Characterization of a virus variant produced by L cells persistently infected with lymphocytic choriomeningitis virus

Christine Stocker,† Liliana Martínez Peralta,‡ Thomas Kratzberg,§ Frauke Lohmann and Michael Bruns*

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse 52, D-20251 Hamburg, Germany

Continuous cultivation of murine L cells infected with lymphocytic choriomeningitis virus strain Armstrong leads to production of L(Arm) cells, which produce a predominantly cell-associated attenuated variant, the L(Arm) virus. The relatively few infectious particles that are released have lost the ability to form plaques on L cells and to cause illness in mice even if inoculated intracerebrally. Based on equal protein Mr, antigenicity and protein kinase activity, essentially identical results were obtained for the purified Armstrong and L(Arm) viruses. There was also no difference in production and release of particles with the potential to cause homologous interference. Such particles consisted of two types, one of which was highly susceptible to u.v.-irradiation, the other was highly resistant. In the case of the L(Arm) virus interfering particles, it appears that the u.v.-irradiation-susceptible forms represented infectious virus. Purified L(Arm) virus particles contained considerable quantities of subgenomic forms of (small) S- and (large) L-RNA and their complementary counterparts, which all appeared to be replicated autonomously in an unenriched manner.

Introduction

In a mouse persistently infected with lymphocytic choriomeningitis (LCM) virus high concentrations of infectious virus are maintained in all tissues despite continuous losses due to excretion and natural decay (Taub, 1939; Buchmeier et al., 1980; Lehmann-Grube et al., 1983). However, the proportion of cells that participate in the infectious process is low and infectious virus titres do not exceed certain levels. The immune system is not involved (Volkert & Hannover-Larsen, 1965; Lehmann-Grube, 1984), and in all probability the virus regulates its own replication.

To understand the underlying mechanism, the LCM virus carrier mouse needs to be studied. Models that are less complex such as L cell carrier cultures provide a suitable system for this. The cells are of murine origin and replication of the virus is efficiently regulated. In previous communications we have described L(Arm) cells, which are L cells persistently infected with LCM virus, strain Armstrong (Lehmann-Grube et al., 1969; Bruns et al., 1990). Here, we present biological and biochemical data on their viral product, designated the L(Arm) virus, and the replication behaviour of their gene products after acute infection of L cells. Similar variants may play a role in persistent LCM virus infection of the mouse.

Methods

Cells and viruses. NCTC clone 929 L cells were cultivated with Eagle’s MEM supplemented with non-essential amino acids (Lockhart & Eagle, 1959), 5% calf serum, and antibiotics. LCM virus strain E350, which is usually called Armstrong although its origin is uncertain (Lehmann-Grube, 1984), had been plaque purified. L(Arm) virus was obtained from L(Arm) cells (Bruns et al., 1990). P.f.u. and interfering particles (IP) in L cells and LD50 and 50% infectious dose (ID50) in mice were determined as described (Lehmann-Grube, 1971; Lehmann-Grube & Ambrassat, 1977; Popescu et al., 1976). The ID50 represented the virus titre where 50% of the mice that survived the first intracerebral virus application survived a second lethal dose of LCM virus. L(Arm) virus was quantified as immunofluorescence p.f.u. in L cells grown on cover slips under methylcellulose-containing medium using a rabbit antiserum raised against the virus (Gschwender et al., 1976) and a fluorescein isothiocyanate-coupled goat antiserum raised against rabbit immunoglobulin.

Purification of virus. Two days after infection of L cells with Armstrong virus, or 48 h after subcultivation of L(Arm) cells, culture fluids were collected and clarified by low-speed centrifugation. Thereafter, virus was concentrated by precipitation with 4% polyethylene glycol 40000 and 6% NaCl and purified in 0 to 40% linear Urografin (Schering AG) gradients as already described for the
preparation of the WE strain of LCM virus (Bruns et al., 1983). To separate particles with different biological activities, the virus was enriched for infectious virus [p.f.u. for Armstrong virus, immunofluorescence p.f.u. for L(Arm) virus] relative to IP by ultracentrifugation for 18 h in 26.5% Urografin at 4 °C at 38000 r.p.m. in a SW50 Beckman rotor as described by Martínez Peralta et al. (1981) and confirmed for L(Arm) virus particles by Bruns et al. (1990).

