Genetic Analysis of In Vivo-Selected Viral Variants Causing Chronic Infection: Importance of Mutation in the L RNA Segment of Lymphocytic Choriomeningitis Virus

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Viral variants with different biological properties are present in the central nervous systems (CNS) and lymphoid tissues of mice persistently infected with lymphocytic choriomeningitis virus (LCMV). Viral isolates from the CNS are similar to the original Armstrong LCMV strain and induce potent virus-specific T-cell responses in adult mice, and the infection is rapidly cleared. In contrast, LCMV isolates derived from spleens of carrier mice cause persistent infections in adult mice. This chronic infection is associated with low levels of antiviral T-cell responses. In this study, we genetically characterized two independently derived spleen variants by making recombinants (reassortants) between the spleen isolates and wild-type (wt) LCMV and showed that the ability to persist in adult mice and the associated suppression of T-cell responses segregates with the large (L) RNA segment. In addition, we analyzed a revertant (isolated from the CNS) derived from one of the spleen variants. By comparing the biological properties of three reassortants that contained the same S segment but had the L segment of either the original wt Armstrong LCMV, the spleen variant derived from it, or the CNS revertant derived from the spleen variant, we were able to show unequivocally that biologically relevant mutations occurred in the L segment not only during generation of the spleen variant from wt LCMV but also in reversion of the spleen variant to the wt phenotype. Thus, our results showed that (i) genetic alterations in the L genomic segment were involved in organ-specific selection of viral variants, and (ii) these mutations profoundly affected the ability of LCMV to cause chronic infections in adult mice.

The selective pressures involved in the emergence of viral variants and the genetic changes that occur in variants selected in vivo in their natural hosts have not been fully defined. We have recently documented the importance of host tissues in the selection of viral variants during chronic lymphocytic choriomeningitis virus (LCMV) infection (2, 3). Our studies have shown that LCMV undergoes mutation during chronic infection in its natural host and there is organ-specific selection of viral mutants. LCMV isolates with different biological properties are present in the central nervous systems and lymphoid tissues of persistently infected mice. LCMV clones isolated from brains of carrier mice behave like the original Armstrong LCMV strain and induce potent virus-specific cytotoxic T-lymphocyte (CTL) and delayed-type hypersensitivity (DTH) responses in adult mice, and the infection is rapidly cleared. In striking contrast, LCMV clones derived from spleens of carrier mice cause persistent infections in adult immunocompetent mice. This chronic infection is associated with suppressed virus-specific T-cell responses. A molecular analysis of these organ-specific variants will be of value not only in understanding the mechanisms of viral persistence and immunosuppression, but it will also provide insight into the genetic basis of the organ-specific selection.

The LCMV genome consists of two segments of single-stranded RNA—a large (L) segment of ~7 kilobases and a small (S) segment of 3.4 kilobases (4, 5, 19–21). The L RNA segment codes for a large protein, L (molecular weight [MW], ≥200,000), that is believed to be the viral transcrip-

tase-replicase. The S segment codes for the three major structural proteins: the internal nucleocapsid protein (NP; MW, 63,000) and the two surface glycoproteins (GPs) GP-1 (MW, 43,000) and GP-2 (MW, 36,000) that are derived from a common precursor polypeptide, GP-C. After coinfection of cells with two different LCMV strains, recombinants (reassortants) are generated by reassortment of genome segments. This permits genetic analysis of LCMV pathogenicity and allows one to determine whether the biological properties of a particular variant are due to mutations in the L or S segment (18).

As a first step towards analyzing the genetic differences between the organ-specific variants, we made reassortants between the variants and wild-type (wt) LCMV and tested the abilities of these reassortants to suppress T-cell responses and persist in adult mice. In this study, we analyzed two independently derived spleen variants and a brain revertant derived from one of the variants. Our results show that mutations in the L RNA segment of LCMV were involved in the organ-specific selection and that these genetic alterations affect the ability of LCMV to establish persistent infections in adult immunocompetent mice.

MATERIALS AND METHODS

Mice. Four- to six-week-old BALB/cByJ mice purchased from Jackson Laboratory, Bar Harbor, Maine, were used in all experiments.

