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Competitive Selection In Vivo by a Cell for One Variant over Another: Implications for RNA Virus Quasispecies In Vivo

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Infidelity of genome replication of RNA viruses leads to the generation of viral quasispecies both in vitro and in vivo. However, the biological significance of such generated variants in vivo is largely unknown and controversial. To study this issue, we continued our evaluation of the tropism of a lymphocytic choriomeningitis virus (LCMV) variant termed clone 13 with its parental virus clonal pool ARM 53b (wild-type parent) for neuronal cells in vivo. Earlier in vivo and in vitro studies noted that the wild-type virus contained a Phe at glycoprotein (GP) residue 260 which correlated with neuron tropism compared with LCMV variants containing a Leu at residue 260 which showed selected tropism for cells of the immune system (C. F. Evans, P. Borrow, J. C. de la Torre, and M. B. A. Oldstone, J. Virol. 68:7367–7373, 1994; L. Villarete, T. Somasundaram, and R. Ahmed, J. Virol. 68:7490–7496, 1994). Here we (i) evaluated the ability of the viral variants with either a Phe or Leu at GP residue 260 to replicate in vivo in the spleen, liver, or brain, (ii) analyzed the ability of these viruses to compete against each other for cell (neuron)-specific selection following a single viral inoculation of different ratios of both viruses, and (iii) utilized genetic reassortants of both viruses to test their ability to replicate in neurons in vivo. We found that viral variants containing either a Phe or Leu at GP residue 260 were equally capable of replicating in neurons, but when inoculated together, neurons selected for the viral population containing Phe at GP residue 260 over viruses containing a Leu at this position. This was in contrast to selection in the liver and spleen that favored viruses with Leu and not Phe at GP residue 260. Analysis of inoculations with viral reassortants indicated that genes encoded on the short RNA (the GP and nucleoprotein, not the L [polymerase] and Z proteins that are encoded by the large RNA) were associated with neurotropism. Since the nucleoprotein sequences of wild-type Armstrong and clone 13 are identical, it is likely that specific cytoplasmic factors of the neurons play a fundamental role in the selection of virus with Phe at GP residue 260.

High mutation rates operating during replication of RNA viruses allow for the generation of viral variants with the abilities to infect a variety of tissues (cell types) within a host, infect a variety of hosts, and establish persistent infections. Recent interest has focused on the study of RNA virus populations as quasispecies (8, 10), especially the molecular basis of their error-prone replication and large population sizes. Our concern has not been on whether these mutations in the viral genome occur but rather whether these changes are of biological significance and are associated with pathogenicity (2, 3, 5, 6, 11, 16, 18, 20, 23, 25). For example, we (2, 3, 5, 11) and others (1, 15, 27) have plotted the emergence of lymphocytic choriomeningitis virus (LCMV) variants during the course of persistent viral infection. Viral variants were found with different biological properties; one variant predominated in the central nervous system in neurons (11, 19, 27) and another predominated in the immune system in lymphocytes and macrophages (1–3, 5, 11, 15, 26, 27). Analysis of multiple variants indicated that a single amino acid change in the viral glycoprotein (GP), a Phe-to-Leu (F→L) mutation at residue 260 correlated with selection of variants in lymphocytes, macrophages, and dendritic cells and these variants caused immunosuppression when inoculated intravenously into adult mice. Inoculation of wild-type (wt) LCMV Armstrong (ARM) strain (Phe at residue 260) into newborn mice resulted in the selection and amplification of variants with Leu at GP residue 260 in lymphoid tissues by 4 to 5 weeks after infection. The prototype variant which has tropism for cells of the immune system is called LCMV clone 13 (Cl 13) (1, 11, 15). Inoculation of 2×10^6 PFU of Cl 13 intravenously into adult immunocompetent mice aborted cytotoxic T-lymphocyte responses and led to viral persistence (1–3, 5, 15, 20, 24, 26, 27). In contrast, inoculation of a similar amount (2×10^6 PFU) of wt ARM resulted in the generation of a cytotoxic T-lymphocyte response that cleared virus infection.

When newborn mice were injected with wt ARM, the mice had wt (Phe at residue 260) virus in neurons throughout the animal's life (11, 27). As a consequence of the virus-neuron interaction, learning and behavioral deficiencies (12) associated with chemical imbalance of several neurotransmitters occurred (7, 12, 14).

