Virus-Induced Immunosuppression: Kinetic Analysis of the Selection of a Mutation Associated with Viral Persistence†

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Infection of neonatal mice with lymphocytic choriomeningitis virus (LCMV) strain Armstrong (ARM) results in a lifelong persistent infection. Viral variants (cytotoxic T lymphocyte [CTL] negative, persistence positive [CTL⁻ P⁺]) can be isolated from the lymphoid tissues of such mice. Adult mice inoculated with these CTL⁻ P⁺ viruses fail to generate sufficient cytotoxic T lymphocytes to clear the acute infection and become persistently infected. By contrast, inoculation of a similar dose of the parental ARM virus (CTL⁺ P⁻) into adult mice leads to the generation of a vigorous virus-specific CTL response that clears the infection. Sequence analysis revealed a phenylalanine (Phe)-to-Leucine (Leu) change at amino acid 260 of the viral glycoprotein (GP) as a marker for variant viruses with the CTL⁻ P⁺ phenotype. An RNA PCR assay that detects the variant GP sequence and thus allows kinetic studies of the selection of the Leu at position 260 was developed. We found that although CTL⁻ P⁺ viruses are known to be lymphotropic, mature T and B cells were not required for the generation and selection of the Leu at GP amino acid 260. Kinetically, in mice infected at birth with LCMV ARM, as early as 3 weeks postinfection the Phe-to-Leu change was found in virus in the serum. By 5 weeks, viral nucleic acid obtained from peritoneal macrophages, spleen, lymph nodes, and liver showed the Phe-to-Leu change. At 2 months postinfection, the Leu change was detected in virus from the thymus, heart, lung, and kidney. By contrast, virus replicating in the central nervous system showed only minimal levels of the Leu change by 4 months and as long as 1 year postinfection. In vitro studies showed that the parental LCMV ARM CTL⁺ P⁻ virus replicates more efficiently and outcompetes CTL⁻ P⁺ virus in a cultured neuronal cell line, indicating that differential growth properties in neurons are likely the basis for the selection of the parental virus over the CTL⁻ P⁺ variant in the brain.

Lymphocytic choriomeningitis virus (LCMV) is an arenavirus that is a natural pathogen of mice. The outcome of infection with this virus is dependent on the virus isolate, dose, and route of inoculation and the age and strain of the mice. Intraperitoneal (i.p.) or intravenous inoculation of up to 2 × 10⁶ PFU of LCMV strain Armstrong (ARM) into adult mice causes an acute infection which is cleared by 7 to 10 days postinfection (7, 19). Viral clearance is mediated by a virus specific CD8⁺ cytotoxic T-lymphocyte (CTL) response (9, 11, 25; reviewed in references 7, 24, and 36), and because of this host response, LCMV ARM is referred to as a CTL⁺ P⁻ (CTL-positive, persistence-negative) virus. By contrast, mice infected with LCMV within 24 h of birth are unable to mount a protective CTL response because of thymic deletion of LCMV-reactive T cells and thus become persistently infected for life (7, 18, 24). By in situ hybridization analysis, viral nucleic acid is detected throughout the body, and infectious virus can be isolated from many different tissues (30). These mice, while not mounting anti-LCMV CTL responses, are able to mount effective CTL responses to other RNA and DNA viruses (20, 34).

Several viral isolates derived from adult mice which were persistently infected since birth by LCMV ARM show differences in their biological properties compared with the parental virus (2, 3). These variants, the prototype of which is the clone 13 isolate, are not cleared after intravenous inoculation of 2 × 10⁶ PFU into adult mice but are able to establish a persistent infection (referred to as CTL⁻ P⁺ viruses). This is due to the

fact that the CTL response generated following infection with these virus variants is abortive and is insufficient to clear the infection. Additionally, not only is the LCMV-specific CTL activity suppressed, but mice persistently infected with clone 13 exhibit a generalized immunodeficiency and when subsequently infected by other pathogens mount impaired immune responses (4, 20, 34, 35).

