

Genetic Basis of Viral Persistence: Single Amino Acid Change in the Viral Glycoprotein Affects Ability of Lymphocytic Choriomeningitis Virus to Persist in Adult Mice

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Summary

This study has identified a single amino acid change in the viral glycoprotein that profoundly affects the ability of lymphocytic choriomeningitis virus (LCMV) to persist in its natural host. Adult immunocompetent mice infected with a variant of the Armstrong strain, spleen isolate clone 13 (svA/svA), harbor virus for several months and exhibit suppressed T cell responses. In contrast, adult mice infected with a reassortant virus (svA/wtA) that contains the L segment of the spleen variant and the S segment of the parental wt Armstrong, make potent LCMV-specific CTL responses and clear the infection within 2–4 wk. These two viruses, spleen variant clone 13 and the reassortant svA/wtA, are identical in their noncoding regions and show no amino acid changes in any of their viral genes except for one substitution in the glycoprotein. The reassortant virus svA/wtA has a phenylalanine at amino acid residue 260 of the glycoprotein, whereas the spleen variant clone 13 has a leucine at this position. This study constitutes one of the first reports defining the genetic basis of viral persistence at the whole animal level, and identifying a single mutation that markedly increases the ability of a virus to persist in its natural host.

Successful resolution of a viral infection depends upon a critical balance between the extent of viral spread and replication, and the magnitude of the host's immune response. We have been studying infection of mice with lymphocytic choriomeningitis virus (LCMV)¹ as a model system to understand the host and viral determinants that lead to viral clearance or persistence (1–4). Infection of immunocompetent adult mice with the Armstrong strain of LCMV induces a potent antiviral T cell response and virus is eliminated within 2 wk. This clearance is mediated by CD8⁺ virus-specific cytotoxic T lymphocytes (4–7). In contrast, infection of adult mice with a naturally selected isolate of Armstrong, spleen variant clone 13, results in a disseminated infection, with virus persisting for several months (1, 2). This chronic infection is associated with suppressed T cell responses and susceptibility to opportunistic infection (1, 2, 8).

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 (9–12). The L RNA segment codes for a large protein, L (molecular mass 250 kD), that is be-

lieved to be the viral polymerase, and also contains a second open reading frame, designated Z, that encodes for a protein of ~10–12 kD. The S segment codes for the three major structural proteins: the internal nucleocapsid protein (NP; 63 kD) and the two surface glycoproteins GP-1 (43 kD) and GP-2 (36 kD) that are derived from a common precursor polypeptide, GP-C. After coinfection of cells with two different LCMV strains, recombinants are generated by reassortment of genome segments. This permits genetic analysis of LCMV pathogenicity (9, 13).

In this study, we have made reassortants between the parental Armstrong strain and the spleen variant clone 13 and examined their biological properties. Using this genetic approach in combination with complete sequence analysis of the S segment, we show that a single amino acid change in the viral glycoprotein profoundly affects the ability of LCMV to persist in adult mice.

Materials and Methods

Mice 4–6-wk-old BALB/cByJ mice purchased from The Jackson Laboratory (Bar Harbor, ME) were used in all experiments.

Virus The origins of the Armstrong CA1371, Pasteur CIPV 76001, and the Traub strains of LCMV used in this study have been

¹ *Abbreviations used in this paper:* L, large; LCMV, lymphocytic choriomeningitis virus; S, small; sv, spleen variants; wt, wild type.

previously described (1, 2). The variant, clone 13, was isolated from the spleen of an 8-wk-old BALB/c LCMV carrier mouse infected at birth with strain Armstrong CA1371. The laboratory virus stocks of the Armstrong, Pasteur and Traub strains of LCMV are referred to as wild type (wt). All LCMV stocks used in this study (wt, spleen variant clone 13, and the reassortants) were triple plaque purified on Vero cells, and then stocks were grown in BHK-21 cells. Virus stocks at the passage 1 or 2 level were used in all experiments.

Generation of Reassortants. Coinfection of BHK-21 cells and the procedures used to screen the progeny for reassortants were as previously described (2).

Determination of Virus Titers. Infectious LCMV was quantitated by plaque assay on Vero cell monolayers as previously described (1).

CTL Assay. LCMV-specific CTL activities in spleens and lymph nodes were determined by a 6-h ⁵¹Cr-release assay as previously described (1).

Sequence Analysis. The complete S segment of the parental Armstrong strain and spleen variant clone 13 was sequenced by the primer extension method using virus specific oligonucleotides (14). Either 1–3 µg of viral RNA extracted from purified virus or 50 µg of total infected cell RNA was used for the sequencing reactions.