Quantification of viral proteins. Proteins were quantified according to the Lowry method with BSA as a reference standard.

Titeration of complement-fixing antigen. For the complement fixation (CF) test the Microtiter System was employed, the final volume in each reaction well being 0.125 ml. The unit of CF antigen is defined as the quantity that fixes approximately one unit of complement in a reaction mix containing sensitized sheep red blood cells, two units of complement, and excess antibodies in the form of rabbit immune serum raised against LCM virus (Gschwendner et al., 1976).

Measurement of viral protein kinase. The method of Strand & August (1971) with minor modifications was employed. Ten μg of purified virus were taken up in 200 μl buffer at pH 7.5 (5 μmol Tris-HCl, 2 μmol dithiothreitol, 4 μmol MgCl₂) containing 0.5%, NP-40 and 2 μCi [3-32P]ATP (400 Ci/mmol). After incubating the mixture for 30 min at 37 °C, the enzymatic process was blocked by adding 200 μl of 40% trichloroacetic acid. The radioactivity in the acid-insoluble material was measured and calculated as decays per minute (d.p.m.).

Synthesis of strand-specific hybridization probes. Following essentially the protocols of Sambrook et al. (1989) we transferred the SalI fragments of plasmid MS19 and S113 (Southern et al., 1987; Salvato et al., 1988) into the mult-cloning site of the vector pSP71, downstream of a bacteriophage T7 or SP6 promoter (Melton et al., 1984; Tabor & Richardson, 1985). Similarly, a fragment obtained from pZORF.1 (Salvato & Shimomaye, 1989; Salvato et al., 1992) by use of restriction enzymes HindIII and EcoRI was inserted into the mult-cloning site of pSP71. The resulting vectors pT7-NP37, pSP6-GP17 (see Fig. 4a) and pT7-Z52 (see Fig. 5a) were linearized at the HindIII, BamHI, and EcoRI sites, respectively, and transcribed into radioactive RNA probes with T7 or SP6 RNA polymerase in the presence of [32P]CTP (800 Ci/mmol) (DuPont-NEN). About 100 ng of each RNA were synthesized with activities in the range 4 to 5 x 10⁵ c.p.m./pmol.

RNA extraction and hybridization. RNA from cells was extracted with guanidinium thiocyanate according to Chirgwin et al. (1979). It was treated with glyoxal at 5 min at 60 °C and then for 15 min at 50 °C and subsequently separated by electrophoresis in 1.2% agarose with 0.01 M-NaH₂PO₄ at pH 7.0 (McMaster & Carmichael, 1977). The blotting conditions employed have been described previously (Bruns et al., 1988).

Results

Biological properties of L(Arm) virus

After purification of L(Arm) virus in Urografin gradients its properties were compared with those of the original Armstrong virus (Table 1). Relative to amount of protein the numbers of IP, antigenicity as measured by the CF test, and protein kinase activity (which, in the case of LCM virus leads to phosphorylation of NP; Howard & Buchmeier, 1983; Bruns et al., 1986; unpublished), were essentially identical. In contrast, marked differences were apparent with regard to infectivity, as measured both in mice and L cells, and to mouse pathogenicity. For L(Arm) virus, plaques on L cells did not form and clinical signs in mice were not apparent even for virus injected intracerebrally. Titration of Armstrong virus using an immunological focus assay and its comparison with conventional plaquing methods has already been described (Battegay et al., 1991).

To measure the multiplication of infectious L(Arm) virus and the corresponding IP, L(Arm) cells were seeded in Petri dishes and both entities in the culture fluid were enumerated at 24 h intervals (Fig. 1). By comparison with Armstrong virus, L(Arm) virus differed in three respects: it grew more slowly, more IP than immunological focus units were observed, and these were of lower infectivity than those of Armstrong virus. The interfering capacity of L(Arm) virus was also lower than that of Armstrong virus, again in agreement with the results of Battegay et al. (1991).