The molecular basis by which clone 13 induces a persistent infection has been mapped. LCMV has an ambisense, bisegmented RNA genome that encodes at least four proteins. The small (S) segment encodes the viral nucleoprotein (NP) and the glycoprotein precursor (GP-C), which is cleaved to give GP-1 and GP-2. The large (L) segment encodes the putative viral polymerase (L gene) and a small zinc-binding protein, the Z protein. When the complete RNA sequences of the parental ARM strain and the variant clone 13 were compared, five nucleotide changes were found, resulting in only two amino acid coding changes: amino acid 260 in the viral GP (phenylalanine [Phe] to leucine [Leu]) and amino acid 1079 in the putative polymerase (lysine to glutamate) (22, 27, 28). Studies with reassortant viruses suggested that the nucleotide changes on both the S and L segments are important for the persistence phenotype (21, 22). Examination of more than 20 other CTL⁻ P⁺ variants of ARM that were also isolated from lymphoid tissue and caused persistent infection of adult mice showed that the Leu at amino acid 260 of the GP is associated with the persistence phenotype and therefore is a good marker for variants of LCMV ARM that are able to cause persistent infection (1, 27).

Here we report results of the use of an RNA PCR technique that enabled us to analyze viral RNA from mice persistently infected with ARM since birth and to examine the kinetics of the appearance of the GP Phe-to-Leu mutation in a variety of

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tissues during the course of persistent infection. We note that the Phe-to-Leu mutation is found early in cells and tissues of the immune system but is rarely found in cells of the central nervous system. We also studied the influence of T and B cells on the selection of this mutation and found that the kinetics of the Phe-to-Leu change in the GP are similar in cells or tissues harvested from immunocompetant and immunodeficient (athymic and SCID) mice. Further, we note that ARM (CTL$^+$ P$^-$) has a replicative advantage over clone 13 (CTL$^-$ P$^+$) in two cultured neuronal cell lines, GT1-7 and PC12.

MATERIALS AND METHODS

Virus strains. LCMV ARM 53b is a triple-plaque-purified isolate of ARM CA 1371 (14) and was passaged in baby hamster kidney (BHK) cells. Clone 13 was isolated from the spleen of a mouse infected at birth with ARM (3). The plaque purification procedure and preparation of stock virus in BHK cells have been described elsewhere (14).

Infection of mice. BALB/WEHI, athymic BALB/WEHI, and C.B. 17-SCID/SCID mice were bred in the closed breeding colony of The Scripps Research Institute. Neonatal BALB/WEHI and C.B. 17-SCID/SCID mice were infected within 18 h of birth by intracerebral or i.p. injection of 1,000 PFU of ARM. Similar results were obtained with both intracerebral and i.p. injections. Six- to eight-week-old athymic mice were injected intravenously with 2 × 10^6 PFU of ARM. At various times postinfection, four to six mice were sacrificed, and organs were removed and frozen at −70°C until RNA extraction was done. To elicit peritoneal macrophages, mice were injected i.p. with 3 ml of 3.85% thioglycolate; 3 days later, they were sacrificed, the peritoneal cavity was washed with RPMI medium, and the cells were collected and frozen at −70°C.

RNA preparation. Organs or cells were homogenized in 2 ml of 4 M guanidine thiocyanate–50 mM Tris (pH 8.5)–10 mM EDTA–0.5% sarcosyl; 220 μl of 2 M sodium acetate (pH 4.0) and 440 μl of CHCl$_3$-isoamyl alcohol (49:1) were then added and mixed vigorously, and then 2 ml of acid phenol (phenol-H$_2$O, 1:1) was added. After a 10-min incubation on ice, the mixture was centrifuged at 10,000 × g for 20 min at 10°C. The aqueous phase was transferred to a new tube, an equal volume of isopropanol was added, the mixture was placed at −20°C for at least 1 h, and the 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$_2$O, and the optical density at 260 nm was measured to determine the concentration. To analyze viral RNA in the serum of infected mice, the animals were bled at various times postinfection and their serum was used to infect BHK cells. After 48 to 72 h, the BHK cells were collected and RNA was extracted as described above.