Results

Generation of Reassortants between wt Armstrong and Armstrong Spleen Variant Clone 13. The Armstrong spleen variants (sv) cannot be distinguished from the parental wild type (wt) Armstrong virus either by reactivity to a panel of mAbs or on the basis of hybridization with wt Armstrong-specific cDNA probes (2; and our unpublished data). However, the Armstrong-specific cDNA probes can differentiate between Armstrong and other LCMV strains such as the Pasteur strain

and the Traub strain (2, 15). Therefore, the strategy outlined in Table 1 was used to make reassortants between the parental wt Armstrong (wtA/wtA; this notation indicates L segment of wt Armstrong and S segment of wt Armstrong) and the Armstrong spleen variant clone 13 (svA/svA). Briefly, reassortants were first made between Armstrong clone 13 and Pasteur, and wt Armstrong and Traub, and then these reassortants were crossed to obtain the desired virus. Table 1 describes the cross that was done to obtain the reassortant svA/wtA (L segment of sv Armstrong and S segment of wt Armstrong), and the data showing the expected hybridization patterns are shown in Fig. 1.

Sequence Comparison between Spleen Isolate Clone 13 (svA/svA) and the Reassortant svA/wtA. The spleen variant clone 13 (svA/svA) and the reassortant svA/wtA share a common L segment (derived from clone 13) but contain different S segments. To determine the precise location of the genetic differences between these two viruses their S segments were sequenced by the primer extension technique as described in Materials and Methods. Sequence analysis of the S segments of the two viruses revealed only two nucleotide differences between the parental wt Armstrong and its spleen variant clone 13. There were no changes in the nucleoprotein gene, and only two changes (at nucleotide positions 855 and 1298) in the glycoprotein (GP-C) gene. Both of these were U → C changes; the one at position 855 resulted in a phenylalanine → leucine change, whereas the one at residue 1298 was a silent one (see Figure 2). These findings are in agreement with the results of Salvato et al. who had previously sequenced the complete S segment of clone 13 (16). Thus, our results show that the spleen variant clone 13 (svA/svA) and the reas-

Table 1. Strategy Used for Making Reassortant between Parental Wild Type Armstrong (wtA) and Armstrong Spleen Variant Clone 13 (svA)

Cross*	Progeny clones†	Reactivity with Armstrong-specific cDNA probes‡	
		L specific	S specific
svA/wtP	svA/wtP	+	-
x	wtT/wtA	-	+
wtT/wtA	wtT/wtP	-	-
	svA/wtA	+	+

* The cross was done between two reassortants svA/wtP and wtT/wtA. The following notation is used to indicate the genotype: svA/wtP indicates a reassortant with L segment of spleen variant Armstrong and S segment of wt Pasteur; and wtT/wtA indicates a reassortant with L segment of wt Traub and S segment of wt Armstrong.

† The genotypes of the four possible progeny clones (the two parents and the two reassortants) can be identified by their characteristic reactivity pattern with Armstrong-specific L and S cDNA probes. Thus, the desired reassortant, svA/wtA, containing the L segment of spleen variant Armstrong and S segment of wt Armstrong can be easily identified.

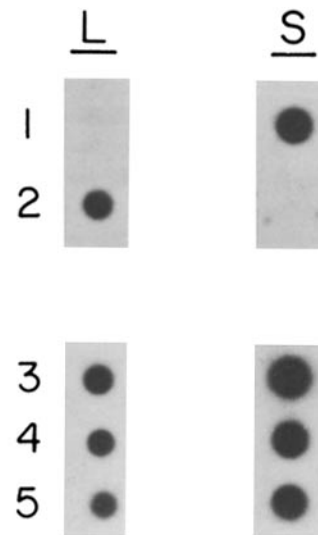


Figure 1. Identification of reassortant containing L segment of Armstrong spleen variant clone 13 and S segment of parental wt Armstrong (svA/wtA genotype). Refer to Table 1 for details of the cross done to obtain the svA/wtA reassortant. Total RNA was extracted from infected BHK-21 cells by the guanidinium thiocyanate–CsCl procedure. The RNA samples were denatured with formaldehyde, and 5.0 µg of RNA dotted onto nitrocellulose paper with a 96-hole Bio-Dot apparatus. The filter was hybridized with ³²P-labeled cDNA probes specific for the L and S segments of the wt Armstrong strain. (Lane 1) Reassortant wtT/wtA showing no hybridization with the L probe but positive hybridization with the S probe; (lane 2) reassortant svA/wtP showing positive signal with the L probe but no hybridization with the S probe; (lanes 3–5) three progeny clones with the svA/wtA genotype showing positive hybridization with both L and S probes.