Here we evaluated first, whether Cl 13 given to newborn mice was neurotropic and able to replicate in the central nervous system, and second, when mixtures of Cl 13 and wt ARM were given as a single inoculation at birth, whether neuronal cells selected out one viral population over the other for in vivo replication. We show that Cl 13 is able to infect and replicate in neurons in vivo. However, when both Cl 13 and wt ARM are inoculated together into newborn mice, their neurons select for a population of virus bearing a Phe at GP residue 260 in preference over those that have a Leu at GP residue 260. Utilization of wt ARM and Cl 13 reassortants (15) indicates that the genes or regulatory sequences encoded by the short (S) RNA, the GP or nucleoprotein (NP) map with neuronal selection. Since the NP gene of the wt ARM and of Cl 13 is identical (21), these results indicate that neurons select for a subset of LCMV on the basis of their GP during persistent

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infection. It is likely that interactions of unique neuronal cellular factors in association with the two base changes in the GP gene, one of which encodes for a single amino acid in the GP open reading frame of the virus, are responsible for this selection.

MATERIALS AND METHODS

Virus strains. LCMV ARM 53b (ARM) is a triple-plaque-purified isolate of Armstrong CA 1371 and was passaged in baby hamster kidney (BHK) cells. Cl 13 was originally isolated from the spleen of a 2-month-old mouse infected at birth with ARM and was also triply plaque purified. The plaque purification procedure, preparation of stock virus in BHK cells, quantitation of viral stocks by plaquing, and sequences of LCMV ARM and LCMV Cl 13 have been described elsewhere (3, 9, 20, 21). The ARM/Cl 13 reassortants were triply plaque purified on BHK cells and characterized as described previously (15).

Infection of mice. BALB/Wehi mice were bred in the closed breeding colony of The Scripps Research Institute. Mice were infected within 18 h of birth by intracerebral (i.c.) inoculation of either 1,000 PFU ARM, Cl 13, reassortants S RNA ARM/L RNA Cl 13, S RNA Cl 13/L RNA ARM, or mixtures of ARM and Cl 13 at ratios of 1:5 and 1:10, respectively. At 3 and 5 months postinfection, three mice per group were sacrificed. Spleens, livers, and brains were removed and frozen at −70°C until RNA extraction was done.

RNA preparation. RNA was prepared as described previously (11). Briefly, organs were homogenized in 2 ml of a solution containing 4 M guanidine thiocyanate, 50 mM Tris (pH 8.5), 10 mM EDTA, and 0.5% sarcosyl. Sodium acetate (2 M, pH 4.0, 220 μl) and $CHCl_3$-isoamyl alcohol (49:1, 440 μl) were added and mixed vigorously. Two milliliters of acid phenol (phenol-H_2O [1:1]) was added, and the mixture was then incubated on ice for 10 min. After centrifugation at 10,000 × *g* for 20 min at 10°C, the aqueous phase was transferred to a new tube and an equal volume of isopropanol was added. The mixture was frozen at −20°C for at least 1 h, and RNA was pelleted at 10,000 × *g* for 20 min at 4°C. The pellet was resuspended in 300 μl of TE (10 mM Tris [pH 7.9], 0.1 mM EDTA) and reprecipitated with 600 μl of ethanol. The RNA was resuspended in H_2O, and the optical density at 260 nm was measured to determine the concentration.

RNA PCR and *Mnl*I digestion. The PCR and *Mnl*I digestion procedures were done as reported by Evans et al. (11). Briefly, purified RNA (0.5 μg) was transcribed into cDNA with 50 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories [BRL]) and random hexamers (2.5 μM) as the primers (20-μl reaction volume). PCR was performed on the cDNA product by adding (100-μl final volume) 2.5 U of *Taq* polymerase (BRL), $MgCl_2$ (2 mM final concentration), and primers specific for LCMV GP. Reaction mixtures were incubated for 45 cycles, with 1 cycle consisting of 1 min at 95°C and then 1 min at 60°C. Twenty microliters of the PCR mixture was digested with *Mnl*I (NEB, 3 U) and the products were run on 1% SeaKem ME–1% Nusieve GTG agarose gels (FMC Bioproducts). The DNA was visualized by ethidium bromide staining. The sizes of the PCR products were determined by comparison with a 1-kb DNA ladder (BRL). Quantitation of the bands was done with a Molecular Dynamics densitometer.