Table 1. Properties of LCM virus strains L(Arm) and Armstrong in comparison

<table>
<thead>
<tr>
<th>Activity</th>
<th>Unit*</th>
<th>L(Arm)</th>
<th>Armstrong</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infectivity</td>
<td>ID₅₀ (mouse)</td>
<td>30</td>
<td>8.5</td>
</tr>
<tr>
<td>Infectivity</td>
<td>p.f.u.</td>
<td>&lt;†</td>
<td>6.9</td>
</tr>
<tr>
<td>Infectivity</td>
<td>IPFU</td>
<td>32</td>
<td>N.D.‡</td>
</tr>
<tr>
<td>Mouse pathogenicity</td>
<td>LD₅₀</td>
<td>&lt;</td>
<td>7.4</td>
</tr>
<tr>
<td>Interference</td>
<td>IP</td>
<td>5.3</td>
<td>5.4</td>
</tr>
<tr>
<td>Antigenicity</td>
<td>CF unit</td>
<td>30</td>
<td>3.6</td>
</tr>
<tr>
<td>Protein kinase</td>
<td>d.p.m.§</td>
<td>3.7</td>
<td>3.7</td>
</tr>
</tbody>
</table>

* Expressed as log₁₀ per μg of protein of purified virus.
† Below detectability.
‡ N.D., Not determined.
§ d.p.m., Amount of radioactive phosphorus incorporated into viral protein.

Fig. 1. Replication of infectious and interfering L(Arm) virus in L(Arm) cells. Freshly seeded L(Arm) cells in 5 cm-Petri dishes were incubated at 37 °C. At intervals, samples from the culture fluid were titrated on L cells for immunofluorescence p.f.u. (■) and IP (●). In comparison, the supernatants of L cells, infected with Armstrong virus at an m.o.i. of 1, were obtained 24, 48, 72 and 96 h after infection and the virus titres, measured as log₁₀/ml, were 6.29 p.f.u./4.37 IP, 8.55 p.f.u./6.74 IP, 7.50 p.f.u./5.83 IP, and 6.93 p.f.u./4.55 IP, respectively.
LCM virus variants

Fig. 2. Effect of u.v.-irradiation on infectious and interfering L(Arm) virus. L(Arm) virus from cell culture fluid was concentrated and purified by ultracentrifugation in a Urografin gradient. It was dispensed in 1 ml samples into 5 cm-Petri plates, exposed to u.v.-light (Mineralight, Ultraviolet Products) at a distance of 10 cm, and thereafter titrated for immunofluorescence p.f.u. (■) and IP (○).

Fluorescence p.f.u. were produced, and the final yield of particles was considerably lower (Fig. 1).

Physical and functional separation of IPFU and IP L(Arm) virus

The effects of u.v.-irradiation on p.f.u. and IP of the WE strain of LCM virus are well known (Welsh & Pfau, 1972; Popescu et al., 1976; Lehmann-Grube et al., 1983). In contrast to its infectivity, the interfering potential is extraordinarily stable, and both properties are lost with first order kinetics. We have repeated these experiments and confirmed the previous findings. However, the effect of u.v.-irradiation on Armstrong virus differed. Again, infectivity was rapidly lost, but the diminution in interfering potential did not follow a first order rate. Rather, a steep decline preceded a gradual inactivation, indicating that there were two types of interfering units (data not shown). Such experiments with L(Arm) virus gave similar results (Fig. 2), indicating that approximately one half of the interfering activity was u.v.-irradiation-sensitive whereas the other half was highly resistant.

For further analysis, purified L(Arm) virus was fractionated by a shallow Urografin gradient, designed to enrich infectious and interfering virus relative to each other (Martinez Peralta et al., 1981; Bruns et al., 1990). Fractions were titrated for both immunofluorescence p.f.u. and IP before and after u.v.-irradiation. As shown in Fig. 3, there were two peaks of interfering virus: one that was localized in the denser portions of the gradient, was u.v.-irradiation-sensitive and associated with immunofluorescence p.f.u. whereas the other was u.v.-irradiation-resistant and relatively free of infectivity. Thus, of the two types of IP that were detected in association with L(Arm) virus, one was linked with infectivity, which opened up the possibility, that the infectious particle itself could interfere or, alternatively,
Table 2. Effect of u.v.-irradiation of L(Arm) virus on its potential to interfere with the replication of Armstrong virus