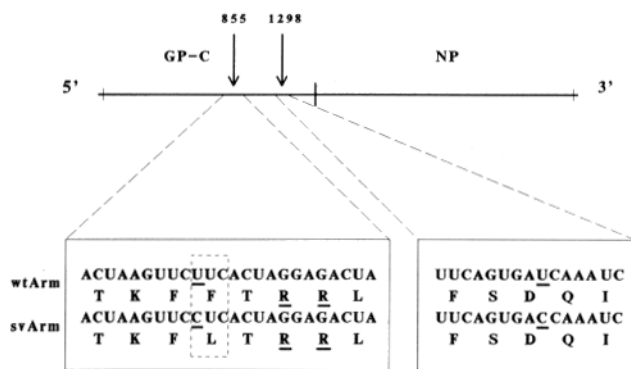


Figure 2. Identification of sequence differences between the S segments of parental wt Armstrong (wtArm) and spleen variant clone 13 (svArm). The phenylalanine to leucine change seen in svArm is close to the putative proteolytic cleavage site (RR) of the viral glycoprotein (19).

sortant (svA/wtA) are identical in all genes except for a single amino acid change in the glycoprotein. There were also no nucleotide differences between these two viruses (clone 13 and svA/wtA) in the noncoding regions.

Biological Studies with the Reassortants. The spleen isolate clone 13 and the reassortant svA/wtA were tested for their ability to induce LCMV-specific CTL responses and to cause persistent infections in adult BALB/c mice. The parental wt Armstrong virus was also included in these experiments. As shown in Table 2 spleen isolate clone 13 caused a persistent infection in adult mice and high levels of virus were present in several tissues tested. In contrast, the reassortant svA/wtA, which differs from clone 13 by only a single amino acid in the glycoprotein, was eliminated from most tissues by day 15 and from all organs tested by day 30 post-infection. Also, the amount of svA/wtA present in tissues and serum of mice 8 d post-infection was 100–1,000-fold lower than the level of clone 13. These results clearly show the biological importance of the Phe → Leu change in the viral glycoprotein. This conclusion is further strengthened by the LCMV-specific CTL

Table 3. LCMV-specific CTL Response of Adult mice

Virus genotype (L/S segment)	Mouse No.	Percent specific ⁵¹ Cr release from BALB Cl7 (H-2 ^d) targets at E/T ratios of:			
		LCMV infected			Uninfected
		5.5:1	16.6:1	50:1	50:1
wtA/wtA	1	33	58	83	3
	2	40	70	84	11
	3	27	50	81	5
svA/svA	1	0	0	4	4
	2	2	6	20	1
	3	0	3	11	0
svA/wtA	1	14	49	62	0
	2	16	47	64	0
	3	10	40	59	2

Adult BALB/c mice were infected intravenously with 10⁶ PFU of the indicated virus and CTL response in the spleen checked at 8 d post-infection.

data shown in Table 3. Adult mice infected with clone 13 contained low levels of LCMV-specific CTL in the spleen, whereas mice infected with svA/wtA contained high levels of antiviral CTL activity.

Our previous studies have shown that genetic changes in the L segment contribute to the clone 13 phenotype (2). The data shown in Tables 2 and 3 also suggest this since the reassortant svA/wtA was not exactly like the parental wt Armstrong virus (wtA/wtA). The level of svA/wtA virus at 8 d post-infection was 10–100-fold higher than that of wtA/wtA. Also, the CTL response induced by wtA/wtA was slightly

Table 2. Viral Persistence in Adult Mice

Virus genotype (L/S segment)	LCMV titer (log ₁₀ PFU/organ or ml)*														
	Day 8 post-infection					Day 15 post-infection					Day 30 post-infection				
	Serum	Spleen	Lung	Liver	Brain	Serum	Spleen	Lung	Liver	Brain	Serum	Spleen	Lung	Liver	Brain
wtA/wtA	<1.6	<2.6	2.8	<2.6	<2.6	<1.6	<2.6	<2.6	<2.6	<2.6	<1.6	<2.6	<2.6	<2.6	<2.6
svA/svA	5.9	6.4	7.1	7.5	5.7	5.7	5.8	6.4	6.3	6.2	4.4	4.7	6.0	5.8	6.0
svA/wtA	3.5	4.1	4.0	4.9	3.9	<1.6	<2.6	2.6	<2.6	3.0	<1.6	<2.6	<2.6	<2.6	<2.6

Adult BALB/c mice were infected intravenously with 10⁶ PFU of the indicated virus.