RNA PCR and MnlI digestion. Purified RNA (0.5 μg) was transcribed into cDNA, using 50 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) and random hexamers (2.5 μM) as primers in a 20-μl reaction volume. PCR was performed on the cDNA product by adding (100 μl, final volume) 2.5 U of Taq polymerase (Boehringer Mannheim), MgCl$_2$ to a final concentration of 2 mM, and primers specific for LCMV GP: primer 1 (5′-TGGACAGGCTCAGATGGCAAGA-3′), which hybridizes at nucleotide 705 of the GP cDNA, and primer 2 (5′-CTCA AAGCAGCCTTGTTGTAGTC-3′), which hybridizes at nucleotide 1066 of the GP cDNA. Reaction mixtures were incubated for 1 min at 95°C and 1 min at 60°C for 45 cycles. MnlI (4 U; New England Biolabs) digestion of 20 μl of the

PCR mixture was carried out, and the products were run on 1% Seakem ME–1% NuSieve GTG agarose gels (FMC Bioproducts) and visualized by ethidium bromide staining. The sizes of the PCR products were determined by comparison with a 100-bp ladder (Bethesda Research Laboratories). The proportion of PCR product cleaved by MnlI was quantitated by densitometric scanning using NIH IMAGE software.

CTL$^+$ P$^-$ and CTL$^-$ P$^+$ growth in GT1-7 and PC12 cells and their competition in GT1-7 cells. The mouse GT1-7 cell line (23) was grown in Dulbecco's modified Eagle's medium containing penicillin, streptomycin, 1% glutamine, 6% heat-inactivated fetal bovine serum, and 6% heat-inactivated horse serum. These cells were derived from a hypothalamic neuronal tumor and retain neuronal characteristics when grown in tissue culture. PC12 cells were derived from a rat pheochromocytoma clone and have been used extensively as a model of neuronal differentiation (12, 15). PC12 cells were grown in supplemented RPMI 1640 medium as reported previously (15). BHK cells were grown in Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin, 1% glutamine, 0.7% glucose, 5% 2× tryptose phosphate broth, 3.5% heat-inactivated fetal bovine serum, and 3.5% heat-inactivated calf serum. GT1-7, PC12, and BHK cells were infected with ARM or clone 13 at a multiplicity of infection (MOI) of 3. At 0, 24, 48, and 72 h postinfection, the supernatants were collected and viral titers were determined by plaque assay on Vero cells as described previously (14). In competition experiments, GT1-7 cells were infected with ARM or clone 13 alone at an MOI of 3 or with a 1:5 mixture of ARM and clone 13 with ARM at an MOI of 1. Supernatants were collected at 72 h postinfection, frozen at −70°C, and then used to infect more GT1-7 cells. In this manner, the viruses were passaged six times through the GT1-7 cells. At each passage, the cells were harvested, and RNA was extracted and analyzed by the RNA PCR-MnlI technique described above.

RESULTS

The nucleotide difference at position 855 in LCMV ARM and clone 13 can be distinguished by RNA PCR. Examination of the LCMV GP cDNA nucleotide sequence coding for the Phe-to-Leu amino acid 260 change in clone 13 revealed that the U-to-C change at nucleotide 855 creates a cleavage site for the restriction enzyme MnlI in the cDNA of clone 13 (Fig. 1A). This finding was used to design an RNA PCR strategy that would enable us to detect the presence of the Leu at amino acid 260 in total cellular RNA extracted from infected cells or tissue (Fig. 1B). RNA is first transcribed into cDNA by using Moloney murine leukemia virus reverse transcriptase and random hexamers as primers. The primers chosen for PCR hybridize near amino acid 260 of the viral GP and upon amplification using Taq polymerase generate a 362-bp DNA product. MnlI digestion of the clone 13 PCR product results in cleavage into two DNA fragments of 202 and 160 bp, whereas MnlI does not recognize the DNA sequence of the ARM PCR product (Fig. 1B). To test this method, ARM and clone 13 were grown in BHK cells, and total cellular RNA was purified and analyzed by the RNA PCR-MnlI technique (Fig. 2). The PCR product obtained from ARM-infected cells was not cleaved with MnlI, whereas MnlI completely digested the clone 13 PCR product into two fragments of 202 and 160 bp. RNA PCR-MnlI cleavage of a 1:1 mixture of ARM and clone 13 RNA resulted in partial digestion of the PCR product by MnlI (Fig. 2). Thus, this RNA PCR-MnlI method can be used to detect the presence of a leucine at amino acid 260 of LCMV in
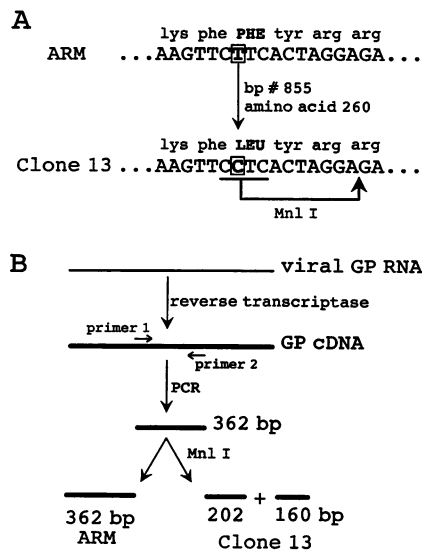
FIG. 1. LCMV ARM and clone 13 can be distinguished by RNA PCR-*MnlI* digestion. (A) In the viral GP, the T at nucleotide 855 of ARM cDNA is changed to a C in the clone 13 cDNA, resulting in the generation of an *MnlI* restriction site in the viral cDNA. (B) Strategy for the RNA PCR *MnlI* technique that distinguishes ARM RNA from clone 13 RNA (see Materials and Methods).

