Molecular Determinants of Macrophage Tropism and Viral Persistence: Importance of Single Amino Acid Changes in the Polymerase and Glycoprotein of Lymphocytic Choriomeningitis Virus

MEHRDAD MATLOUBIAN, SUHAS R. KOLHEKAR,† T. SOMASUNDARAM,
AND RAFI AHMED*

*Department of Microbiology and Immunology, University of California at Los Angeles
School of Medicine, Los Angeles, California 90024-1747*

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This study documents that the immunosuppressive lymphocytic choriomeningitis virus (LCMV) variant, clone 13, shows a specific predilection for enhanced infection of macrophages both in vitro and in vivo and that single amino acid changes in the viral polymerase and glycoprotein are responsible for macrophage tropism. The growth difference seen between variant clone 13 and the parental Armstrong strain was specific for macrophages, since both clone 13 and Armstrong grew equally well in fibroblasts and neither isolate infected lymphocytes efficiently. Complete sequencing of the clone 13 genome, along with genetic analysis, showed that a single amino acid change in the polymerase (K→Q at position 1079) was the major determinant of virus yield in macrophages. This was proven unequivocally by comparing the sequences of parental and reassortant viruses, which were identical at all loci except for the single mutation in the polymerase gene. This finding was further strengthened by showing that reversion at this site back to lysine (Q→K) resulted in loss of macrophage tropism. In addition, an independently derived macrophage-tropic variant of LCMV, clone 28b, had a K→N mutation at the same position. Thus, these results show that substitution of the positively charged amino acid K with a neutral amino acid (either Q or N) at residue 1079 of the polymerase resulted in enhanced viral replication in macrophages. In addition to the polymerase change, a mutation in the glycoprotein was also associated with macrophage tropism. This single amino acid change in the glycoprotein (F→L at position 260) did not affect virus yield per macrophage but was critical in determining the number of macrophages infected. Our previous studies have shown that the same two mutations in the polymerase and glycoprotein are essential for establishing a chronic infection in adult mice. Since the same mutations confer macrophage tropism and ability to persist in vivo, these studies provide compelling evidence that infection of macrophages is a critical determinant of viral persistence and immune suppression.

The outcome of a viral infection depends on 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) as a model system to understand the host and viral determinants that lead to viral clearance or persistence (2, 4, 5, 20). Infection of immunocompetent adult mice with the Armstrong strain of LCMV induces a potent antiviral T-cell response, and virus is eliminated within 2 weeks. 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 (2, 4). This chronic infection is associated with suppressed T-cell responses and susceptibility to opportunistic infection (4, 5, 33, 35).

The cell types infected in vivo by the parental Armstrong strain and its variant, clone 13, during the early stages of infection have not been fully defined (8). An understanding of these early virus-cell interactions may provide a cellular basis for the different outcomes of infection (i.e., acute versus chronic) seen in adult mice challenged with these isolates. As a first step toward addressing this issue, we have examined the tropism of these two LCMV strains for various cell types (primary macrophages, lymphocytes, and fibroblasts). In this

study, we document that variant clone 13 shows a specific predilection for enhanced infection of macrophages both in vitro and in vivo and that single amino acid changes in the viral polymerase and glycoprotein are responsible for this macrophage tropism.

MATERIALS AND METHODS

Mice. Six- to 8-week-old BALB/c ByJ and C57BL/6 mice were purchased from Jackson Laboratory, Bar Harbor, Maine.

Virus. The Armstrong CA1371 strain of LCMV was used in these studies (4). The variant, clone 13, was isolated from the spleen of an 8-week-old BALB/c LCMV carrier mouse infected at birth with Armstrong. Clone 28b was isolated from the spleen of a 12-week-old BALB/c LCMV carrier mouse infected at birth with Armstrong. Revertants 1a-1 and 15a-1 were isolated from the brains of adult mice infected with clone 13. All LCMV stocks used in this study (Armstrong, clone 13, and the reassortants) were triple plaque purified on Vero cells, and the stocks were grown in BHK-21 cells. Virus stocks at the passage one or two 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 (5, 22).

Determination of virus titers. Infectious LCMV was quantitated by plaque assay on Vero cell monolayers as previously described (4).

