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Molecular Basis of Organ-Specific Selection of Viral Variants during Chronic Infection

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Viral variants of different phenotypes are present in the central nervous system (CNS) and lymphoid tissues of carrier mice infected at birth with the Armstrong strain of lymphocytic choriomeningitis virus. The CNS isolates are similar to the parental virus and cause acute infections in adult mice, whereas the lymphoid isolates cause chronic infections associated with suppressed T-cell responses. In this study, we provide a molecular basis for this organ-specific selection and identify a single amino acid change in the viral glycoprotein that correlates with the tissue specific selection and the persistent and immunosuppressive phenotype of the variants. This phenylalanine (F)-to-leucine (L) change at position 260 of the viral glycoprotein was seen in the vast majority (43 of 47) of the lymphoid isolates, and variants with L at this residue were selected in spleens of persistently infected mice. In striking contrast, isolates with the parental sequence (F at residue 260) predominated (48 of 59 isolates) in the CNS of the same carrier mice. Complete nucleotide sequence analysis of the major structural genes of several independently derived (from different mice) spleen isolates showed that these variants were >99.8% identical to the parental virus. In fact, the only common change among these spleen isolates was the F→L mutation at residue 260 of the glycoprotein. These results show that an RNA virus can exhibit minimal genetic drift during chronic infection in its natural host, and yet a single or few mutations can result in the organ-specific selection of variants that are markedly different from the parental virus.

The genetic changes and selective pressures involved in the emergence of viral variants in vivo are not well understood. To address this fundamental issue, we have been studying the evolution of lymphocytic choriomeningitis virus (LCMV) in its natural host (2, 13). Our previous studies have documented the importance of host tissues in the selection of viral variants during chronic LCMV infection. We have shown that LCMV isolates of different phenotypes predominate in the central nervous system (CNS) and lymphoid tissue of carrier mice infected at birth with the Armstrong strain of LCMV. Most of the CNS isolates are similar to the parental Armstrong strain and induce potent virus-specific cytotoxic T-lymphocyte (CTL) and delayed-type hypersensitivity responses in adult mice, and the infection is cleared within 2 weeks. In contrast, LCMV clones derived from the lymphoid tissue of carrier mice cause persistent infections in adult mice associated with suppressed T-cell responses and susceptibility to opportunistic infections (3, 4, 26). To determine the genetic basis of the organ-specific selection and to understand the mechanism of viral persistence and immune suppression, we have conducted a molecular analysis of these naturally selected LCMV variants.

The LCMV genome consists of two segments of single-stranded RNA: a large (L) segment of 7.2 kb and a small (S) segment of 3.4 kb (4, 18, 20, 22, 23). The L RNA segment codes for a large protein, L (molecular size, 250 kDa), that is believed to be the viral polymerase, and also contains a second open reading frame, designated Z, that encodes for a protein of approximately 10 to 12 kDa. The S segment codes for the three major structural proteins: the internal nucleocapsid (63 kDa) and the two surface glycoproteins GP-1 (43 kDa) and GP-2 (36 kDa) that are derived from a common precursor polypeptide, GP-C.

In this study, we identify a single amino acid change in the viral glycoprotein that correlates with the organ-specific selection and the persistent and immunosuppressive phenotype of the variants. This phenylalanine (F)→leucine (L) change is seen in >95% of the lymphoid isolates, and mutants with L at residue 260 of the viral glycoprotein are selected in the spleens of carrier mice, whereas LCMV isolates with the parental genotype (F at residue 260) predominate in the CNS.

MATERIALS AND METHODS

Mice. BALB/cByJ mice were purchased from Jackson Laboratory, Bar Harbor, Maine. Neonatally infected carriers were made by injecting 10^4 PFU of LCMV intracerebrally into mice within 24 h of birth. The congenital carrier colony was derived from these neonatally infected mice and bred at the University of California at Los Angeles.

Virus. The Armstrong CA 1371 strain of LCMV was used in these studies (3). The virus was triple plaque purified on Vero cells, and stocks were grown in BHK-21 cells. This original laboratory stock of CA 1371 will be referred to as the parental Armstrong strain. The carrier colony was originally started by infecting 1-day-old mice with this strain. The CNS and lymphoid isolates obtained from LCMV carrier mice were also plaque purified three times. Virus stocks (grown in BHK cells) at passage 1 or passage 2 levels were used in subsequent experiments. The biological properties of the various isolates are extremely stable in tissue culture, and we have had no reversion of the phenotype during the plaque purification on Vero cells or growth in BHK cells.