a mixture of viral populations and in the presence of cellular RNA.

Kinetics of the selection of a leucine at GP amino acid 260 in vivo. The RNA PCR-*MnlI* technique was used to study the kinetics of the selection of GP Leu-260 in different tissues of mice infected at birth with ARM. At various ages, mice were sacrificed, RNA was extracted from tissues, and RNA PCR-*MnlI* digestion was performed. To determine whether virus with a Leu at GP amino acid 260 was present in serum, mice were eye bled and their serum was used to infect BHK cells to amplify the virus. After 72 h, BHK cells were harvested, total RNA was extracted, and RNA PCR-*MnlI* digestion was performed. Since even after five sequential passages of ARM in BHK cells the GP Phe-260-to-Leu change was not detected (data not shown), analysis of serum-borne virus amplified through BHK cells should allow accurate determination of which amino acid is present at GP amino acid 260 in the serum.

An example of the kinetics of the Leu-260 change is shown
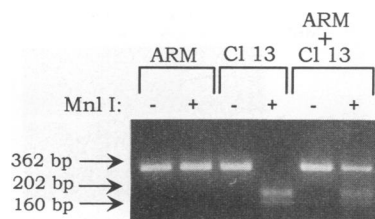

FIG. 2. Distinguishing LCMV ARM and LCMV clone 13 by detection of the GP amino acid 260 difference by RNA PCR *MnlI* digestion. RNA from BHK cells infected with LCMV ARM or clone (Cl) 13 and a mixture of equal amounts of RNA from BHK cells infected with either ARM or clone 13 were subjected to RNA PCR *MnlI* digestion as described in Materials and Methods. *MnlI* cleaves the 362-bp RNA PCR product coding for Leu at GP amino acid 260 to produce two bands of 202 and 160 bp.

in Fig. 3. Five-week-old mice infected at birth with ARM showed an *MnlI*-cleavable PCR product in RNA from serum, activated peritoneal macrophages, lymph nodes, spleen, and liver (Fig. 3A); in 11-week-old mice, the change was also found in the thymus, heart, and lung and at minimal levels in kidney (Fig. 3B). The kinetics of the GP amino acid 260 change are summarized in Fig. 4. At all time points and in all cell types tested, viral RNA sequences were always detected by RNA PCR, but the amount cleavable by *MnlI* varied with age and tissue type. The Leu at GP amino acid 260 was detected first at 3 weeks in serum samples and by 5 weeks appeared in thioglycolate-elicited peritoneal macrophages, lymph nodes, spleen, and liver. By 2 months, it was detected in the thymus, heart, and lung, and at a low level in the kidney. By 4 months, very low levels of the GP Leu-260 change were detected in brain tissue, and even after 1 year only minimal amounts of the Leu change were noted, despite the high proportion of variant virus containing this change found in various other organs and serum (data not shown). Because of the redundancy of the genetic code, other mutations in the parental ARM virus sequence could result in a Leu at amino acid 260 in the GP that would not result in the generation of an *MnlI* site. One of these, UUC to UUA, would be detectable by cleavage of the RNA PCR product with the restriction enzyme *MseI*; however, such analysis of tissues at various time points never detected the presence of this mutation. In addition, sequence analysis of more than 20 other CTL⁻ P⁺ variants (1, 28) detected only the U-to-C change at nucleotide 855 that produces the *MnlI* restriction site analyzed in this report.