Virus. The origins of the Armstrong CA1371 and Pasteur CIPV 76001 strains of LCMV used in this study have been previously described (2, 3, 5). The spleen variants (clones 13

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TABLE 1. CTL response and virus level in adult mice infected with the wt or spleen variants of LCMV[a]

Virus strain	% Specific ^{51}Cr release from LCMV-infected BALB Cl-7 (H-2^d) targets at an E/T ratio of:			LCMV titer ($\log_{10}$ PFU/sample of tissue or ml of serum) in:					
	50:1	16.6:1	5.5:1	Spleen	Serum	Liver	Lung	Kidney	Salivary gland
wt Armstrong	87	84	46	<2.4	2.0; <1.6	2.4; 2.3	2.6; <2.0	<2.0; <2.0	<2.0; <2.0
wt Pasteur	69	44	19	<2.4	2.0; <1.6	2.3; 2.4	3.7; 3.1	3.0; 2.6	3.3; <2.0
Spleen variant Armstrong 13	22	12	4	6.8	5.4; 5.8	7.3; 7.4	7.2; 7.0	7.0; 6.6	5.9; 5.4
Spleen variant Armstrong 12a	16	6	2	6.5	5.3; 5.4	7.1; 7.4	6.5; 6.9	6.4; 6.3	6.2; 5.6

[a] Adult BALB/c mice were infected i.v. with 2 × 10⁵ PFU of the indicated virus, and CTL responses and virus titers were checked at 8 days postinfection. Two mice were infected with each virus, and their spleens were pooled for the CTL assay and virus titer determination. The amounts of virus in other organs were determined for each mouse individually. E/T ratio, Effector/target ratio.

and 12a) were isolated from the spleens of 8-week-old BALB/c LCMV carrier mice infected at birth with strain Armstrong CA1371. Clones 13 and 12a are independently derived variants isolated from different mice. Clone 403a is a revertant of clone 13 and was isolated 172 days postinfection from the brain of a BALB/c mouse infected with clone 13 as an adult.

The laboratory virus stocks of the Pasteur and Armstrong strains of LCMV are referred to as wt. All LCMV stocks used in this study—wt, spleen isolates, and brain isolates— 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. It should be pointed out that the LCMV variants breed true in tissue culture; the biological properties of the various isolates are extremely stable, and we had no reversion of the phenotype during plaque purification and growth in tissue culture. This allowed us to do the genetics for mapping the biological differences seen in vivo.

Determination of virus titers. Infectious LCMV was quantitated by plaque assay on Vero cell monolayers as previously described (3).

Generation of reassortants. Genetic reassortants between two LCMV strains were made as previously described (17, 18). Subconfluent monolayers of BHK-21 cells were coinfected with the two parental LCMV strains at multiplicities of infection ranging between 1 and 3 PFU per cell. At 48 h postinfection, the supernatants were harvested, and progeny virus was obtained by plaque formation on Vero cells. All plaques were recloned on Vero cells, and individual stocks were made by one passage in BHK-21 cells. The genotype of the progeny was determined by Northern (RNA) hybridizations with cDNA probes specific for the L and S segments of the Armstrong strain as previously described (17, 18). Briefly, monolayers of BHK-21 cells (2 × 10⁶ cells per flask) were infected with the indicated virus at a multiplicity of infection of 0.2 PFU per cell, and total RNA was extracted from infected cells 40 to 44 h later. The cells were lysed with guanidinium thiocyanate, and total RNA was recovered after pelleting through cesium chloride (7). The RNA samples (50 μg each) were denatured with glyoxal, separated on the basis of size by electrophoresis in an agarose gel, transferred to a nitrocellulose filter, and hybridized with ³²P-labeled cDNA probes specific for both the L and S segments of LCMV.

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

DTH response. LCMV-specific DTH responses were monitored by increases in footpad thickness. Adult BALB/c mice were injected intravenously (i.v.) with 10⁶ PFU of LCMV

and at the same time in the right footpad with 10⁶ PFU of virus in a 0.03-ml volume. DTH response was monitored by measuring the increase in the thickness of the right footpad compared with that of the left footpad (injected with medium only).

Immunofluorescence. Frozen tissue sections (6 μm) were fixed stepwise in 95% ethanol-absolute ether (1:1 by volume) and 95% ethanol and then washed in phosphate-buffered saline. To check for viral antigen, the sections were stained with polyclonal anti-LCMV guinea pig serum (1:100 dilution), followed by fluorescein-conjugated rabbit anti-guinea pig immunoglobulin G (1:100 dilution; Organon Teknika, Malvern, Pa.). The polyclonal anti-LCMV guinea pig serum used recognizes all of the major structural proteins of LCMV and shows no reactivity with uninfected cells or tissues from uninfected mice.

RESULTS

Generation of reassortants between spleen variants and wt virus. Two spleen variants, clones 13 and 12a, derived from the wt Armstrong CA1371 strain were chosen for genetic analyses. Clones 13 and 12a are independently derived variants (different mice) isolated form the spleens of 2-month-old carrier mice infected at birth with the wt Armstrong strain (3). Each of the two Armstrong spleen variants was crossed with the wt Pasteur strain of LCMV. The Pasteur strain was chosen because (i) cDNA probes specific for the wt Armstrong L and S segments hybridize with the Armstrong spleen variants but not with the Pasteur strain and thus can be used to screen the progeny for reassortants, and (ii) adult mice infected with the wt Pasteur strain (similar to the wt Armstrong strain) generate vigorous CTL and DTH responses and clear the infection within 2 weeks (in contrast, the Armstrong spleen variants persist for >6 months). The amount of virus present in various tissues and the CTL response following infection of adult mice with the wt LCMV strains or the spleen variants are shown in Table 1.