Virus titration. Infectious LCMV was quantitated by

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plaque assay on Vero cell monolayers as previously described (3).

Generation of reassortants. Coinfection of BHK-21 cells and the procedures used to screen the progeny for reassortants were as previously described (4).

CTL assay. LCMV-specific CTL activities in spleens and lymph nodes were determined by a 6-h ^{51}Cr release assay as previously described (3).

Sequence analysis. The L and S segments of the parental Armstrong strain and the spleen variants were sequenced by the primer extension method, using virus-specific oligonucleotides (11, 14, 19). Either 1 to 3 μg of viral RNA extracted from purified virus or 50 μg of total infected cell RNA was used for the sequencing reactions.

Oligonucleotide hybridization. Matching sets of oligonucleotides specific either for the parental Armstrong sequence or the spleen variant clone 13 sequence were used to determine the viral RNA sequence of the CNS and lymphoid isolates at nucleotide residues 855 and 1298. The pairs of oligonucleotides used were as follows: for nucleotide residue 855, Armstrong-specific 5′-GTCTCCTAGTGAAGAACTTA-3′ and clone 13-specific 5′-GTCTCCTAGTGAGGAACTTA-3′, and for residue 1298, Armstrong-specific 5′-TTCGATTTGA TCACTGAAGT-3′ and clone 13-specific 5′-TTCGATTTGG TCACTGAAGT-3′. Total RNA was extracted from infected BHK-21 cells by the guanidinium thiocyanate-CsCl procedure (8). The RNA samples were denatured with formaldehyde, and 5 μg of RNA was dotted onto nitrocellulose paper with a 96-hole Bio-Dot apparatus. Duplicate filters of each viral RNA sample were hybridized with ^{32}P-labeled oligonucleotides that were specific either for the parental Armstrong sequence or for the spleen variant clone 13 sequence. The pairs of oligonucleotides used differ at only one residue, and washing conditions were used such that a single mismatch resulted in complete loss of hybridization. To determine the amount of viral RNA in each sample, an additional filter was hybridized with a ^{32}P-labeled cDNA probe that hybridizes equally well with the S RNA segment of both parental Armstrong virus and the spleen variant clone 13 (4, 23).

RESULTS

Genetic analysis of one spleen isolate, clone 13, has shown that biologically important mutations occurred in both the L and S RNA segments (4, 14). Replacing the L or S segment of clone 13 with the parental Armstrong L or S segment profoundly affected its ability to persist and cause immune suppression in adult mice (Table 1). The precise changes in the L segment of clone 13 have not yet been identified, but complete sequence analysis of the S segment of clone 13 has revealed two differences (at nucleotide positions 855 and 1298) in the glycoprotein gene compared with the parental Armstrong virus (14, 19). Both of these were U→C changes; the one at position 855 resulted in an F→L change at amino acid residue 260, whereas the one at position 1298 was silent (Fig. 1). There were no mutations in the nucleoprotein gene, the intergenic region, or the noncoding regions at the 5′ and 3′ ends of the S segment.

It was of interest to determine whether mutations seen in the glycoprotein gene of this one particular spleen isolate would be found in other lymphoid isolates, and more importantly to ascertain whether these genetic changes were involved in the organ-specific selection of LCMV variants. To address this question, the sequences of several independently derived spleen and brain isolates obtained from BALB/c carrier mice infected at birth with the Armstrong strain were determined. Sequences at the two sites showing changes (positions 855 and 1298) were analyzed by oligonucleotide hybridization (see Materials and Methods). This technique allows rapid screening of a large number of viral isolates. Using this method, we analyzed >100 LCMV brain and spleen isolates. Many of these isolates were independently derived (i.e., isolated from different carrier mice). The silent U→C change at nucleotide position 1298 was not seen in any of the brain or spleen isolates, indicating that this was a random mutation in clone 13 and unlikely to be of any biological significance (data not shown). In contrast, the mutation at nucleotide position 855 (amino acid residue 260)

TABLE 1. Genetic evidence showing that mutations in the S and L segments of spleen isolate clone 13 affect ability to persist in adult mice