Where does the GP amino acid 260 change first appear? The leucine at GP amino acid 260 was detected early in cells and tissues of the immune system (macrophages, spleen, lymph nodes), suggesting that this mutation may give a growth advantage to the virus in one or more cell types of the immune system. To address whether the variant is first selected in lymphocytes in the spleen, CD4⁺, CD8⁺, and B220 cell populations from the spleen were isolated by fluorescence-activated cell sorting and analyzed by RNA PCR-*MnlI* digestion. In these isolated populations (>97% pure), the Leu at GP amino acid 260 was not detected until 2 months postinfection, compared with its presence in intact spleens at 5 weeks postinfection, suggesting that the variant may first be found in cells other than lymphocytes in the spleen (data not shown). To show that mature T and/or B cells are not necessary to obtain the GP amino acid 260 change, adult athymic and neonatal SCID mice were infected with ARM and then examined for the selection of the GP variant. Since CD8⁺ cells are required for viral clearance (9, 11, 25, 36), athymic mice infected as adults become persistently infected with ARM. At various times postinfection, the athymic and SCID mice were sacrificed and RNA was isolated from peritoneal macrophages, lymph nodes, and spleen. At 6 weeks postinfection, viral RNA PCR products from athymic and SCID peritoneal macrophages were cleaved by *MnlI* (also seen in lymph nodes and spleen; data not shown), in contrast to the ARM virus used to inoculate the mice (Fig. 5). This finding indicates that the GP amino acid 260 variant can be initially selected in a cell type other than mature lymphocytes.

Differential growth abilities of the parental CTL⁺ P⁻ virus and the CTL⁻ P⁺ variant in neuronal cells. In mice persistently infected with LCMV since birth, virus within the brain is harbored in neurons (16, 26). Since minimal amounts of virus with the GP Phe-260-to-Leu change were found in the brain, the replication of ARM (CTL⁺ P⁻) and the replication of clone 13 (CTL⁻ P⁺) were compared in two neuronal cell lines. GT1-7 and PC12 cells were infected with ARM or clone 13 at
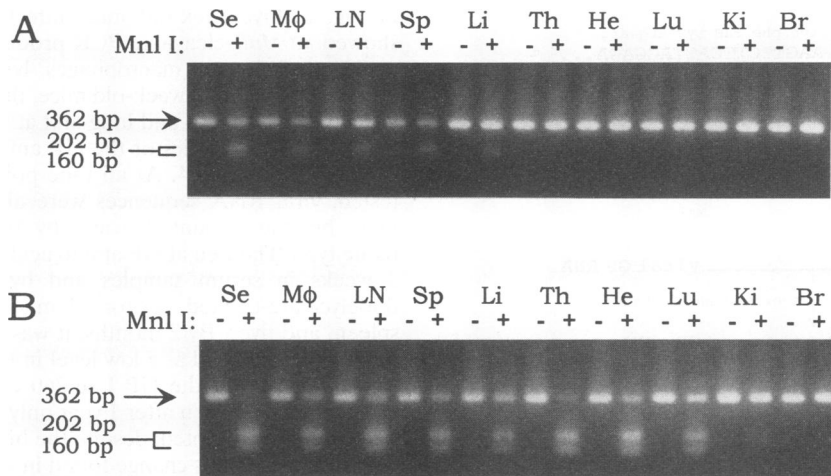
FIG. 3. Detection of the Phe-to-Leu change at amino acid 260 of GP in mice infected at birth with LCMV ARM. RNA was isolated from the serum (Se), activated peritoneal macrophages (Mφ), lymph nodes (LN), spleen (Sp), liver (Li), thymus (Th), heart (He), lung (Lu), kidney (Ki), and brain (Br) of mice infected at birth with LCMV ARM and sacrificed at 5 weeks (A) or 11 weeks (B) of age. RNA-PCR *MnlI* digestion was performed to detect the Phe-to-Leu change at amino acid 260 of GP as described in Materials and Methods.