The genotype of the progeny clones derived from the crosses between the two spleen variants and the wt Pasteur strain was determined by Northern analysis (Fig. 1). A summary of the genetic crosses is shown in Table 2. We were successful in obtaining the necessary reassortants (Fig. 1 and Table 2). There is no evidence of intramolecular recombination in LCMV, and the only known mechanism of genetic exchange is by reassortment of entire genome segments (4). RNA-RNA recombination has been shown to occur with certain single-stranded RNA viruses, such as foot-and-mouth disease virus (11), poliovirus (8), and mouse hepatitis virus (12). However, this is usually a rare event,
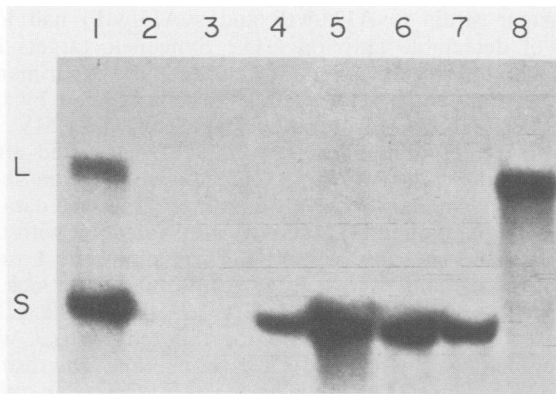
FIG. 1. Analysis of LCMV genotype by Northern hybridization with Armstrong strain-specific cDNA probes. Total RNA from infected and control BHK-21 cells were extracted by the guanidinium thiocyanate-CsCl procedure. The RNA samples (50 μg) were denatured with glyoxal, separated on the basis of size by electrophoresis in an agarose gel, and then transferred to a nitrocellulose filter. The filter was hybridized with ^{32}P-labeled cDNA probes specific for the L and S segments of the Armstrong strain. Lanes 1, spleen variant Armstrong clone 13 showing positive hybridization with both L- and S-specific probes (svA13/svA13 genotype); 2, uninfected BHK-21 cells; 3, wt Pasteur strain showing minimal or no hybridization with either probe (wtP/wtP genotype); 4 to 7, four reassortants showing no hybridization with the L probe but positive hybridization with the S probe (wtP/svA13 genotype). 8, reassortant showing positive hybridization with the L probe but minimal or no hybridization with the S probe (svA13/wtP genotype).

and intramolecular recombinants are detected only under strong selective conditions (e.g., crosses between temperature-sensitive mutants and selection for wt virus). The LCMV reassortants between the spleen variants and the Pasteur strain were derived from crosses with no selective pressures and were obtained at a high frequency (41%). Thus, it was highly unlikely that these reassortants would have also undergone intramolecular recombination. However, this was an important point and would have profoundly affected the interpretation of the biological studies. Therefore, the genotype of these reassortants was further confirmed by hybridization with additional cDNA probes spe-

TABLE 2. Summary of genetic crosses

Cross[a]	No. of clones with indicated genotype[b]			
	wtP/wtP	svA/svA	wtP/svA	svA/wtP
Spleen variant Armstrong 13 × wt Pasteur strain	3	4	4	1
Spleen variant Armstrong 12a × wt Pasteur strain	5	2	4	1

[a] BHK-21 cells were coinfected with the indicated LCMV strains, each at a multiplicity of infection of 3 PFU per cell. Culture fluids were harvested at 36 to 48 h postinfection, and titers were determined on Vero cell monolayers. For each cross, 12 progeny clones was checked. Progeny clones derived from the coinfections were double plaque purified on Vero cell monolayers, and passage 1 stocks were made in BHK-21 cells.

[b] The genotype of the progeny LCMV clones was determined by Northern transfer of viral RNA and hybridization with ^{32}P-labeled cDNA probes specific for the L and S segments of the Armstrong strain (Fig. 1). The following notation is used to designate the genotype: wtP/wtP, L and S segments of wt Pasteur strain; svA/svA, L and S segments of spleen variant Armstrong; wtP/svA, L segment of wt Pasteur strain-S segment of spleen variant Armstrong; svA/wtP, L segment of spleen variant Armstrong-S segment of wt Pasteur strain.

cific for the NP and the GP genes. The S segment of the spleen variants of strain Armstrong hybridized with both NP- and GP-specific cDNA probes of the wt Armstrong strain, whereas the S segment of the Pasteur strain did not hybridize with either probe. We checked the reactivity pattern of the S segment present in the reassortant viruses and found that it exhibited either the strain Armstrong or the strain Pasteur S segment pattern. No altered patterns, such as positive hybridization with the NP-specific probe and no hybridization with the GP-specific probe (or vice versa), indicating possible recombination within the S segment, were detected. Similar hybridization studies were performed with cDNA probes specific for the 3'- and 5'-proximal ends of the L segment. These studies also revealed no major crossovers within the L segment of the reassortants.