Virus and genotype[a]	% Specific ^{51}Cr release from BALB Cl7 (*H-2*^{d}) targets[b]				LCMV titer in serum (log_{10} PFU/ml)[c]		
	LCMV infected			Uninfected, 50:1			
	5.5:1	16.6:1	50:1		8	15	30
Parental Armstrong, A/A	33	59	82	6	<1.6	<1.6	<1.6
Spleen variant clone 13, svA/svA	0	3	11	1	5.9	5.7	4.4
Reassortant C406a1, svA/A	13	45	61	1	3.5	<1.6	<1.6
Reassortant T800, A/svA	20	41	59	3	3.3	<1.6	<1.6

[a] Adult BALB/c mice were infected intravenously with 10^6 PFU of the indicated virus. The following notation is used to indicate genotype: A/A, virus with both L and S segments of parental Armstrong; svA/svA, virus with both L and S segments of spleen variant clone 13; svA/A, reassortant with L segment of spleen variant clone 13 and S segment of parental Armstrong; A/svA, reassortant with L segment of parental Armstrong and S segment of Clone 13.

[b] CTL response was checked 8 days postinfection at the indicated E/T ratios. Data are averages for three mice per group.

[c] Mice were eye-bled on the indicated days postinfection, and the titer of infectious LCMV in the serum was determined by a plaque assay on Vero cells. Data are averages for three to six mice per group.

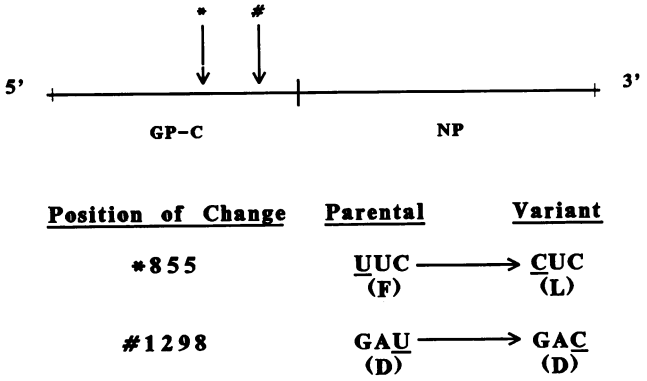

FIG. 1. Sequence differences between the S segments of parental Armstrong and spleen variant clone 13. The S segments (3,376 bases) of these two viruses show only two nucleotide changes, both in the glycoprotein gene. The mutation at position 855 results in an F→L change, whereas the mutation at position 1298 is silent. There are no nucleotide changes in the nucleoprotein gene, the intergenic region, or the noncoding regions at the 5′ and 3′ ends of the S segment.

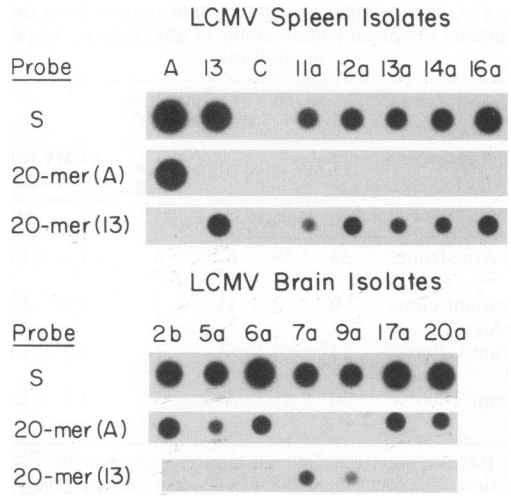

FIG. 2. Identification of nucleotide residue 855 of the viral glycoprotein gene by hybridization with oligonucleotides specific for the parental Armstrong or spleen variant clone 13 sequence. Total RNA was extracted from infected BHK-21 cells by the guanidinium thiocyanate-CsCl procedure. The RNA samples were denatured with formaldehyde, and 5 μg of RNA was dotted onto nitrocellulose paper in a 96-hole Bio-Dot apparatus. Triplicate filters of each sample were made and hybridized with a ^{32}P-labeled cDNA probe (S) that hybridizes with the small RNA segment of both Armstrong and clone 13 RNA and with ^{32}P-labeled oligonucleotides (20-mers) that specifically hybridize with either the parental Armstrong (A) or clone 13 (13) RNA, respectively. The two oligonucleotides used (clone 13 specific, 5′-GTCTCCTAGTGAGGAACTTA-3′; Armstrong specific, 5′-GTCTCCTAGTGAAGAACTTA-3′) differ at only one residue, and washing conditions were used such that a single mismatch resulted in complete loss of hybridization. Sample C designates RNA from uninfected BHK cells. Samples 11a, 12a, 13a, 14a, and 16a are independently derived (from different mice) spleen isolates from five 2-month-old LCMV carrier mice; samples 2b, 5a, 6a, 7a, 9a, 17a, and 20a are brain isolates obtained from the same five carrier mice.