an MOI of 3 PFU per cell. As shown in Fig. 6A for GT1-7 cells, both viruses replicate in these neuronal-like cells, but titers of ARM were significantly higher than titers of clone 13 at 24, 48, and 72 h postinfection. Similar results were noted with ARM or clone 13 replication in PC12 cells (data not shown). By contrast, no significant differences in the replication of ARM and clone 13 in BHK cells were observed (Fig. 6B). We then studied the ability of CTL⁻ P⁺ virus to compete with CTL⁺ P⁻ virus in GT1-7 cells. Cells were infected with a 1:5 ratio of ARM to clone 13, and after 72 h, the supernatant was used to infect more cells. After six sequential passages in this manner, the viral RNA in the cells at passages 2, 4, and 6 was analyzed by the RNA PCR-*MnlI* technique. As seen in Fig. 7, when the GT1-7 cells were initially infected with a 1:5 mixture of ARM and clone 13, by the fourth passage only minimal amounts of PCR product were cleaved by *MnlI*, and by the sixth passage no cleavage of the PCR product was detected. This finding indicates that the ARM virus had successfully outcompeted clone 13 for growth in the GT1-7 cells. After six sequential

passages of ARM in GT1-7 cells, the Phe-260-to-Leu change in the GP was not detected (Fig. 7). We noted, however, that when clone 13 was sequentially passaged in GT1-7 cells, by the sixth passage a minor amount of RNA PCR product was no longer cleaved by *MnlI*, indicating that a nucleotide change in the codon for GP amino acid 260 had occurred in this viral population. Since the proportion of virus containing this change in the sixth passage was very minor, it is not likely that this accounts for the ability of ARM to outcompete clone 13 for growth in the GT1-7 cells.

DISCUSSION

In this report we have examined the kinetics of the selection of a viral variant containing an amino acid change associated with viral persistence and immunosuppression. This was made possible by an RNA PCR-*MnlI* digestion technique that allowed us to detect a single amino acid change in the parental virus, LCMV ARM (CTL⁺ P⁻), in infected tissues. We found that there is a strong selection for the Phe-to-Leu change at amino acid 260 of the viral GP in most tissues analyzed with

FIG. 4. Kinetics of the appearance of the Phe-to-Leu GP amino acid 260 change in mice infected at birth with LCMV ARM. At various times after infection of neonatal mice with LCMV ARM, RNA was isolated from serum (Se), activated peritoneal macrophages (Mφ), lymph nodes (LN), spleen (Sp), liver (Li), thymus (Th), heart (He), lung (Lu), kidney (Ki), and brain (Br) and analyzed by RNA PCR *MnlI* digestion as described in Materials and Methods. +, >15% of the PCR product was cleaved by *MnlI*; (+), up to 15% of the PCR product was cleaved; −, cleavage of the PCR product by *MnlI* was not observed; ND, not determined.
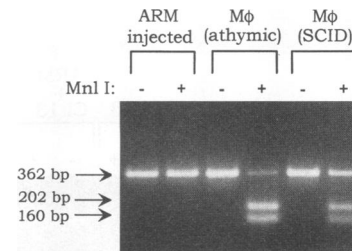

FIG. 5. The Phe-to-Leu GP amino acid 260 change occurs in the absence of mature T and B cells. RNA PCR analysis was performed on RNA from peritoneal macrophages (Mφ) isolated from athymic mice infected as adults with LCMV ARM 6 weeks previously, from peritoneal macrophages from 6-week-old SCID mice infected at birth with LCMV ARM, or from the ARM viral stock used to inoculate the athymic and SCID mice. Total cellular RNA was prepared and analyzed by RNA PCR-*MnlI* digestion as described in Materials and Methods.
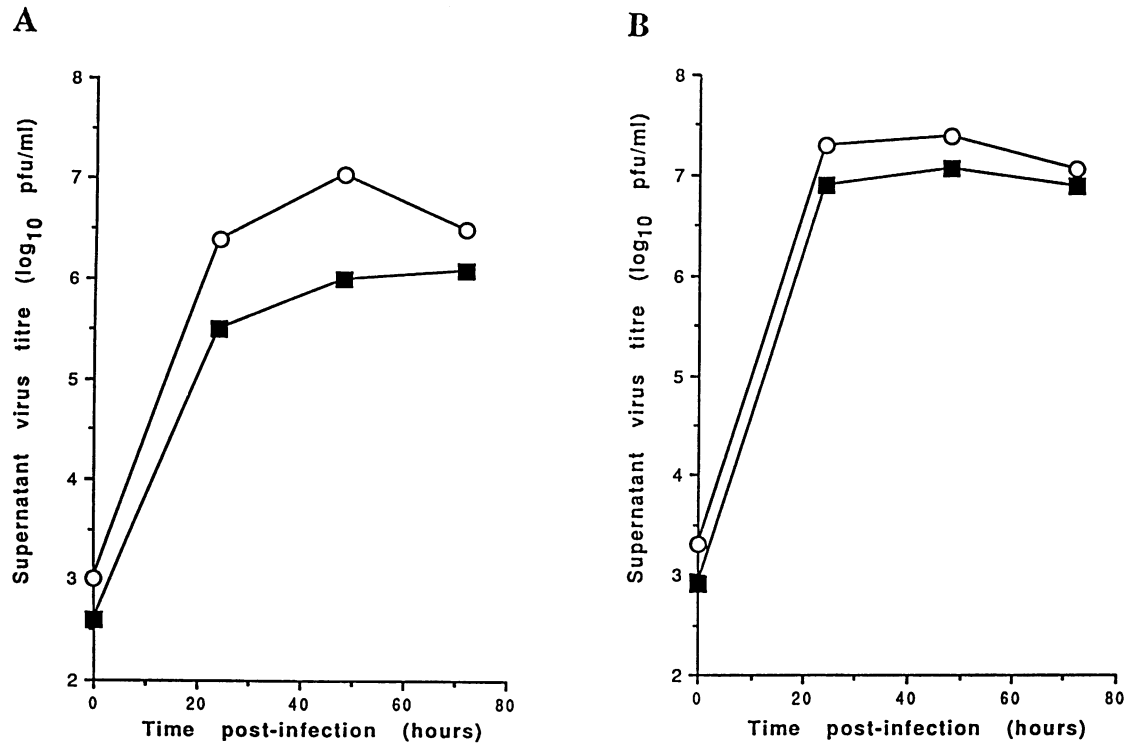
A