Biological studies with the reassortants. The genetically characterized reassortants (svA13/wtP, wtP/svA13, svA12a/wtP, and wtP/svA12a) and the three parental viruses (wtP/wtP, svA13/svA13, and svA12a/svA12a) were tested for their ability to induce LCMV-specific CTL and DTH responses and to cause persistent infections in adult BALB/c mice. The importance of these T-cell responses in clearing LCMV infections has been documented by us as well as by several other investigators (1, 6, 10, 14, 16, 22). Antiviral antibody and natural killer cells do not play a decisive role in elimination of LCMV infections. Therefore, in our studies we focused on virus-specific CTL and DTH responses.
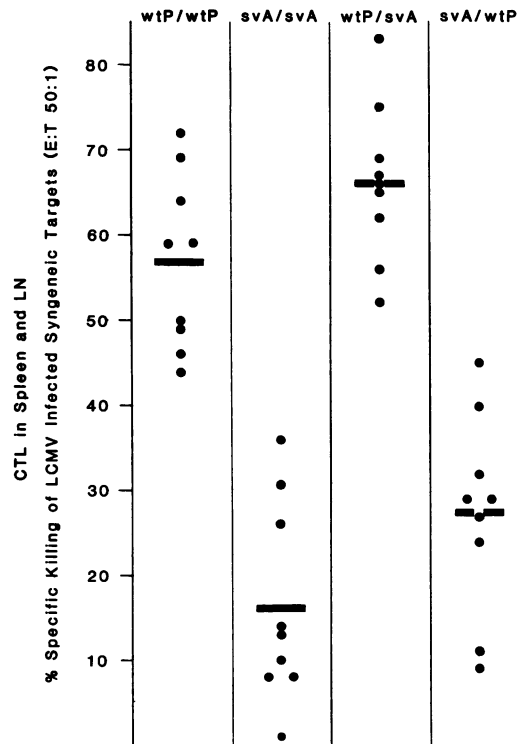

FIG. 2. LCMV-specific CTL response. Adult BALB/c mice were infected i.v. with 10⁶ PFU of the indicated LCMV strain or reassortant, and LCMV-specific CTL activity was determined at 8 days postinfection. The data presented are from nine separate experiments. In each experiment (represented by a dot), spleen and lymph node (LN) cells from 2 to 4 mice infected with the indicated virus were pooled and tested for CTL activity in a 6-h ^{51}Cr release assay. Between 18 and 36 mice were injected with each virus, and a horizontal bar denotes the average for each group. E:T, Effector-target ratio.

TABLE 3. Secondary (memory) LCMV-specific CTL response[a]

Virus genotype (L/S segment)	LU/10⁶ spleen and lymph node cells at		
	Day 58	Day 102	Day 158
wtP/wtP	19.5	27.0	26.5
svA/svA	1.6	1.4	2.9
wtP/svA	28.3	38.0	35.7
svA/wtP	5.6	6.2	7.0

[a] Adult BALB/cByJ mice infected with the indicated viruses were sacrificed at 58, 102, and 158 days postinfection. Mononuclear cells from spleens and lymph nodes (2 to 4 mice per group) were stimulated in vitro with the homologous virus for 5 days, and virus-specific CTL activity was determined by a ⁵¹Cr release assay. One lytic unit (LU) is defined as the number of effector cells required for 30% specific ⁵¹Cr release from LCMV-infected syngeneic targets.