was found in the vast majority of the lymphoid isolates (Fig. 2 and Table 2). Of the 47 spleen isolates tested, 43 (>90%) had L at residue 260 of the glycoprotein gene and only 4 isolates had the parental Armstrong sequence (F at residue 260). The presence of this F→L mutation in the spleen isolates was further confirmed for several of these isolates by directly sequencing the viral RNA in this region by the primer extension method. Strikingly, the brain isolates showed the reverse pattern; the majority (48 of 59, or 81%) of the isolates still retained the original Armstrong glycoprotein sequence (Fig. 2 and Table 2). The data in Table 2 also show that the parental Armstrong strain that was used to initiate the chronic infection was reasonably homogeneous and that all 20 clones analyzed had F at residue 260 of the viral glycoprotein. These results clearly document the importance of this F→L mutation in the organ-specific selection of viral variants during persistent LCMV infection.

TABLE 2. Organ-specific selection of LCMV variants with leucine at residue 260 of viral glycoprotein during chronic infection[a]

Origin of LCMV isolates	Total no. analyzed	No. with phenylalanine at residue 260 of glycoprotein	No. with leucine at residue 260 of glycoprotein
Parental Armstrong	20	20	0
Carrier brain	59	48	11
Carrier spleen	47	4	43

[a] LCMV isolates were obtained from the brains and spleens of BALB/c carrier mice infected at birth with the parental Armstrong strain. The viruses analyzed were isolated from 1- to five-month-old LCMV carrier mice. Fifteen carrier mice were used in this analysis, and therefore many of the LCMV isolates were truly independently derived (i.e., isolated from different carrier mice). The CNS and lymphoid isolates of LCMV were plaque purified three times on Vero cells, and then stocks were grown in BHK-21 cells. The sequence at amino acid residue 260 of viral glycoprotein was determined by the oligonucleotide hybridization technique shown in Fig. 2 and further confirmed for some isolates by direct sequencing of the viral RNA by the primer extension method.

We next determined whether acquisition of this F→L change in the viral glycoprotein correlated with the ability to persist in adult mice and suppress T-cell responses. As shown in Fig. 3, the vast majority (24 of 29) of LCMV isolates with L at residue 260 were present at high levels (>10^4 PFU per spleen) in the spleens of adult mice at 8 days postinfection, and these mice contained low levels of LCMV-specific CTL. In contrast, most (15 of 17) of the isolates with F were similar to the parental Armstrong strain, and adult mice infected with these isolates contained low levels of virus (<10^2 PFU per spleen) and exhibited a potent LCMV-specific CTL response. By day 15 postinfection, all 17 isolates containing F had been eliminated from adult mice, whereas 24 of 29 variants with L were present at high levels (>10^4 PFU) in the serum and spleen (data not shown). These mice continued to harbor virus for several months. These results clearly establish a strong correlation between the F→L amino acid change in the glycoprotein and the persistent and immunosuppressive phenotype of the spleen isolates. Figure 3 also shows the strong correlation that exists between LCMV-specific CTL response and viral clearance.