<table>
<thead>
<tr>
<th>Dilution of L(Arm) virus</th>
<th>L(Arm) virus*</th>
<th>U.v.-irradiated</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>5.81 1.26</td>
<td>5.93 2.16</td>
<td>6.50 4.46</td>
</tr>
<tr>
<td>1:2</td>
<td>6.80 5.07</td>
<td>7.43 7.43</td>
<td></td>
</tr>
<tr>
<td>1:4</td>
<td>6.80 5.07</td>
<td>7.43 7.43</td>
<td></td>
</tr>
<tr>
<td>1:8</td>
<td>6.80 5.07</td>
<td>7.43 7.43</td>
<td></td>
</tr>
<tr>
<td>L(Arm) virus</td>
<td>7.43 7.43</td>
<td>7.43 7.43</td>
<td></td>
</tr>
<tr>
<td>not present</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Virus from L(Arm) culture fluid was concentrated 100-fold by ultracentrifugation. The resulting preparation was divided into two portions, one of which was u.v.-irradiated so as to inactivate all infectivity. Both were diluted and to each was added 10⁶ p.f.u. Armstrong virus. The mixtures were added to 10⁶ L cells and then left at room temperature for 30 min. The cells were rinsed three times and then incubated for 44 h at 37 °C, after which the culture fluids were titrated for infectious yield.

† Dilution of irradiated and unirradiated concentrated L(Arm) virus.

‡ Log₁₀ p.f.u./ml.

could maintain interfering components co-packaged during the encapsidation process.

Inhibition of production of Armstrong wild-type virus by L(Arm) IPFU or IP

The possibility that infectious particles have interfering activity was explored by testing the ability of L(Arm) virus to inhibit the multiplication of standard Armstrong LCM virus before and after u.v.-irradiation treatment. L(Arm) virus was concentrated 100-fold by ultracentrifugation, but was not further purified in order to avoid loss of infectivity. The resulting preparation was divided into two parts, one of which was exposed to u.v.-light. Subsequently, both portions were serially diluted and equal volumes were mixed with identical quantities of fully infectious Armstrong virus. L cells were infected with the mixtures or with Armstrong virus alone and incubated for 44 h. Thereafter, the various culture supernatants were titrated for p.f.u. to assay the amount of Armstrong virus present. The results (Table 2) revealed an essentially linear dose-response relationship between u.v.-inactivated L(Arm) virus and the yield of infectious Armstrong virus. In contrast, the inhibition of Armstrong virus by various quantities of non-irradiated L(Arm) virus did not indicate a linear relationship, instead addition of the less diluted samples caused disproportionately large reductions in yield. We interpret this result to mean that the infectious progeny of the multiplying (untreated) L(Arm) virus may exert homologous interference, which would be compatible with the existence of two kinds of IP in L(Arm) virus differing in infectivity and density.

Fig. 4. Replication and transcription products from S-RNA of L(Arm) virus. (a) Scheme for construction of radioactive RNA molecules for binding to viral genomic RNAs and their mRNAs. The model depicts the ‘ambisense’ strategy of standard LCM virus (Bishop, 1991) for replication from S- into VC-S-RNA and vice versa (double arrow) and for transcription (single arrows) into mRNAs for NP (from left to right) and GPC (from right to left). The RNAs of LCM virus (empty boxes with small black boxes inside to indicate intergenic regions) are presented together with the viral cDNAs (■) for the synthesis of complementary radioactive RNA molecules produced by the plasmids pSP6-GP17 or pT7-NP37, to recognize GPC-mRNA and S-RNA or NP-mRNA and VC-S-RNA, respectively; the small horizontal arrows above the plasmids display the direction of RNA synthesis. The scale on top illustrates the size in bp. (b) RNAs of L(Arm) cells (lane 1), L cells acutely infected with Armstrong virus (lane 2), purified L(Arm) virus (lane 3), and purified Armstrong virus (lane 4) were separated by electrophoresis in agarose gels. RNA was blotted onto nitrocellulose and hybridized with radioactive probes transcribed either from pSP6-GP17 for detecting viral genomic S-RNA and GPC-mRNA (left side) or from pT7-NP37 for detecting genomic-sized viral complementary S-RNA and NP-mRNA (right side). Bands were made visible by autoradiography.