Whole-animal body sectioning and use of ^{32}P-labeled LCMV cDNA probe. The whole-animal body sectioning and cDNA probing procedures were done as reported elsewhere (4, 17). Briefly, mice were anesthetized with ether, exsanguinated, embedded in 3.5% carboxymethylcellulose, and frozen by immersion in dry ice-ethanol. The carboxymethylcellulose blocks were cut with a LKB cryomicrotome, model 2258, until the appropriate plane for sectioning was obtained. Thirty-micron-thick sections were cut, collected on 3M Scotch tape (no. 688), and used for nucleic acid hybridization. A ^{32}P-labeled, nick-translated DNA probe, with a specific activity of 1×10^8 to 10×10^8 cpm/μg of DNA, was used to detect viral nucleic acid in the whole-animal body section.

RESULTS

The immunosuppressive lymphotropic and macrophagetropic variant LCMV Cl 13 is able to replicate in the brain. wt LCMV ARM and the variant Cl 13 were tested for their ability to replicate in the brains of mice infected at birth with either virus. As seen in Fig. 1, both the wt ARM and Cl 13 variant replicated in the central nervous system. Further, viral nucleic acid was detected in other tissues of the mice inoculated with either ARM or Cl 13. By contrast, in tissues of uninfected control animals, viral nucleic acid was not detected. Infectious virions were produced in both ARM- and Cl 13-infected animals since 10^4 to 10^5 PFU of virus per gram of brain tissue were detected. The amounts of virus found following ARM or Cl 13 inoculation were equivalent. Neurons were determined to be the cells infected within the brain by immunohistochemistry with monoclonal antibodies against the viral NP or in situ hybridization with an LCMV NP probe, followed by ultrastructural microscopic analysis (13, 14, 17, 19, 27; data not shown).

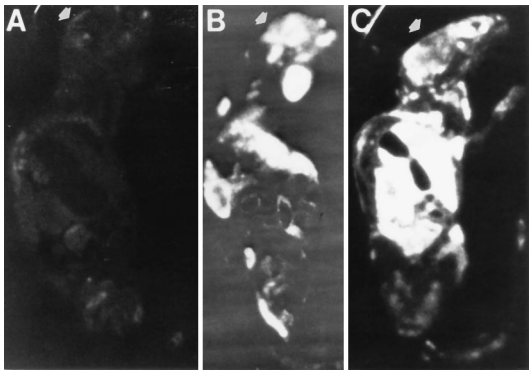

FIG. 1. LCMV nucleic acid sequences in the brains of 4-month-old mice that had been infected with Cl 13 (B) or wt ARM (C) at birth. Thirty-micron-thick whole-body sections were incubated with the ^{32}P-labeled LCMV NP probe (see Materials and Methods). Panel A shows a corresponding section from a 4-month-old uninfected mouse with the ^{32}P-labeled LCMV NP probe. Note that following infection of newborns, both LCMV Cl 13 and wt ARM replicated in the brain (indicated by the arrows) as well as other tissues. Electron microscopic studies indicated that viral materials in the brain were restricted to neurons (19).

Detection of specific replication of wt ARM and Cl 13 variant in tissues in vivo. wt ARM and the variant Cl 13 can be distinguished molecularly in vivo by an RNA PCR technique (11). The wt ARM Phe-to-Leu change at amino acid 260 of the GP in Cl 13 results from a U-to-C change at nucleotide 855 in the viral RNA (15, 20). This coding change creates a cleavage site in the Cl 13 cDNA for the restriction enzyme *Mnl*I. RNA from infected tissues can be reverse transcribed into cDNA, and then PCR amplification of the region surrounding amino acid 260, followed by digestion of the PCR product with *Mnl*I, can be performed. By using primers as specified in Materials and Methods, the RNA from tissues infected with wt ARM is detected as a 362-bp PCR product, whereas RNA from tissues infected with virus containing a Leu at GP amino acid 260 is detected as two DNA fragments of 202 and 160 bp. Because of the redundancy of the genetic code, other mutations in the ARM viral sequence could result in a Leu at amino acid 260 in the GP that would not result in the generation of an *Mnl*I site. However, no evidence for other nucleotide changes in the GP amino acid 260 codon has been described (1, 11, 20).