The data presented in Fig. 3 provide strong evidence linking the F→L change with persistence in adult mice. However, the correlation was not absolute, and five of the 29 isolates (~17%) tested had not acquired the persistent phenotype despite having the F→L mutation. These results are not totally unexpected since we have shown (Table 1) that the complete spleen variant phenotype is due to mutations in both the S and L viral RNA segments (4, 14). Thus, it is likely that the five isolates shown in Fig. 3 represent an intermediate stage in their transition from the parental virus to the spleen isolate phenotype; these isolates have acquired the F→L change in the viral glycoprotein, but the critical mutation(s) in the L segment is yet to occur. A prediction based on the results shown in Table 1 and Fig. 3 is that all F→L variants derived from a reassortant (svA/A) that contains the L segment of spleen variant clone 13 and the S segment of parental Armstrong should behave exactly like clone 13. In other words, isolates derived from carrier mice infected with the reassortant svA/A should show a perfect correlation between the F→L mutation and the persistent and immunosuppressive phenotype, since the biologically relevant change in the L segment is already present in the parental virus. This was tested by obtaining isolates from BALB/c mice infected at birth with the reassortant svA/A. As shown in Table 3, there was a complete correlation between the F→L mutation and acquisition of the persistent and immunosuppressive phenotype.

To determine the extent of genetic drift in variants isolated from carrier mice, we sequenced the complete S segment of several independently derived spleen isolates (i.e., isolated from different carrier mice). As summarized in Table 4, these

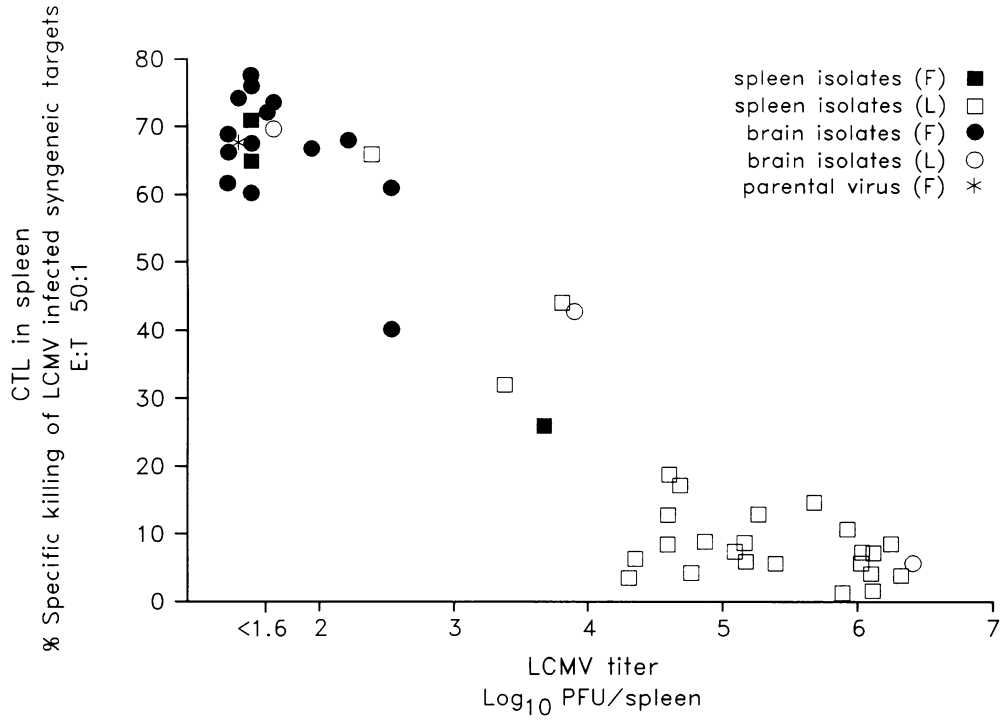

FIG. 3. Correlation between F→L mutation in the glycoprotein and the ability of LCMV variants to persist in adult mice. Two-month-old BALB/c mice were infected intravenously with 10^6 PFU of the indicated LCMV brain or spleen isolate containing either F or L at residue 260 of viral glycoprotein. The CTL activity in the spleens of mice infected with a particular viral isolate is plotted against the titer of infectious LCMV in the spleens of the same group of mice. Data are averages for three to four mice per group. Virus titer and LCMV-specific CTL activity in spleen were checked eight days after infection, which is the peak of the primary CTL response.

spleen isolates exhibited a surprisingly high level of conservation and showed >99.8% overall identity at the nucleotide level with the S segment of the parental Armstrong virus. In fact, the only common change among these five spleen isolates was the F→L mutation at residue 260 of the viral glycoprotein. Sequence analysis of the L segments of these variants also indicated a similar level of conservation; no mutations have been detected in the 2,500 nucleotides of the polymerase gene that have been sequenced so far (data not shown). These results show that LCMV undergoes minimal genetic drift during chronic infection in vivo and that a single or few mutations can result in the organ-specific selection and emergence of variants with profoundly altered biological properties.