(i) LCMV-specific CTL response. Adult (4- to 6-week-old) BALB/cByJ mice were injected i.v. with 10⁶ PFU of the reassortant or parental virus, and the primary CTL response was measured on days 3, 5, 8, and 11 postinfection. The level of CTL activity at day 8 (day of peak response) is shown in Fig. 2. Mice infected with the wt Pasteur strain generated a potent CTL response, whereas mice infected with the Armstrong spleen variants, clones 12a and 13, contained low levels of detectable LCMV-specific CTL in the spleen and lymph nodes. The reassortants containing the L segment of the wt Pasteur strain and the S segment of the spleen variant (wtP/svA12a and wtP/svA13) behaved like the wt Pasteur strain and induced high levels of LCMV-specific CTL. Five reassortants of this genotype (L segment of the wt and S segment of the spleen variant)—three between the wt Pasteur strain and svA13 and two between the wt Pasteur strain and svA12a—were tested, and all showed the same phenotype, i.e., high CTL response upon infection of adult mice. In contrast, mice infected with the reassortants containing the L segment of the spleen variant and the S segment of the
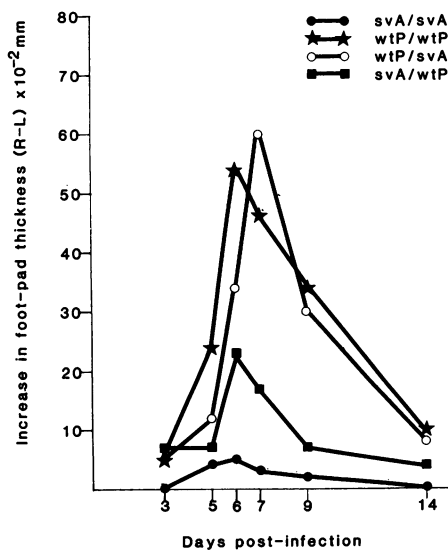
wt Pasteur strain (svA12a/wtP and svA13/wtP) had low levels of detectable antiviral CTL. Syngeneic targets infected with the spleen variants were used in the experiments shown in Fig. 2. This shows that targets infected by the variants are recognized by CTL induced by wt LCMV. In addition, we tested the reactivity of CTL generated after infection with svA/wtP on targets infected with this reassortant and found similar low levels of killing. Thus, the data in Fig. 2 showing limited CTL activity after infection with the spleen variants and the reassortants containing the L segment of the variants represent fewer CTL and not a lack of appropriate targets. The secondary LCMV-specific CTL response of mice infected with the different viruses was checked on days 58, 102, and 158 postinfection. The results are shown in Table 3. As in the primary CTL response, mice infected with the spleen variant (clone 13) or the reassortant containing the L segment of the spleen variant showed suppressed secondary CTL activity against LCMV.

(ii) LCMV-specific DTH response. Mice infected with the wt Pasteur strain and the reassortant containing the S segment of the spleen variant (wtP/svA13) exhibited strong virus-specific DTH responses, whereas mice infected with the spleen variant (clone 13) or the reassortant containing the L segment of the variant (svA13/wtP) had lower DTH responses (Fig. 3).

(iii) Virus levels in tissues. The amounts of infectious virus present in various organs (spleen, lymph nodes, lung, liver, kidney, brain, etc.) of mice infected with parental and reassortant viruses were determined by plaque assay on Vero cells, and viral antigen was checked by immunofluorescent staining of frozen tissue sections. Adult mice in-

FIG. 3. LCMV-specific DTH response. Adult BALB/c mice were injected i.v. (10⁶ PFU) and at the same time in the right footpad (10⁶ PFU) with the indicated LCMV strain or reassortant. DTH response was monitored by measuring the increase in the thickness of the right (R) footpad compared with that of the left (L) footpad (injected with medium only). Each point represents the mean value of five mice infected with the virus indicated.
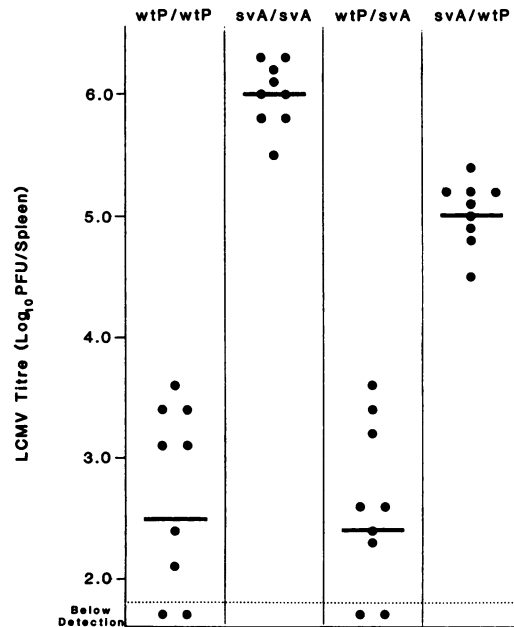