RNAs of L(Arm) virus

In L(Arm) cells there are the standard RNA species (small) S-RNA, their viral complementary form (VC-S-RNA), and S-RNA molecules of subgenomic size (Bruns
LCM virus variants

Fig. 5. Replication and transcription products from L-RNA of L(Arm) virus. (a) Scheme for construction of radioactive RNA molecules for binding to viral genomic L-RNA and Z-mRNA. The model delineates the ‘ambisense’ strategy of standard LCM virus on the right end of the L-RNA (Salvato & Shimomaye, 1989) for its replication into VC-L-RNA and vice versa (double arrow) and for transcription (single arrows) into mRNAs for X (from left to right) and Z (from right to left). The RNAs of LCM virus (empty boxes) are presented together with the viral cDNA (stippled box) for the synthesis of complementary radioactive RNA molecules (■) produced by the plasmid pT7-Z52 to distinguish Z-mRNA and L-RNA, respectively; the small horizontal arrow above the plasmid shows the direction of their synthesis. The scale on top depicts the size in bp. (b) RNA of L(Arm) cells from different origins (Fig. 5b lane 1, Lehmann-Grube et al., 1969; Fig. 5b lane 2, Bruns et al., 1990) and Armstrong virus-infected L cells were compared again by Northern blotting using pT7-Z52 to generate a radioactive probe complementary to Z-mRNA and L-RNA. From the figure it is evident that in addition to the mRNA for the Z protein and the L-RNA, which are present in cells infected with Armstrong virus (Salvato & Shimomaye, 1989; Salvato et al., 1992), RNA molecules of subgenomic size were present in L(Arm) cells.

In further experiments, purified L(Arm) virus was separated by Urografin gradient ultracentrifugation into fractions containing predominantly either infectious or interfering virus. RNA was isolated, separated electrophoretically, and subjected to Northern blotting using probes for genomic S- and L-RNAs and their derivatives. No differences were observed between the two portions of the gradient, i.e. the same RNA species were demonstrated in both samples (not shown). This is particularly true for the upper part of the gradient, from which exclusively non-infectious IP could be isolated. In contrast, immunofluorescence p.f.u. are enriched within the denser fractions of the gradient, which also contain considerable numbers of IP. The data also revealed that L(Arm) virus particles contained mRNA for the Z protein as recently described for Armstrong standard virus (Salvato et al., 1992). In addition, RNA species from different sources (lanes 1 and 2) and L cells acutely infected with standard Armstrong virus (lane 3) were separated in an agarose gel and subjected to Northern blotting as described in Fig. 4. For the analysis of viral RNA molecules pT7-Z52 was introduced to generate radioactive RNA complementary to L-RNA or Z-mRNA.

We have now determined which of these are enclosed in L(Arm) virus particles. L(Arm) virus and standard Armstrong virus, both from culture fluid, were concentrated and purified by ultracentrifugation in Urografin gradients, and RNAs from both sources as well as from L(Arm) cells and L cells acutely infected with Armstrong virus were identified by Northern blotting. The results in Fig. 4 show that, just as L(Arm) cells, L(Arm) virus contained subgenomic RNA molecules. Similar ones were not found in Armstrong virus or associated with acutely-infected L cells. Corresponding results were obtained with radioactive probes detecting molecules that were complementary to genomic and subgenomic viral RNAs. There was a further finding in these experiments: although highly purified, Armstrong as well as L(Arm) virus particles contained genomic sized viral complementary S-RNA. A Northern blot does not lend itself readily to quantification, but there appeared to be similar amounts of complementary and genomic RNA molecules in Armstrong and L(Arm) viruses.

In the next analysis (Fig. 5) the RNAs from L(Arm) cells from different origins (Fig. 5b lane 1, Lehmann-Grube et al., 1969; Fig. 5b lane 2, Bruns et al., 1990) and Armstrong virus-infected L cells were compared again by Northern blotting using pT7-Z52 to generate a radioactive probe complementary to Z-mRNA and L-RNA. From the figure it is evident that in addition to the mRNA for the Z protein and the L-RNA, which are present in cells infected with Armstrong virus (Salvato & Shimomaye, 1989; Salvato et al., 1992), RNA molecules of subgenomic size were present in L(Arm) cells.