TABLE 3. Biological characterization of spleen isolates derived from reassortant svA/A[a]

Virus	Amino acid at residue 260 of glycoprotein	% Specific ^{51}Cr release from BALB Cl7 (*H-2*^{d}) targets (E/T, 50:1)		LCMV titer in serum (log_{10} PFU/ml)	
		LCMV infected	Uninfected	8[b]	15
Parental svA/A	F	57	0	3.5	<1.6
Spleen isolate					
24	L	7	1	5.2	5.3
26	L	2	0	5.7	5.5
28	L	6	0	5.7	5.4
33	L	4	0	5.9	5.6
36	L	9	0	5.7	5.6
32	F	59	1	3.6	<1.6

[a] Adult BALB/c mice were infected intravenously with 10^6 PFU of the indicated virus. CTL response and virus titer were checked 8 days after infection. Data are averages for two to four mice per group. The spleen isolates used in this study were obtained from 15-day-old BALB/c mice infected at birth with the reassortant svA/A.

[b] Days postinfection.

TABLE 4. Minimal genetic drift in LCMV variants selected in spleens of carrier mice

Spleen isolate[a]	No. of nucleotide changes[b]		% Identity with parental Armstrong virus	
	Nucleoprotein gene (1,677 bases)	Glycoprotein gene (1,497 bases)	Nucleotide level	Amino acid level
M10.1A	1 (1)	4 (2)	99.84	99.71
M2.1A	1 (1)	5 (3)	99.81	99.62
T5.10A	1 (1)	4 (3)	99.84	99.62
t1b	1 (1)	3 (3)	99.87	99.62
Clone 13	0	2 (1)	99.93	99.90

[a] Obtained from three BALB/c carrier mice. Clone 13 was isolated from the spleen of a 2-month-old carrier mouse infected at birth with the parental Armstrong virus, isolate M10.1A was obtained from a 3-month-old congenitally infected carrier mouse (third generation), and isolates M2.1A, T5.10A, and t1b were obtained from spleens of different congenitally infected carriers. The congenital carrier colony was originally started by infecting 1-day-old BALB/c mice with Armstrong virus. All spleen isolates were triple plaque purified prior to use.

[b] Sequencing of the spleen isolates and Armstrong virus was done by the primer extension method using specific oligonucleotides. Numbers in parentheses represent number of amino acid changes.

DISCUSSION

This study provides a molecular basis for the organ-specific selection of LCMV variants during chronic infection. Our results identify a single amino acid change in the viral glycoprotein that is found in viral isolates obtained from lymphoid tissue but is absent in viral isolates derived from CNS of the same mice. This F→L change is seen in >95% of the lymphoid isolates, and mutants with L at position 260 of the viral glycoprotein are selected in spleens of persistently infected mice, whereas LCMV isolates with the parental sequence (F at residue 260) predominate in the CNS.

It is of particular interest that the lymphoid isolates exhibit minimal genetic drift. Complete sequence analysis of the major structural genes of several independently derived spleen isolates shows that these variants are >99.8% identical to the parental virus. In fact, the only common change among these spleen isolates is the F→L mutation at residue 260 of the glycoprotein. These results show that, contrary to dogma, chronic infection with an RNA virus does not necessarily result in extensive genetic variation (12, 24). However, one or few mutations are sufficient to result in the organ-specific selection of variants that show major biological differences compared with the parental virus. These results emphasize the importance of selection and the role of host tissues in the emergence of viral variants in vivo.

The significance of this F→L change was confirmed by making reassortants between a spleen isolate, clone 13, and the parental Armstrong virus. Reassortant svA/A (L segment of clone 13 and S segment of parental Armstrong) is unable to persist in adult mice and is eliminated within 2 to 3 weeks. In contrast, clone 13 (svA/svA), which differs from svA/A by only this single F→L change in the glycoprotein, persists in adult mice for several months. It is worth noting that in an earlier study in which reassortants between clone 13 and the Pasteur strain of LCMV were tested, the reassortant svA/P (L segment of clone 13 and S segment of the Pasteur strain) was almost as persistent as clone 13 in adult mice. In retrospect, this result is not surprising since the Pasteur strain, similar to the spleen variants of Armstrong, has L at residue 260 of the glycoprotein (19). Thus, the different phenotypes of svA/A and svA/P are most likely due to the sequence difference at position 260 of the glycoprotein (L for Pasteur and F for Armstrong).

