind passage. Supernatants of LCMV ARM 53b (m.o.i. of 0.01 p.f.u. per cell) in 500 μl DMEM and 500 μl of the supernatants to be tested for interfering activity. Titrs were carried out as described above.

**Preparation of FU stock solutions, determination of FU toxicity for BHK-21 cells and procedures for infections in the presence of FU have been described previously (Grande-Pérez et al., 2002; Ruiz-Jarabo et al., 2003). At least 80% cell survival was scored after the FU treatments performed in the present study, in agreement with previous determinations (Sierra et al., 2000). LCMV was considered extinguished when no RT-PCR-amplifiable material and no infectivity (limit of detection ≤33 p.f.u. ml\(^{-1}\)) could be observed in the cell-culture supernatant after three blind passages of the undiluted viral population in BHK-21 cells in standard culture medium, in the absence of mutagens (Pariente et al., 2001; Sierra et al., 2000). In addition, extinction was also ascertained in a cellular extract of the BHK-21 cells after the first blind passage. Supernatants used to interfere were preincubated with 10 μg RNase A ml\(^{-1}\). A sample of LCMV with DMEM was pretreated in the same way before the infection.

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**Viral inactivation.** Virus-containing supernatants were irradiated with 0.2 J cm\(^{-2}\) using a UV 254 nm Energy Cross-linker CL-508 (UVtec Ltd). As a control, 10-fold dilutions of LCMV were treated in the same manner, and no infectivity remained at any dilution (0 to 10\(^{-10}\)). As an additional control, DMEM supplemented with 10% FCS, 2% L-glutamine, 0.52% glucose and 50 μg gentamicin ml\(^{-1}\) was irradiated under the same conditions, and no effect on the growth of BHK-21 cells was observed. Also, the FMDV-specific mAb SD6 was irradiated under the same conditions, without any effect on its FMDV neutralization activity.

**Table 2.** Type of mutations found in LCMV and the position-dependent frequency context in mutations A to G of all the viral supernatants of designs 1 and 2

<table>
<thead>
<tr>
<th>Mutation</th>
<th>L</th>
<th>Z</th>
<th>GP</th>
<th>NP</th>
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<tbody>
<tr>
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<td>0 1</td>
<td>0 2</td>
<td>1 0</td>
<td>0 2</td>
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<tr>
<td>A→G</td>
<td>0 5</td>
<td>0 4</td>
<td>1 0</td>
<td>1 0</td>
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<tr>
<td>A→U</td>
<td>0 6</td>
<td>0 9</td>
<td>0 1</td>
<td>3 4</td>
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<tr>
<td>G→A</td>
<td>0 2</td>
<td>0 1</td>
<td>1 1</td>
<td>0 0</td>
</tr>
<tr>
<td>G→C</td>
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<td>0 13</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>G→U</td>
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<td>0 21</td>
<td>0 1</td>
<td>2 0</td>
</tr>
<tr>
<td>C→A</td>
<td>0 1</td>
<td>0 0</td>
<td>1 0</td>
<td>0 0</td>
</tr>
<tr>
<td>C→G</td>
<td>2 9</td>
<td>1 21</td>
<td>0 1</td>
<td>1 1</td>
</tr>
<tr>
<td>C→U</td>
<td>1 3</td>
<td>0 1</td>
<td>1 1</td>
<td>0 0</td>
</tr>
<tr>
<td>U→A</td>
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<td>0 70</td>
<td>4 13</td>
<td>1 7</td>
</tr>
<tr>
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<td>0 2</td>
<td>0 1</td>
<td>0 0</td>
</tr>
<tr>
<td>U→G</td>
<td>7 141</td>
<td>17 111</td>
<td>3 20</td>
<td>12 46</td>
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<td>Transversions</td>
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<td>0 44</td>
<td>5 4</td>
<td>3 2</td>
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<td>17 155</td>
<td>9 24</td>
<td>15 48</td>
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<td>51 136</td>
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<tr>
<td></td>
<td>33 948</td>
<td>30 012</td>
<td>38 500</td>
<td>35 500</td>
</tr>
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</table>

**Nucleotide position**

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<th>Mutation</th>
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<th>C</th>
<th>G</th>
<th>T</th>
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</thead>
<tbody>
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<td>−2</td>
<td>0.32</td>
<td>0.23</td>
<td>0.23</td>
<td>0.20</td>
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<tr>
<td>−1</td>
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<td>0.15</td>
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<tr>
<td>+1</td>
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<td>0.27</td>
<td>0.28</td>
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<tr>
<td>+2</td>
<td>0.32</td>
<td>0.23</td>
<td>0.23</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Viral RNA was sampled from the culture supernatant of BHK-21 cells persistently infected with LCMV, subjected to either experimental design 1 or 2 as analysed in Table 1, in the absence (−FU) or presence (+FU) of FU in the culture medium. Details are given in Methods. In the upper part of the table, changes of nucleotide found in the L, Z, GP and NP gene fragments sequenced are indicated. Numbers represent the total mutations found for each nucleotide change. The most frequent changes found are highlighted in bold and underlined. In the lower part of the table, position-dependent frequencies are given as percentages (per unit).
extracted with Trizol (Invitrogen) following the manufacturer’s protocol. RNAs were amplified by two-step RT-PCR using ThermoScript reverse transcriptase (Invitrogen) and a reverse (antisense) primer at 60 °C for 45 min, followed by PCR with Pfu DNA polymerase (Promega). Molecular clones corresponding to the Z gene (residues 7–402) and part of the L (residues 3662–4268), GP (residues 247–759) and NP (residues 2223–2743) genes were obtained by ligation of cDNA into the pGEM-T Easy vector (Promega) or into the pCR 4Blunt-TOPO vector (Invitrogen) and transformation into Escherichia coli DH5α cells. DNA from positive colonies was amplified with a TempliPhi amplification kit (GE Healthcare) following the manufacturer’s protocol. RNAs were amplified by two-step RT-PCR using extracted with Trizol (Invitrogen) following the manufacturer’s protocol and sequenced. Primer sequences are available upon request. Clones obtained in pGEM-T Easy vector were sequenced with T7 and SP6 promoter primers whereas pCR 4Blunt-TOPO clones were sequenced with T7 and M13R promoter primers. Sequences were determined with at least twofold coverage.