FIG. 4. Mice infected with reassortant containing the L segment of the spleen variant have high levels of virus in the spleen. Adult BALB/c mice were infected i.v. with 10⁶ PFU of the indicated LCMV strain or reassortant, and the virus titers in the spleens were determined at 8 days postinfection. The data presented are from nine separate experiments. In each experiment (represented by a dot), spleen cells from 2 to 4 mice infected with the indicated virus were pooled and the amount of virus was quantitated by a plaque assay on Vero cells. A horizontal bar indicates the average value for each group.
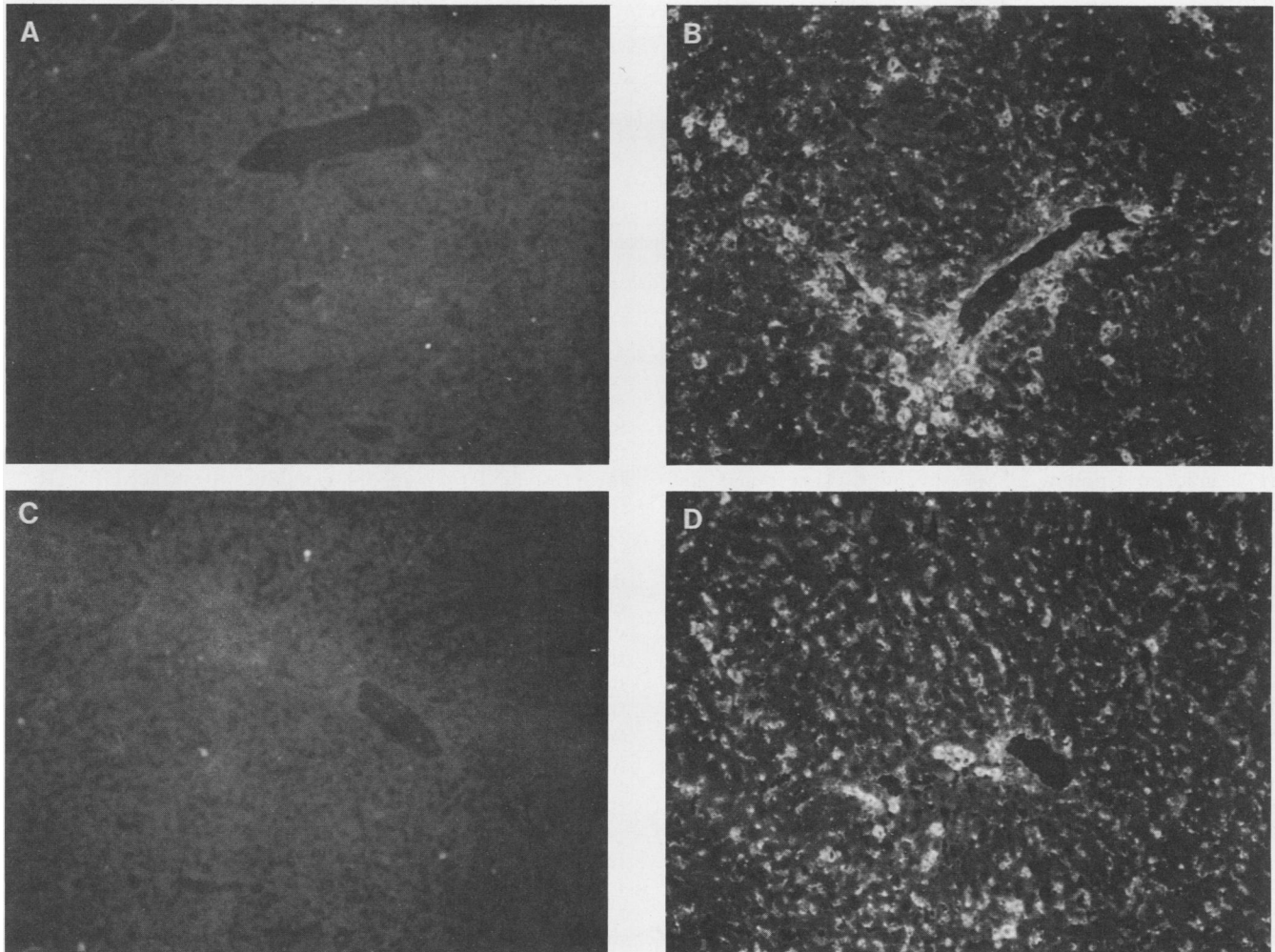
FIG. 5. Mice infected with reassortant containing the L segment of the spleen variant have high levels of viral antigen in the liver. Panels: A, wt Pasteur, wtP/wtP; B, spleen variant Armstrong clone 13, svA/svA; C, reassortant with S segment of clone 13, wtP/svA; D, reassortant with L segment of clone 13, svA/wtP. Liver samples were taken at 8 days postinfection. Magnification, ×73. The levels of infectious LCMV ($\log_{10}$ PFU/g) in the four liver samples shown were as follows: wtP/wtP, 2.6; svA/svA, 7.2; wtP/svA, <2.4; svA/wtP, 5.9.

fected with the wt Pasteur strain or the wtP/svA13 and wtP/svA12a reassortants eliminated the virus rapidly (within 2 weeks). In contrast, the spleen variants and the reassortants containing the L segment of the spleen variants (svA13/wtP and svA12a/wtP) persisted for several months. The titers of infectious virus in the spleens are shown in Fig. 4, and immunofluorescent staining of liver sections is shown in Fig. 5. Although the amounts of infectious virus in mice infected with the svA/wtP reassortants were lower than those seen upon infection with the spleen variants, these reassortants containing the L segment of the spleen variants persisted in the kidneys of adult mice for the same duration (>6 months) as the spleen variants.

Genetic analysis of a revertant derived from spleen variant clone 13. The LCMV spleen variants revert to the wt phenotype after growth in the central nervous system in vivo (2). One of these revertants (clone 403a) was isolated from the brain of a mouse infected 172 days earlier with clone 13. This clone 13 revertant is similar to the original wt Armstrong strain (Table 4). Adult mice infected with the revertant made a potent LCMV-specific CTL response and cleared the infection within 8 to 10 days.