The spleen isolates and the parental Armstrong virus grow equally well in mouse fibroblasts, but the spleen isolates show enhanced replication in macrophages and lymphocytes (13). This selective growth advantage in cells of the immune system explains the emergence of these variants in spleens of carrier mice. In contrast to the selection of variants containing the F→L mutation in the spleen, the parental genotype predominates in the CNS. Previous studies have shown that although a variety of cell types in the CNS can be infected by LCMV, infection is confined predominantly to neurons in neonatally infected carrier mice (10, 17). Thus, growth potential in neurons is likely to be an important determinant in the selection of viral variants in the CNS. Experiments are currently in progress to compare the ability of the various isolates to replicate in neurons in vitro as well as in vivo. The results presented in this report strongly suggest that the parental virus grows at least as well as, if not better than, the variants (with the F→L mutation) in the CNS. In this context, it is worth noting that the parental Armstrong virus was originally maintained by brain→brain passage in mice before a clone was obtained by plaque purification (9). Thus, the parental Armstrong virus most likely represents a brain-adapted strain of LCMV.

The F at residue 260 of the parental Armstrong virus is encoded by a UUC codon. This can change to a codon specifying L by three different single-base substitutions; UUC→CUC, UUC→UUG, or UUC→UUA. In the LCMV variants that we analyzed, this F→L change always occurred by a U→C mutation. This is somewhat surprising, but it should be noted that of the three possibilities, two are transversions (C→G and C→A) whereas the U→C change is a transition. Since transitions occur more readily than transversions, this may explain our findings (25). Also, studies on genetic variation in measles virus during chronic infection in vivo have shown a high frequency of U→C mutations in the measles genome (7).

How does the F→L mutation in the glycoprotein have such a profound effect on the selection of variants in lymphoid tissue? It is worth noting that the F→L mutation is close to the putative cleavage site of the viral glycoprotein (6). Proper proteolytic cleavage is essential for infectivity, and alterations in the processing or stability of the viral glycoprotein are likely to affect the growth rate of the virus. It is currently not known whether cleavage of the LCMV glycoprotein is mediated by a viral or cellular protease(s). If a cellular protease(s) is involved, it might be possible to explain our findings on the basis of different proteases being present in different cell types (for example, macrophages versus neurons). Clearly, additional experiments are needed to resolve this issue. Nevertheless, it is clear from our studies that this single amino acid change determines the selection of variants in an organ-specific manner.

The F→L mutation also correlates with the persistent and immunosuppressive phenotype of the LCMV variants. The enhanced growth capability of these variants in lymphocytes and macrophages is a likely explanation for the ability of the spleen variants to establish chronic infections in adult mice (13). The observed immune suppression may also be due to increased growth in lymphoid tissue and greater dissemination of the virus. The F→L change in the glycoprotein is not within any of the CTL epitopes, and therefore it is unlikely that the low level of LCMV-specific CTL response detectable in mice infected with the spleen isolates is due to altered recognition of T-cell epitopes (16, 21). In fact, the spleen isolates are recognized both in vitro and in vivo by LCMV-specific CTL induced by the parental Armstrong virus. H-2-matched targets infected by the variants are efficiently killed by LCMV-specific CTL in vitro, and transfer of these CTL into mice infected with the variants results in rapid elimination of virus in vivo (1, 4).

It will be of interest to identify the mutations in the L segment that affect viral persistence and the tissue specific selection. About 2,500 nucleotides of the polymerase gene of several spleen isolates have been sequenced so far, and no changes have been detected. This result further attests to the extreme conservation of the LCMV genome during chronic infection.

In conclusion, this study shows that a virus can exhibit minimal genetic drift during chronic infection in its natural host and yet a single or few mutations can result in the organ-specific selection of variants that are markedly different from the parental virus. These results document the role of host tissues in virus evolution and emphasize the importance of selection in the emergence of viral variants in vivo. These observations suggest a possible mechanism by which viral variants emerge in nature and provide a frame-

work for understanding the evolution of other viruses, in particular viruses that cause chronic infections (15).

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