Nucleotide positions are given in the viral (genomic) sense, and refer to the consensus genomic sequence determined previously [Grand-Pérez et al., 2005b; GenBank accession numbers AY847351 (L) and AY847350 (S)]. To calculate the mutation frequency of mutant spectra, repeated mutations were counted only once. Shannon entropy was calculated with the formula Sn = −Σ(p_i × ln p_i)/ln N, in which p_i is the frequency of each sequence in the clones analysed and N is the total number of sequences compared (Volkenstein, 1994).

**Quantification of the L genome segment.** Total RNA from samples was extracted with Trizol (Invitrogen). Reverse transcription of the genomic segment L (viral RNA polymerase) was performed with ThermoScript RT (Invitrogen) at 65 °C for 60 min with primer L4268R (antisense). RNA corresponding to the L genomic segment was quantified by two-step RT-PCR with the LightCycler Fast Start DNA Master SYBR green I kit (Roche Applied Science). An LCMV RNA fragment spanning nucleotides 3662–4268 (transcript from a molecular DNA clone) was used as a standard.

**Nucleotide pool analysis by HPLC.** The procedure of Pogolotti & Santi (1982) was used with minor modifications. Cell monolayers (in 100 mm-diameter dishes) were washed with PBS, treated with 600 µl 0.6 M trichloroacetic acid and incubated on ice for 10 min. The supernatants were extracted with 1,1,2-trichlorotrifluoroethane (Sigma) containing 0.5 M tri-n-octylamine (Sigma), vortexed for 10 s and centrifuged 30 s at 12 000 × g. The entire treatment was performed at 0–4 °C, using ice-cold solutions. Nucleotides were separated using a 4.6 × 250 mm Partisil 10 SAX column (Whatman) with a 4.6 × 30 mm Partisil 10 SAX precolumn (Phenomenex), using buffers and elution conditions described by Airaksinen et al. (2003). Chromatograms were analysed using the Unicorn 3.00 software (Amerham Pharmacia Biotech). The values used to convert mAU. ml for UTP, CTP, ATP, GTP and FUTP to pmol were 300.1, 492.1, 182.1, 191 and 323.4, respectively.

**Immunofluorescence samples.** Semiconfluent BHK-21 cell monolayers were infected with LCMV at an m.o.i. of 0.01 p.f.u. per cell or with 1 ml of the sample to be tested. At 24 h p.i., the monolayers were washed and fixed in methanol/acetone at −20 °C. Samples were blocked in PBS, 1 % BSA for 15 min and incubated with anti-LCMV NP antibody 113 (Buchmeier et al., 1981; Weber & Buchmeier, 1988), diluted 1:100 in blocking solution, for 1 h at room temperature. After three washes in PBS, samples were incubated with donkey anti-mouse Alexa-488 secondary antibody (Invitrogen), diluted 1:500 in blocking solution, for 45 min at room temperature. Cell nuclei were counterstained using DAPI (Invitrogen) following the manufacturer’s instructions. Finally, samples were washed three times with PBS and once with distilled water and rinsed with absolute ethanol before mounting with Prolong (Invitrogen). An inverted Axiovert 200 Zeiss fluorescence microscope with a CCFD-monochrome colour camera was used to visualize the samples.

**Electron microscopy.** BHK-21 cell monolayers were infected with LCMV at an m.o.i. of 1 p.f.u. per cell in the presence or absence of FV, or with 1 ml of the supernatants without detectable infectivity in vero cells. At 24 or 96 h p.i., cells were fixed for 90 min at room temperature with 4 % paraformaldehyde and 2 % glutaraldehyde in sodium phosphate buffer (0.1 M pH 7.4) and then post-fixed for 1 h at 4 °C with 1 % OsO4 and 1.5 % K2Fe(CN)6 in H2O. After extensive washing, cells were dehydrated in increasing concentrations of ethanol and embedded in Epon 812 resin. Ultrathin sections were examined and photographed on a JEOL 1010 transmission electron microscope operating at 80 kV.

**Bioinformatic analysis of the molecular clones.** Mutation detection was obtained by comparison of each sequence with the consensus sequence, after multiple sequence alignment (MSA), using the MUSCLE program (Edgar, 2004).

**ACKNOWLEDGEMENTS**

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