* Mice were killed and the virus titer in the various organs and serum was determined by a plaque assay on Vero cells. The data shown are the average of four to six mice per group at each time point.

more (~2-fold higher in lytic units/spleen) than that seen in svA/wtA-infected mice. To further confirm these observations we made the reciprocal reassortant wtA/svA and tested its biological properties. As shown in Table 4, the reassortant wtA/svA was unable to cause a chronic infection in adult mice and induced a potent LCMV-specific CTL response. These results confirm our earlier findings and show unequivocally that biologically relevant mutation(s) have also occurred in the L segment of spleen variant clone 13 (2). The data in Table 4 also re-emphasize the significance of the Phe → Leu mutation in the S segment; the reassortant wtA/svA was present at higher levels (>20-fold) in the serum at day 8 compared with the wt Armstrong, and the CTL response in wtA/svA infected mice was ~2-fold lower (in lytic units/spleen) than the response of wtA/wtA infected mice.

Discussion

The main finding of this study is the identification of a single amino acid change in the viral glycoprotein that profoundly affects the ability of LCMV to persist in adult mice. We have compared the biological properties of two viruses, LCMV spleen isolate clone 13 (svA/svA) and the reassortant svA/wtA, that are identical in their noncoding regions and show no amino acid changes in any of their viral genes except for one substitution in the glycoprotein. Our results show that mice infected with clone 13 (leucine at amino acid residue 260 of glycoprotein) contained low levels of LCMV-specific CTL in the spleen and were unable to clear the virus, whereas mice infected with the reassortant svA/wtA (phenylalanine at residue 260) exhibited high levels of LCMV-specific CTL and the infection was cleared within 2–4 wk.

The spleen isolate clone 13 also contains a second change in the glycoprotein gene at nucleotide residue 1298 (see Fig. 2). Although the contribution of this mutation to the clone 13 phenotype cannot be completely ruled out, it is highly

unlikely since this nucleotide change is a silent one that does not alter the amino acid sequence. Moreover, our preliminary results show that this silent U → C change at residue 1298 is not found in other spleen isolates that are biologically similar to clone 13. In contrast, the U → C change at position 855 that results in the Phe → Leu mutation is present in all LCMV spleen isolates that show the clone 13 phenotype (Ahmed, R., et al., unpublished data).

The spleen variant clone 13 was isolated from mice originally infected with the parental wt Armstrong virus (1). Thus, the results of this study provide unequivocal proof that the naturally selected Phe → Leu mutation is biologically relevant. We have preliminary data suggesting that this Phe → Leu mutation allows enhanced growth within lymphoid tissue (Matloubian, M., and R. Ahmed, unpublished data). This enhanced growth capability in cells of the immune system is a likely explanation for the ability of the spleen variant to establish a chronic infection in adult mice. The observed Phe → Leu change is not within any of the CTL epitopes that have been mapped (17, 18); therefore, it is unlikely that the low level of LCMV-specific CTL response detectable in clone 13 infected mice is due to altered recognition of CTL epitopes. It is more likely that this low CTL phenotype is the result of increased growth in lymphoid tissue and greater dissemination of the virus. In this context, it is worth noting that the Phe → Leu mutation is close to the putative cleavage site of the viral glycoprotein (Fig. 2; reference 19). Proper proteolytic cleavage is essential for infectivity, and alterations in the processing of the viral glycoprotein are likely to affect the growth rate of the virus. Experiments are currently in progress to determine if this Phe → Leu change affects either the site or rate of cleavage of the viral glycoprotein.

The results of this study also confirm our earlier findings that biologically relevant mutation(s) occurred on the L segment during the wt Armstrong to clone 13 transition (2). Thus, the complete clone 13 phenotype is due to the Phe

Table 4. Biological Characterization of Reassortant wtA/svA

Virus genotype* (L/S segment)	LCMV-specific CTL in spleen†				LCMV titer in serum (log ₁₀ PFU/ml) [‡]		
	Percent specific ⁵¹ Cr release from BALB C17 (H-2 ^d) targets at E/T ratio of:				Days post-infection		
	LCMV infected			Uninfected			
	5.5:1	16.6:1	50:1	50:1	8	15	30
wtA/wtA	31	57	85	4	<1.6	<1.6	<1.6
svA/svA	3	6	11	4	5.6	5.0	4.6
wtA/svA	23	43	67	2	3.1	<1.6	<1.6

* Adult BALB/c mice were infected intravenously with 10⁶ PFU of the indicated virus.

† CTL response was checked 8 d post-infection. The data shown are the average of three mice per group.

‡ Mice were eye-bled at the indicated times and titer of infectious LCMV in the serum was determined by a plaque assay on Vero cells. The data shown are the average of three to six mice per group.

→ Leu change in the glycoprotein and additional mutation(s) in the L segment. Studies are in progress to identify these changes in the L segment.

Viral determinants of pathogenicity have been identified in many different viruses, and in some instances changes in virulence have been linked to single amino acid or nucleotide

substitutions (20). However, there have been very few studies on the genetic basis of viral persistence at the whole animal level. Our study constitutes one of the first reports identifying a single amino acid change that affects the ability of the virus to persist in its natural host.

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