B

FIG. 6. Infection of neuronal and fibroblast cell lines with ARM or clone 13. GT1-7 (A) and BHK (B) cells were infected at an MOI of 3 PFU per cell with ARM (O) or clone 13 (■), and the infectious virus produced in the supernatant was quantitated at various times postinfection as described in Materials and Methods.

the exception of the brain, in which the parental virus Phe at GP amino acid 260 predominates. We note a similar preference for the parental ARM strain over the variant in two neuronal tissue culture cell lines, indicating that growth advantages in different cell types are likely the basis of the selection of parental or variant viruses.

RNA viruses have high mutation frequencies (13, 29, 33) which lead to the generation of heterogeneous viral populations both in cultured cells and in in vivo infections. For example, variants of vesicular stomatitis virus are generated at a high frequency both in vitro and in vivo (31, 32). The selection of virus variants in vivo likely occurs as a result of a variety of selective pressures that exist within an organism,

depending on properties of the virus and the specific host cells it infects. In particular, the immune system can contribute to the selection of viral variants, as seen with viruses such as human immunodeficiency virus and Epstein-Barr virus (5, 10).

LCMV is an excellent model with which to study the generation and selection of viral variants. LCMV productively infects most tissues (7, 19), and organ-specific variants which alter host control of the virus have been isolated and characterized (2, 3). Analysis of variants isolated from the spleens of mice infected at birth with LCMV ARM showed that a Phe-to-Leu amino acid change at position 260 of the viral GP was associated with the ability to cause persistent infection in adult mice (1, 22, 27, 28). Why the Phe-to-Leu change at GP
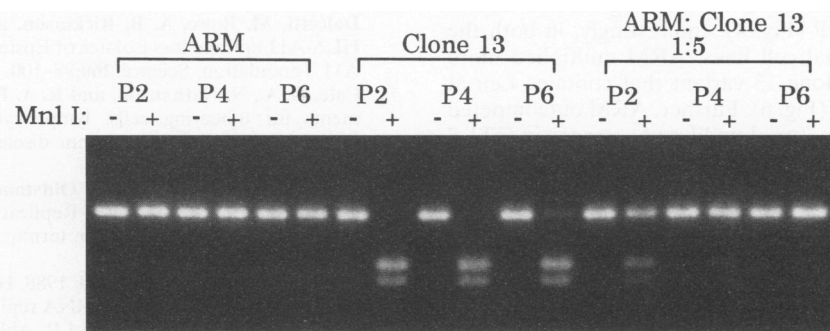
FIG. 7. Competition of ARM versus clone 13 in the mouse neuronal GT1-7 cell line. ARM alone, clone 13 alone, or a 1:5 mixture of ARM and clone 13 was used to infect GT1-7 cells; the viruses released into the supernatants were sequentially passaged six times in GT1-7 cells. After the second (P2), fourth (P4), and sixth (P6) passages, RNA from the infected cells was subjected to RNA PCR-MnlI digestion as described in Materials and Methods.