* Corresponding author.

† Present address: National Institute of Virology, Pune, India.

Infection of macrophages in vitro with LCMV. Resident peritoneal macrophages and alveolar macrophages were obtained from 6- to 8-week-old BALB/c ByJ mice. Resident peritoneal macrophages from C57BL/6 mice were also used, with similar results. Isolation of peritoneal macrophages was as previously described (21). Alveolar macrophages were obtained by intratracheal lavage of lungs with phosphate-buffered saline containing 1 mM EDTA (18). Preparations of both types of macrophages were >95% pure as determined by staining with macrophage-specific monoclonal antibody F4/80 (6). Macrophages were plated at 2×10^5 cells per well in 16-mm-diameter wells, and 16 h after incubation at 37°C the nonadherent cells were washed and removed. The adherent cells were infected with LCMV at a multiplicity of infection of 0.25 PFU per cell in a volume of 0.2 ml. After 1 h of virus adsorption at 37°C, the inoculum was removed and 2 ml of fresh Eagle's minimal essential medium with 7% fetal calf serum was added. Supernatant was harvested from separate wells at various times and stored at -70°C for plaque assay on Vero cells.

Immunofluorescence and immunohistochemistry. For immunofluorescence assay, macrophages grown on glass coverslips were fixed in acetone and stained with polyclonal anti-LCMV guinea pig serum and then with fluorescein-conjugated rabbit anti-guinea pig immunoglobulin G (Cooper Biomedical, Inc., Malvern, Pa.). The polyclonal anti-LCMV guinea pig serum used recognizes all the major structural proteins of LCMV and shows no reactivity with uninfected macrophages or tissues from uninfected mice. Duplicate coverslips were used to determine the proportion of LCMV-infected macrophages by counting at least 5,000 cells in 10 to 15 different fields. Double staining for both viral antigen and macrophage marker F4/80 was done on acetone-fixed 6- μ m frozen liver sections. Sections were sequentially incubated with rat monoclonal antibody F4/80, fluorescein-conjugated goat anti-rat serum, guinea pig anti-LCMV serum, and rhodamine-conjugated goat anti-guinea pig serum. For determination of the infection pattern in spleen tissue, frozen sections (6 μ m) were fixed in acetone and stained with polyclonal anti-LCMV guinea pig serum and then by mouse-adsorbed biotinylated goat anti-guinea pig immunoglobulin G. Infected cells were visualized by the addition of avidin-biotin-peroxidase complexes (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif.) and 3-amino-9-ethylcarbazole (AEC) (Sigma, St. Louis, Mo.) as substrate. Tissue sections were then counterstained with Mayer's hematoxylin. The mouse-adsorbed goat anti-guinea pig antibody showed no reactivity with spleen tissues from uninfected mice.

Northern analysis. Total RNA was extracted from a million macrophages by the acid-guanidinium thiocyanate-phenol-chloroform method (10). RNA samples, equivalent to 5×10^5 macrophages each, were denatured with formamide, separated on the basis of size by electrophoresis in a denaturing agarose-formaldehyde gel, transferred to a nitrocellulose filter, and hybridized with 32 P-labeled single-stranded RNA probes. The construct for RNA probe synthesis was made by cloning a cDNA, obtained from reverse transcription of the LCMV Armstrong S segment, between the SP6 and T7 promoters of the pGem3 Riboprobe vector (Promega, Madison, Wis.). This cDNA clone contains nucleotides 34 through 183 of the glycoprotein gene. The resulting RCS-3 plasmid was linearized with the appropriate restriction enzyme, and 32 P-labeled sense or antisense transcripts were made by using either SP6 or T7 RNA polymerase (Promega). Levels of genomic L segment were analyzed by hybridization with a 32 P 5'-end-labeled oligonucleotide complementary to the Z gene. To ensure

comparable loading of RNA samples, filters were stripped and hybridized with an α -actin cDNA probe.

Sequence analysis. The complete L and S segments of the Armstrong and clone 13 strains of LCMV as well as those of their reassortants were sequenced by the primer extension method, using virus-specific oligonucleotides (16). Either 1 to 3 μ g of viral RNA extracted from purified virus or 50 μ g of total infected cell RNA was used for the sequencing reactions.