To determine the genetic basis of reversion to the wt phenotype, reassortants were made between the revertant and the wt Pasteur strain. We were successful in obtaining the reassortant containing the L segment of the revertant and the S segment of the wt Pasteur strain. These data are shown in Fig. 6. The biological properties of the reassortant containing the L segment of the revertant and the S segment of the wt Pasteur strain (designated svrA/wtP) were compared with wtA/wtP and svA/wtP; these three reassortants have the same S segment (derived from the wt Pasteur strain) but contain the L segment of wtA, svA, or svrA. Thus, by using these reassortants one can directly compare the L segments of the original wt Armstrong strain, the spleen variant derived from it, and the revertant derived from the spleen variant (wtA → svA → svrA). Adult mice were infected with the three reassortants, and CTL responses and virus clearance were monitored (Fig. 7). Mice infected with the reassortant (wtA/wtP) containing the L segment of the wt Armstrong strain showed a good CTL response and contained no detectable or trace amounts of virus in their tissues at 8 days postinfection. Consistent with the results shown earlier (Fig. 2, 4, and 5), mice infected with the reassortant

TABLE 4. Biological characterization of revertant of spleen variant clone 13[a]

Virus strain[b]	% Specific ^{51}Cr release from BALB Cl-7 (H-2^d) targets (E/T ratio, 50:1)			LCMV titer ($\log_{10}$ PFU/g) of tissue or ml of serum			
	Uninfected	wt Armstrong infected	Clone 13 infected	Serum	Spleen	Liver	Lung
wt Armstrong	3	76	82	<1.6	2.4	<2.4	2.4
sv Armstrong 13	2	7	6	4.9	6.3	7.3	7.2
svr Armstrong 13	0	69	62	<1.6	2.4	<2.4	<2.4

[a] Adult BALB/c mice were infected i.v. with 2 × 10⁵ PFU of the indicated virus. CTL responses and virus titers were checked at 8 days postinfection. The data shown are averages of two mice per group.

[b] sv, Spleen variant; spleen variant revertant (svr) Armstrong 13, revertant (clone 403a) derived from clone 13.

containing the L segment of the spleen variant (svA/wtP) had low levels of CTL activity and contained high amounts of virus in the serum, spleen, and liver. The reassortant with the L segment of the revertant (svrA/wtP) behaved like the wt; it induced a potent LCMV-specific CTL response and was rapidly eliminated. These results show that a critical mutation(s) occurred on the L segment of clone 13, resulting in reversion to the wt phenotype.

DISCUSSION

The main findings of this study are (i) that a mutation(s) in the L RNA segment of LCMV has a profound effect on the ability of the virus to cause chronic infection in adult mice and (ii) that genetic alterations in the L segment were involved in the organ-specific selection of viral variants. We genetically characterized two independently derived spleen variants by making recombinants (reassortants) between the spleen isolates and wt LCMV and showed that the ability to persist in adult mice segregates with the L segment. In addition, we analyzed a revertant derived from one of the spleen variants and showed that a mutation(s) in the L segment was involved in reversion to the wt phenotype.

The LCMV genome is composed of two segments of single-stranded RNA. The L segment (~7 kilobases) codes for the viral polymerase (MW, ≥200,000), and the S segment (3.4 kilobases) codes for the three major structural proteins: the internal NP (MW, 63,000) and the two surface GPs, GP-1 (MW, 43,000) and GP-2 (MW, 36,000) (4, 5, 19, 20, 21). Our genetic studies mapping LCMV persistence in adult mice to the L segment suggest that mutations in the polymerase gene may be involved in allowing the variants to replicate more efficiently in lymphocytes or macrophages or both. This enhanced replication in cells of the lymphoid system may be the underlying cause of immunosuppression and chronic infection. It is worth noting that the reassortants containing the S segment of the variant and the L segment of wt LCMV were unable to establish a chronic infection and were cleared as efficiently as wt LCMV. This shows that the major structural proteins of the variant, the NP and the two surface GPs, by themselves have no effect on the ability of the virus to suppress T-cell responses and persist in adult mice. These results suggest that if the variants have an altered tropism it is not likely to be due to changes in the surface GPs of the virus affecting interaction with cellular receptors. Our findings also imply that tissue-specific selection (lymphoid ver-
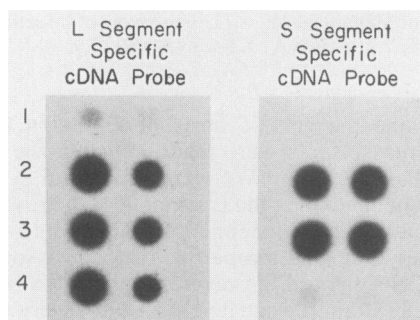