In further experiments, purified L(Arm) virus was separated by Urografin gradient ultracentrifugation into fractions containing predominantly either infectious or interfering virus. RNA was isolated, separated electrophoretically, and subjected to Northern blotting using probes for genomic S- and L-RNAs and their derivatives. No differences were observed between the two portions of the gradient, i.e. the same RNA species were demonstrated in both samples (not shown). This is particularly true for the upper part of the gradient, from which exclusively non-infectious IP could be isolated. In contrast, immunofluorescence p.f.u. are enriched within the denser fractions of the gradient, which also contain considerable numbers of IP. The data also revealed that L(Arm) virus particles contained mRNA for the Z protein as recently described for Armstrong standard virus (Salvato et al., 1992). In addition, RNA species
Fig. 6. Time course of appearance of subgenomic RNA molecules in L cells infected with L(Arm) virus. L cells were infected with L(Arm) virus from cell culture medium and passaged at intervals of 48 h. Spread of infections and appearance of viral RNA were monitored at the ends of the indicated passage numbers by indirect immunofluorescence and Northern blot analysis, respectively. The radioactive probes used to identify NP-mRNA and VC-S-RNA on one hand and L-RNA and Z-mRNA on the other had been transcribed from pT7-NP37 and pT7-Z52, respectively (see Fig. 4 and 5).

migrated to positions between the genomic L-RNA and Z-mRNA species, indicating the presence of shorter L-RNA molecules in L(Arm) virus particles, as also found for L(Arm) cells (not shown).

Replication of subgenomic RNA molecules in L(Arm) cells

Since L(Arm) virus contains smaller RNA molecules that are related to L- and S-RNAs, we wanted to know whether these replicated by themselves or originated from the full-length molecules. L cells were acutely-infected with L(Arm) virus and subcultivated. Cells from each passage were studied with regard to expression of viral antigen using immunofluorescence and viral RNA by Northern blotting. The results (Fig. 6) revealed a slow spread of infectious L(Arm) virus, which only became detectable as cell-associated antigen by the fourth passage (8 days of cultivation). Thereafter, the proportion of infected cells increased relatively rapidly, and by the seventh passage (14 days of cultivation) all cells contained viral antigen.

The first detectable viral RNA was of subgenomic size. It appeared during passage five (when about 25% of the cells contained viral antigen). Although a small amount of full-sized RNA could be demonstrated at passage six (when about 75% of the cells were antigen-positive) subgenomic molecules predominated, as was the case during later passages. No full-sized RNA molecules were...
produced prior to the subgenomic species. The latter species were detected earlier presumably due to the larger quantities made.

**Replication of subgenomic RNA molecules in double-infected cells**

As shown above virus particles containing RNA molecules of subgenomic size are capable of infecting cells and replicating their full-length genome as well as their subgenomic RNA molecules. To support these findings cells that were co-infected by L(Arm) virus and standard Armstrong virus were analysed (Fig. 7). Since Armstrong virus does not produce subgenomic RNAs during acute infections, we investigated whether Armstrong virus could replicate the subgenomic RNA molecules of L(Arm) virus. The reason for doing this experiment arose from the observation that no viral RNA was detectable during the first 24 h of infection with L(Arm) virus, because of its slow propagation (compare also Fig. 6). L(Arm) virus was concentrated 100-fold and thereafter diluted by a factor of 2. These preparations were used together with standard Armstrong virus to infect L cells simultaneously, the latter at an m.o.i. of 1. Twenty-four h later the supernatants were harvested to titrate the p.f.u. of Armstrong virus (Fig. 7, upper panel) and the cells were recovered to isolate the total RNA for analysis by Northern blots. The vector pT7-NP37 was used as a probe for NP-mRNA and VC-S-RNA (Fig. 7, below). The infection with 100-fold concentrated L(Arm) virus alone or in combination with Armstrong virus produced no detectable viral RNA (Fig. 7a and b). After simultaneous infection with half the amount of concentrated L(Arm) virus together with standard virus VC-S-RNA, NP-mRNA and subgenomic RNA molecules were detected. Thereafter, the amounts of RNA molecules increased with decreasing concentrations of L(Arm) virus, as a consequence of the decreasing amount of interfering activity (Fig. 7, c to l). Similarly, the quantities of subgenomic RNA molecules increased, although not in the same proportion as the full-sized genomic RNA molecules. Thus, Armstrong virus, which does not produce subgenomic RNAs during an acute infection, was able to replicate subgenomic RNAs of L(Arm) virus.