The RNA PCR-*Mnl*I digestion technique was used to follow the conversion of wt ARM Phe at amino acid 260 of the GP to a Leu at this position in spleen, liver, and brain tissues of mice infected at birth with ARM. Figure 2 documents that after inoculation of mice with wt ARM at birth, the wt ARM genotype was found exclusively in brain tissue 5 months later. No evidence of a conversion to a Cl 13-like variant (i.e., with a Phe-to-Leu change at GP amino acid 260) occurs in this tissue. By contrast, in vivo conversion of the wt GP with Phe at amino acid 260 to the GP with Leu at amino acid 260 occurred in the spleens and livers of these mice. The proportions of Cl 13-like virus found in these tissues at 5 months were 64 and 68% in the spleen and liver, respectively, but 0% in the CNS (Fig. 3). When the Cl 13 variant was inoculated into newborn mice, at 3 and 5 months postinfection, the Cl 13 genotype was found in the spleen, liver, and brain (Fig. 4). There was no evidence of conversion of the Cl 13 genotype to the wt ARM genotype, as indicated by the absence of any uncleaved 362-bp PCR product (Fig. 4).

wt ARM competes successfully against the Cl 13 variant for

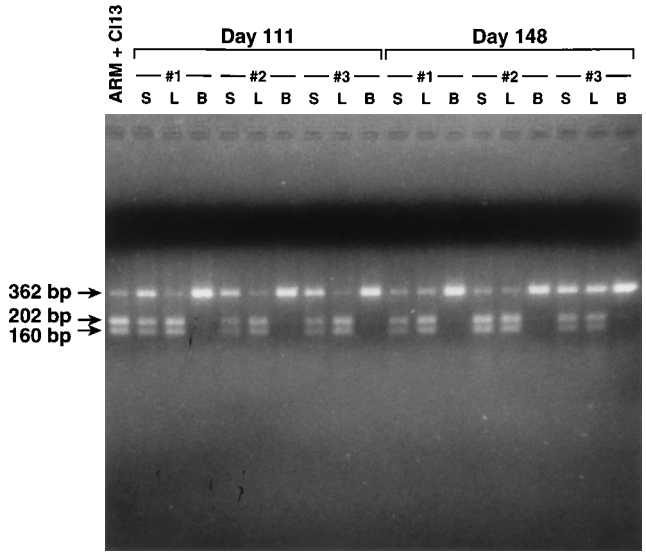

FIG. 2. Over time, LCMV ARM is selected preferentially for replication in the brain. BALB/Wehi mice were inoculated with 1,000 PFU of wt LCMV ARM i.c. within 18 h of birth. Three mice were sacrificed at 111 and 148 days postinfection, and RNA was extracted from the spleen (S), liver (L), and brain (B). RNA PCR and *Mnl*I digestion of PCR products were performed as described in Materials and Methods. The RNA PCR product from ARM virus containing a Phe at position 260 of the GP is 362 bp, whereas *Mnl*I cleaves the 362-bp PCR product coding for Leu at GP amino acid 260 from Cl 13 virus to produce two DNA fragments of 202 and 160 bp.