FIG. 6. Generation of reassortant between the wt Pasteur strain and clone 13 revertant 403a. Total RNA was extracted from infected BHK-21 cells by the guanidinium thiocyanate-CsCl procedure. The RNA samples were denatured with formaldehyde, and 5.0- and 1.6-μg of RNA samples were dotted onto nitrocellulose 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. Lanes: 1, wt Pasteur strain showing minimal or no hybridization with either probe (wtP/wtP genotype); 2, spleen variant clone 13 showing positive hybridization with both probes (svA/svA genotype); 3, revertant (403a) of clone 13 showing positive hybridization with both probes (svrA/svrA genotype); 4, reassortant between the wt Pasteur strain and revertant 403a showing positive hybridization with the L probe and minimal or no hybridization with the S probe (svrA/wtP genotype).
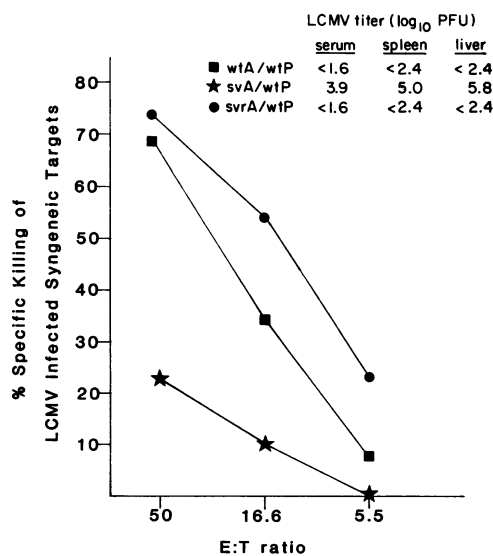

FIG. 7. Genetic mapping of clone 13 revertant: evidence for a biologically relevant mutation(s) on the L segment. Adult mice were infected i.v. with 10⁶ PFU of the indicated reassortant. LCMV-specific CTL activities (in spleens and lymph nodes (LN)) and virus titers were determined at 8 days postinfection. The data shown are averages of 2 to 4 mice per group. E:T ratio, Effector-target ratio.

sus central nervous system) of the variants does not operate at the level of virus adsorption-penetration (i.e., cellular receptors) but at some internal (intracellular) step. However, it should be pointed out that some contribution of the S segment cannot be completely ruled out, since the reassortants (svA/wtP) containing the L segment of the variant and the S segment of the wt were not completely like the parental variants (svA/svA). The peak titer of the svA/wtP reassortant was lower than the spleen variants, and clearance from most tissues except the kidneys was faster than that of the variant. Also, mice infected with the svA/wtP reassortant had higher CTL and DTH responses than did mice infected with the parental spleen variants. This may reflect a critical change in the S segment of the variant, or it may simply be due to the different genetic background of the Pasteur strain. These alternative possibilities are currently being tested by making reassortants between the spleen variants and the wt Armstrong strain from which the variants were derived. These reassortants will allow us to assess more accurately the contribution of changes in the S segment in causing chronic infections in adult mice.

Additional evidence documenting the importance of mutations in the L segment in organ-specific selection and persistence comes from genetic analysis of the revertant derived from spleen variant clone 13. We made a reassortant containing the L segment of the revertant and the S segment of the wt Pasteur strain (svrA/wtP) and compared its biological properties with those of svA/wtP and wtA/wtP; these three reassortants have the same S segment (derived from the wt Pasteur strain) but contain the L segment of the original wt Armstrong strain (wtA), the spleen variant derived from it (svA), or the revertant derived from the spleen variant (svrA). Thus, by using these reassortants one can directly compare the L segments of wtA, svA, and svrA. Both wtA/wtP and svrA/wtP induced a potent CTL response in adult mice and were cleared within 8 days, whereas mice infected with svA/wtP contained high levels of virus and a low detectable CTL response (Fig. 7). These results show unequivocally that a biologically relevant mutation(s) occurred in the L segment, not only during generation of the spleen variant from the wt Armstrong strain but also in reversion of the spleen variant to the wt phenotype (wtA → svA → svrA). In most studies with other viruses, the viral surface GPs or outer capsid proteins were implicated in causing disease (9, 13, 15). The results of this study mapping persistence in adult mice to the L segment, along with those of our earlier report (18) implicating the L segment in virulence for guinea pigs, show that mutations in genes other than those coding for the surface proteins can profoundly modify the virulence of a virus.

Genetic studies of virulence have been done with many different viruses (9, 13, 15). However, there is one aspect of our study that gives it a unique flavor. This relates to the origin of the viral variants we analyzed. This represents one of the few studies in which variants selected in vivo during chronic infection in their natural host were genetically characterized. These studies may provide some insight into the mechanisms by which viral variants emerge in nature.

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