amino acid 260 is associated with an inadequate anti-LCMV CTL response and generalized immunosuppression is not fully understood. The GP has been implicated in binding to the cellular receptor (6), and preliminary evidence suggests that GP amino acid 260 influences the tropism of the virus in vivo (5a). After translation, the GP is cleaved to GP-1 and GP-2 proteins. Interestingly, amino acid 260 of GP is found in GP-1, only six amino acids from the GP cleavage site (8). This position may be important for association of GP-1 and GP-2 on the surface of the virion, or it may affect processing of the GP precursor to GP-1 and GP-2. Thus, a rather conservative (Phe-to-Leu) amino acid change appears to contribute significantly to the ability of the virus to infect various cell types and to be associated with inducing immunosuppression and causing persistent infection in adult mice.

The Phe-to-Leu change at amino acid 260 of the GP results in the generation of an *Mnl*I restriction site in the cDNA sequence, allowing the use of RNA PCR followed by *Mnl*I digestion to detect the presence of this viral variant within a viral RNA population. RNA PCR-*Mnl*I analysis of RNA from various tissues and organs of mice infected at birth with LCMV ARM showed that the Phe-to-Leu changes were similar in immunocompetent and genetically immunoincompetent (SCID and athymic) mice, indicating that the generation and selection of this mutation is not dependent on parental viral replication in mature B or T lymphocytes. The Phe-to-Leu change was detected first in the serum of mice at 3 weeks of age and then in other lymphoid tissues and liver (Fig. 4). By 2 months postinfection, significant amounts of variant virus with the Leu-260 change were detected in heart and lung, with a low level in the kidney. Interestingly, the brain was markedly resistant to the Leu mutation at position 260 of the GP even after 1 year of infection. The discordance of brain with other tissues (lymphoid, liver, heart, lung) and differences in the kinetics of the GP amino acid 260 change in lymphoid tissues compared with heart and lung indicate that it is improbable that blood contamination within tissues accounts for the detection of the GP amino acid 260 change but rather that there is a selective growth advantage for the GP amino acid 260 variant virus in a selected cell type(s) within every tissue in which the variant was detected.

This hypothesis of specific cell selection was tested and confirmed with studies of the growth of $CTL^+ P^-$ and $CTL^- P^+$ viruses in the neuronal GT1-7 and PC12 cell lines. In persistently infected mice, LCMV replicates almost exclusively in neurons, regardless of whether $CTL^+ P^-$ or $CTL^- P^+$ virus is used to initiate infection (16, 26, 34a). Analysis of viral RNA from the brains of mice persistently infected with ARM during the course of infection from its onset at birth to 1 year revealed minimal amounts of viral nucleic acid coding for the Leu at amino acid 260 of the GP (Fig. 4). Interestingly, in both the GT1-7 and PC12 neuronal cell lines, ARM multiplied more efficiently than did the clone 13 variant that contains Leu at amino acid 260 of the GP (Fig. 6). Further, ARM outcompeted clone 13 for growth during serial undiluted passages in GT1-7 cells, even when the initial infection was done with a 5:1 ratio of clone 13 to ARM. By contrast to neurons in the brain, the variant containing a Leu at amino acid 260 shows a selective growth advantage in immune tissues (Fig. 4) (1, 17). Biologically, the enhanced growth of $CTL^- P^+$ virus in cells of the immune system over that observed with $CTL^+ P^-$ virus parallels the observation that adult mice infected with $CTL^+ P^-$ parental ARM generate a CTL response that clears the viral infection, whereas the CTL response in adult mice infected with $CTL^- P^+$ clone 13 is abortive and a persistent viral infection results. A selective growth advantage of CTL^-

P^+ variant virus within a specific cell type of the immune system may enable this virus to impair the immune response in adult animals and prevent the propagation of an effective CTL response, resulting in immunosuppression. We are currently investigating the mechanism by which viruses with a leucine at GP amino acid 260 cause persistent infection and immunosuppression and have found evidence for a differential tropism of clone 13 versus ARM for certain cells of the immune system (5a).

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