RESULTS

In vitro growth phenotype of LCMV Armstrong strain and its variant clone 13. To determine the underlying cellular basis for the ability of clone 13 to cause chronic infection in vivo, its replication was compared with that of parental Armstrong in murine fibroblasts, lymphocytes, and macrophages. Cultures of each cell type were infected with the Armstrong or clone 13 strain of LCMV, and the amount of virus released in the supernatant was quantitated by plaque assay. Data from one representative experiment showing the growth of Armstrong and clone 13 in the three different cell populations are presented in Fig. 1. Both Armstrong and clone 13 grew comparably well in BALB C17 cells, a continuous mouse fibroblast cell line (Fig. 1A). Similar results were seen in other fibroblast cell lines (for example, L-929) as well as in primary mouse embryo fibroblasts; in most experiments both strains grew equally well but in some cases the parental Armstrong strain grew slightly better (data not shown). In contrast, when tested for the ability to replicate in primary lymphocyte cultures, neither of these two strains showed any appreciable growth (Fig. 1B), whereas in the same culture, a lymphotropic LCMV isolate, t.1B, replicated well (19). In primary cultures of resident peritoneal macrophages, clone 13 replicated much better than the parental Armstrong (Fig. 1C); consistently, by day 2 after infection, there were 10- to 20-fold higher levels of infectious virus in supernatants of clone 13-infected macrophages than in the Armstrong-infected ones. Similar differences in growth were seen in cultures of alveolar (Fig. 1D) and bone marrow-derived macrophages (not shown). In all three types of macrophages, clone 13 showed better replication, with ~10- to 20-fold-higher levels of virus than the levels of the parental Armstrong strain. In summary, both Armstrong and clone 13 grew equally well in fibroblasts, whereas neither of them could replicate in lymphocytes. In contrast, in primary macrophages clone 13 consistently grew better than the parental Armstrong strain. Thus, the observed growth differences between clone 13 and Armstrong are specific for macrophages and are not seen in fibroblasts and lymphocytes.

In addition to quantitating the amount of infectious virus as shown in Fig. 1, we also determined the percentage of macrophages that were infected by Armstrong or clone 13. At various times after infection, macrophages were fixed and stained for viral antigen with guinea pig anti-LCMV serum, followed by fluorescein-conjugated rabbit anti-guinea pig immunoglobulin G. In all experiments, at 12 and 24 h postinfection there was a difference in the number of macrophages infected by Armstrong or clone 13. At these early phases of infection, there were two- to threefold more cells infected by clone 13 than by Armstrong. For instance, in one experiment at 24 h postinfection, 60% of the macrophages were infected by clone 13, whereas only 30% were infected by Armstrong. However, by 48 h after infection this difference diminished and by 72 h there was virtually no difference in the number of macrophages infected by the two strains. These results suggest that the Armstrong strain of LCMV is less efficient but not completely restricted in entering and infecting macrophages.

FIG. 1. Growth of LCMV Armstrong and variant clone 13 in different cell types in vitro. BALB C17 cells, a continuous mouse fibroblast cell line (A), primary lymphocytes (B), resident peritoneal macrophages (C), and alveolar macrophages (D), were infected with the indicated LCMV strain and the amounts of infectious virus released in the supernatant were quantitated by plaque assay. Growth of a lymphotropic variant of LCMV, t.1B, in lymphocyte cultures is shown for comparison (panel B).

Replication of the two LCMV strains in macrophages was also compared at the RNA level by Northern blot analysis of steady-state intracellular viral RNA. The LCMV genome consists of two segments of single-stranded RNA, each containing two genes coded in an ambisense polarity; that is, on each segment one gene is coded in the negative sense, whereas the other is coded in the positive sense (7, 32). During replication, a full-length complementary or anti-genomic RNA species of each segment is synthesized. Using single-stranded RNA probes derived from the glycoprotein gene, we analyzed the amount of genomic S segment, anti-genomic S segment, and glycoprotein message in Armstrong- or clone 13-infected macrophages. Levels of genomic L segment were analyzed by hybridization with an oligonucleotide probe. This allowed us to examine both replication (production of genomic and anti-genomic RNA species) and transcription (production of message). At days 1 and 2 after infection, levels of all four different LCMV RNA species examined (genomic S and glycoprotein mRNA, Fig. 2A; anti-genomic S RNA, Fig. 2B; and genomic L RNA, Fig. 2C) were three- to fivefold higher in clone 13-infected macrophages than in the Armstrong-infected ones.