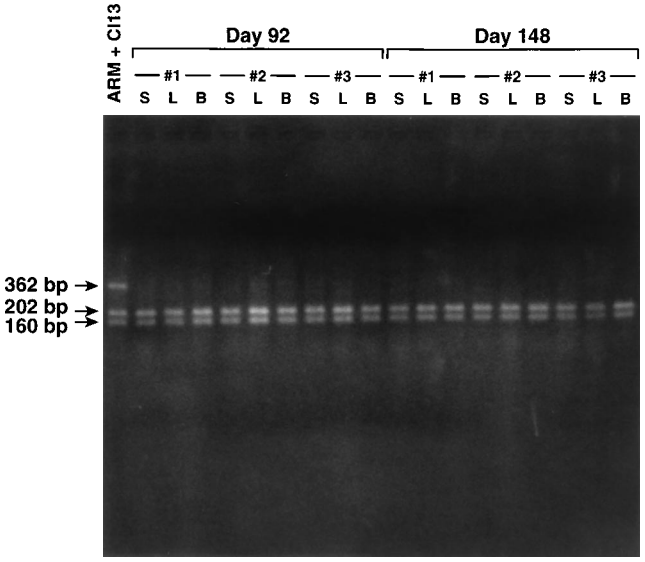

FIG. 4. LCMV with a Leu at the GP amino acid 260 is detected in the brains of mice inoculated at birth with Cl 13. BALB/Wehi mice were inoculated with 1,000 PFU of Cl 13 i.c. within 18 h of birth. Mice were sacrificed at either 92 or 148 days postinfection, and RNA was extracted from the spleen (S), liver (L), and brain (B). RNA PCR and *Mnl*I digestion of PCR products were performed as described in Materials and Methods.

replication in the brain. The above experiments clearly demonstrated that variant virus with Leu at amino acid 260 of the GP was selected in vivo in the livers and spleens but not in the brains of mice infected at birth with the wt (ARM) virus. Yet virus with a Leu at amino acid 260 of the GP was able to replicate in the brain (Fig. 1 and 4). We next determined if neurons would preferentially select wt ARM over Cl 13 in vivo following infection with both viruses. Newborn mice were inoculated with different ratios of ARM and Cl 13 that favored Cl 13 replication, i.e., 1:5 and 1:10 ratios of ARM and Cl 13. As shown in Fig. 5, at both ratios, despite the abundance of Cl 13 inoculated, neuronal cells in the brain selected wt ARM virus, while Cl 13 was selected in the peripheral tissues analyzed. Thus, although the inoculated viral mixture contained only 20% (ARM:Cl 13 1:5) or 10% (ARM:Cl 13 1:10) virus with a Phe at GP amino acid 260, after 150 days, 40 and 37%, respectively, of the virus in the brains of these mice contained the wt Phe at amino acid 260 (Fig. 6).

Analysis of viral genes responsible for in vivo selection of viral variants in specific tissues. To determine the viral genes responsible for the tissue selection in vivo, viral reassortants of

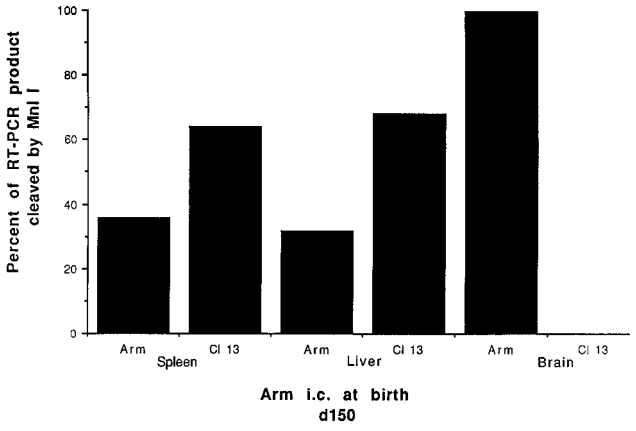

FIG. 3. Quantitation of the average proportion of virus with a Phe at GP amino acid 260 (ARM) or with a Leu at that position (Cl 13-like) in the spleens, livers, and brains of three mice 150 days (d150) after inoculation of ARM i.c. at birth. RNA was extracted from tissues, and RNA PCR, *Mnl*I digestion, and quantitation of cleavage products were performed as described in Materials and Methods. RT-PCR, reverse transcription-PCR.