**Discussion**

Infection of mice before or shortly after birth with LCM virus leads to a carrier state, in which infectivity persists throughout life in essentially all tissues. In these animals considerably more viral RNA and nucleoprotein (NP) are detected than would be expected from the titres of infectious virus (Buchmeier et al., 1980; Southern et al., 1984). It cannot be assumed that the quantities of both RNA and NP are relatively high because (non-infectious) IP are also produced, since the latter are present in low numbers (Popescu & Lehmann-Grube, 1977). Thus, one possibility is that LCM virus carrier mice harbour other viral units that have accounted in concentration measurements of certain viral components, but do not contribute towards infectivity.

The establishment of carrier L cells with the LCM virus strain Armstrong proceeds in three well defined phases (Bruns et al., 1990). Initially, fully infectious virus and IP are produced in characteristic cycles. Then a period follows, in which the virus has lost its plaque-forming potential for L cells but is still pathogenic for mice. Subsequently, virus particles are made that neither cause plaques on L cells nor harm mice even when inoculated intracerebrally. In this final stage, viral antigen is observed by immunofluorescence in essentially every cell. Further, there is marked homologous interference (although infection with vesicular stomatitis virus proceeds unimpeded) and large numbers of IP are synthesized. It is at this stage that L(Arm) virus is produced. If a similar situation pertains in carrier mice, the discrepancy between the quantities of RNA and NP on one hand and infectivity on the other may be explained.

L(Arm) virus released into the culture medium was analysed and compared with standard Armstrong virus. Besides differences in infectious and pathogenic properties the most striking deviation was found in RNA. In contrast to Armstrong virus, L(Arm) virus particles contained many subgenomic S-RNA and, comparatively fewer, L-RNA molecules. Northern blotting revealed that about half of these were complementary, and that probably both the viral and the complementary forms were equally represented in preparations of virus particles. Full-length complementary S- and L-RNAs were also found.

As in L(Arm) virus particles, the numbers of small RNA molecules are high in L(Arm) cells (Bruns et al., 1990). They are relatively lower in other types of LCM virus carrier cultures (Francis & Southern, 1988a), and in the tissues of carrier mice (Francis & Southern, 1988b; own observations), which may result from the concomitant presence of infectious standard virus. Nevertheless, in persistent LCM virus infections subgenomic forms are characteristically found. It could be demonstrated that these subgenomic RNA molecules are synthesized after acute infection of L cells with the L(Arm) virus itself, but so slowly that the first RNA molecules could not be clearly distinguished before the fifth passage. The replication of RNA from the attenuated variant is also delayed and intact RNA molecules could not be detected before the appearance of subgenomic RNA molecules. This indicates that these RNA
constructs are replicated at the same time as the genomic-sized RNAs. On the other hand, after co-infection with L(Arm) virus and the LCM virus strain Armstrong, the other forms were also replicated, but only because of the presence of infectious virus. In this case, it has been shown that the amount of such molecules depended on the amplification of the Armstrong virus. The number of subgenomic RNA molecules increased, but less efficiently than the complete genomes of Armstrong virus. The reason could be that the molecules of subgenomic size could replicate by themselves. Therefore, no enrichment of the truncated RNA molecules was observed, when both particles, the attenuated variant as well as the fully infectious standard Armstrong virus, were present in L cells.

The possibility that the infectious L(Arm) virus acts at the same time as an interfering particle could have eminent consequences for the regulation of virus replication during persistent infection. Whether this phenomenon is the result of point mutations or deletions within the viral genome or is due to co-packaging of interfering components remains to be determined. The demonstration that Arenaviruses contain more than one copy of S- and L-RNA and are frequently diploid supports this concept (Romanowski & Bishop, 1983).

We thank Ms Gesa-Katharina Fuhrmann for excellent technical assistance, and Dr Maria S. Salvato, Department of Pathology, University of Wisconsin Medical School, Madison, Wisconsin, U.S.A. and Dr Peter J. Southern, Department of Microbiology, University of Wisconsin Medical School, Madison, Wisconsin, U.S.A. for providing LCM virus cDNA probes. This work was carried out with a grant from the Deutsche Forschungsgemeinschaft. The Heinrich-Pette-Institut is financially supported by Freie und Hansestadt Hamburg and Bundesministerium für Gesundheit.

References


(Received 26 May 1994; Accepted 5 July 1994)