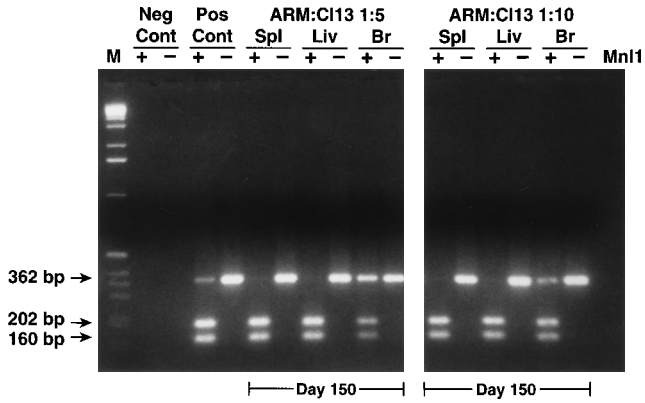

FIG. 5. LCMV ARM successfully competes against LCMV Cl 13 for replication in the brain (neurons). BALB/Wehi mice were inoculated with a mixture of ARM and Cl 13 at ratios of 1:5 and 1:10 and sacrificed 150 days postinfection. Each mouse received a total of 1,000 PFU of virus i.c. within 18 h of birth. RNA was extracted from the spleens (Spl), livers (Liv), and brains (Br) of these mice. RNA PCR and *Mnl*I digestion of PCR product were performed as described in Materials and Methods. Lane M contains 1-kb markers (BRL). Over the lanes, a plus sign indicates treatment of the RNA PCR product with *Mnl*I, whereas a minus sign indicates no *Mnl*I was added. Neg Cont lanes did not have an RNA sample added to the reverse transcription reaction mixture, and the RNA in the Pos Cont lanes was from a liver sample containing both ARM and Cl 13 viruses.

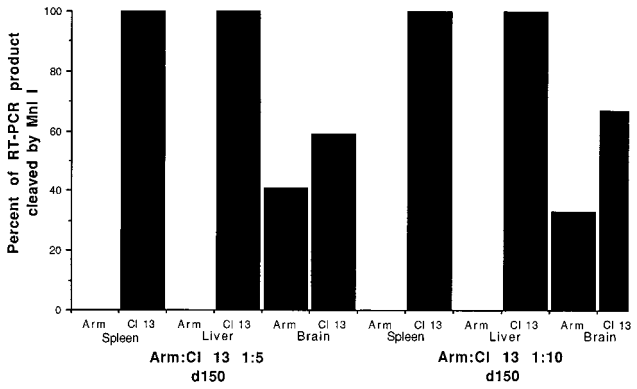

FIG. 6. Quantitation of the average proportion of LCMV ARM and LCMV Cl 13-like viral sequences in the spleens, livers, and brains of three mice 150 days (d150) after inoculation at birth with ARM:Cl 13 at ratios of 1:5 and 1:10. RNA was extracted from tissues, and RNA PCR, *Mnl*I digestion, and quantitation of cleavage products were performed as described in Materials and Methods. RT-PCR, reverse transcription PCR.

wt ARM and Cl 13 were made (15). As seen in Fig. 7, the S RNA of ARM contained the genetic material associated with neurotropism, while the large RNA of ARM did not. Thus, the GP and NP genes, which are encoded on the S RNA, are involved in the neuronal tropism, not the Z and L proteins, which are encoded on the large RNA. Since the NP is identical in ARM and Cl 13, the preferential neuronal selection is associated with ARM GP sequences.

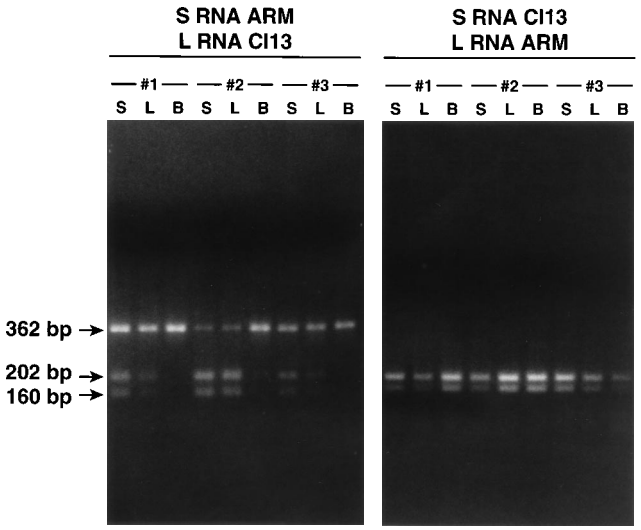

FIG. 7. Use of LCMV ARM and LCMV Cl 13 variant reassortants indicates that genes controlling preferential selection of virus in neurons of the brain reside in the S RNA. (Left) Three BALB/Wehi mice were inoculated with 1,000 PFU of the reassortant S RNA ARM/L RNA Cl 13 within 18 h of birth. Individual mice were sacrificed at 145 days postinfection. RNA was extracted from the spleens (S), livers (L), and brains (B) of these mice. RNA PCR and *Mnl*I digestion of PCR product were performed. ARM and Cl 13-like S RNA sequences were found in the spleens and livers of these mice. Only ARM S RNA sequences were found in the brains of these mice. (Right) Three BALB/Wehi mice were inoculated with 1,000 PFU of the reassortant S RNA Cl 13/L RNA ARM within 18 h of birth. Individual mice were sacrificed at 150 days postinfection. RNA was extracted from the spleens (S), livers (L), and brains (B) of these mice. RNA PCR and *Mnl*I digestion of the PCR product were performed. Cl 13-like S RNA sequences were found in the spleens, livers, and brains of these mice.

DISCUSSION

As with other RNA viruses, during the course of a persistent LCMV infection, variants are generated. We show here that these variants are selected by certain tissues for replication. Similarly, wt virus maintains a preferential selection in certain tissues. Thus, despite inoculation or generation of two viruses, wt ARM and Cl 13, wt virus is selected in neuronal cells while Cl 13 is selected by cells in the liver and spleen.

The LCMV variant Cl 13 is able to replicate in neuronal cells in the absence of wt ARM, but C13 is counterselected when competing with wt ARM in neuronal cells. The amounts of viral nucleic acid sequences, protein, and infectious virus produced by Cl 13 when replicating alone are equivalent to those produced by wt ARM. Yet when mixtures of Cl 13 and ARM are added, ARM is preferentially replicated in the brain (neurons) although not in the liver or spleen. Since there is no block to Cl 13 replication in neurons, when Cl 13 and wt ARM jointly infect these cells, wt ARM has a selective advantage.

While wt ARM is preferentially selected by neurons, Cl 13 is actively selected by liver and spleen cells. Recent work has shown that this selection is also cell specific within the spleen (5). For example, wt ARM replicates in the red pulp preferentially in $F4/80^+$ macrophages of the spleen, while Cl 13 replicates in the white pulp interdigitating dendritic antigen-presenting cells. Destruction of these dendritic professional antigen-presenting cells by anti-LCMV $CD8^+$ cytotoxic T lymphocytes leads to a profound immunosuppression (5).

The reason for the preferential selection of wt ARM over the Cl 13 variant in neurons is not known. Since amino acid 260 of the viral GP is associated with this selection, it is possible that events during infection that depend on the GP are responsible for the selection, including viral attachment, internalization, or assembly. In addition, cellular cytoplasmic factors specific to neurons likely play a role in the selection.

There are multiple reports of a mutation in a virus generating a variant that alters the course of infection in a host. For example, Sitbon et al. (22) showed that the specific selection of a nonlytic viral variant is due to a single-amino-acid change, a leucine to an isoleucine in the envelope region of Friend murine leukemia virus. This change results in the decrease of pathogenicity, because the variant reduces the host's susceptibility to early hemolytic anemia, presumably decreasing the number of infected cells with budding retrovirus. Another example, described by Yoon et al. (28), is a single-base change in encephalomyocarditis virus that is associated with disease. Encephalomyocarditis virus replicates in the beta cells of the pancreas, is lytic, and causes insulin-dependent diabetes mellitus by destroying the beta cells that make insulin. However, a variant, EMC-B, induces interferon production locally in beta cells, thereby disrupting viral replication and preventing disease. Although this variant differs from the pathogenic strain by 14 bp, a single-base change is associated with pathogenicity. These and similar findings differ from our reports in that we are looking at a nonlytic virus generating variants that are preferentially being selected in different tissues.

Persistent infection allows the opportunity for a continuous generation of variants. These variants can then be identified by a specific mutation(s), although the biological significance of these generated variants has been difficult to dissect. The LCMV murine model offers an opportunity to study the generation, selection, and consequences of such variants during the course of infection. In addition to the biological importance in terms of the mechanisms of immunosuppression and maintenance of persistence that we and others have reported (1, 3, 5, 15, 24), it is interesting to consider the molecular basis

of how a virus species is selected for a greater degree of replication in one particular cell type over another. Our results show that infection of mice at birth with an RNA virus leads to the selection of viral variants within the mice and the selective replication of these variants in different tissues. The molecular basis of this selection is currently under investigation.

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