This difference remained even at 3 days postinfection when similar numbers of macrophages were infected by the two LCMV strains (data not shown). These results show that the Armstrong strain of LCMV is inefficient in transcription and replication of its genome in macrophages and that mutation(s) in clone 13 have enhanced its ability to replicate and transcribe its RNA in macrophages.

Replication of Armstrong and clone 13 in vivo. To examine the relationship between enhanced in vitro replication in macrophages and persistence in mice, we compared in vivo growth of Armstrong and clone 13 during the early stages of infection. Adult (6- to 8-week-old) BALB/c ByJ mice were infected intravenously, and at various times (10, 24, 48, and 72 h) after infection the amount of infectious virus in liver, spleen, and lung tissues was quantitated by plaque assay. These three tissues were selected because, upon intravenous injection, they receive the majority of the virus inoculum and are the sites of primary virus seeding (24). The early times (10 to 72 h postinfection) were chosen since the host's primary antiviral T- and B-cell responses have not yet developed and the extent of infection seen during this period reflects the intrinsic ability of

FIG. 2. Northern blot analysis of LCMV RNA in macrophages. Total RNA extracted from resident peritoneal macrophages infected with the indicated strain was separated on an agarose-formaldehyde gel and transferred to nitrocellulose paper as described in Materials and Methods. The same blot was probed with single-stranded RNA probes that were specific either for the genomic S RNA and the glycoprotein mRNA (A) or for the anti-genomic S RNA (B). Levels of genomic L segments (C) were analyzed by hybridization with an oligonucleotide probe. At each time point, duplicate samples are shown for the indicated strain, with each lane representing RNA extracted from an independent macrophage culture. Comparable levels of RNA loading were determined by hybridization with an actin probe. Lanes: Arm, Armstrong infected; Cl. 13, clone 13 infected; U, uninfected. Levels of infectious LCMV ($\log_{10}$ PFU/ml) in each culture were as follows: day 1, Arm, 4.19 and 4.16; clone 13, 5.11 and 4.95; day 2, Arm, 4.20 and 4.20; clone 13, 5.08 and 5.08.

a virus to infect various cell types within a given time (24). Results of a representative *in vivo* growth experiment (Fig. 3) show that clone 13 grew better than Armstrong in all three of the organs tested. As shown in Fig. 3A, as early as day 1 postinfection liver virus titers were >10 -fold higher in clone 13-infected mice than in Armstrong-infected ones. This difference remained at days 2 and 3 postinfection and in all experiments became more pronounced afterwards. To identify cell types infected in the liver, sections from infected mice were stained for viral antigen. By day 3 after infection, mice that were challenged with clone 13 had about three times more infected cells in the liver than mice that received Armstrong. Morphologically, the infection was within small, stellate-shaped sinusoidal cells that resembled liver macrophages (Kupffer cells) more closely than the large polyhedron-shaped hepatocytes (Fig. 4A). The identity of these cells as Kupffer cells was confirmed by double staining the same section for LCMV antigen (Fig. 4A) and for the macrophage-specific marker F4/80 (Fig. 4B) (6). These results show that higher virus titers in liver tissues of clone 13-infected mice (Fig. 3A) during the early stages (days 1 to 3) are primarily due to better infection and replication of this virus in Kupffer cells *in vivo*. At these early times, especially day 1, there was minimal to no infection of hepatocytes. By day 5 after infection a substantial number of hepatocytes were infected by clone 13, whereas very few infected hepatocytes could be detected in Armstrong-infected mice. Hence, it is likely that the initial growth

advantage of clone 13 in liver macrophages allowed this variant to spread to parenchymal cells, resulting in production of more infectious virus.

In the spleen similar differences in infectious virus titer were observed as in the liver. As shown in Fig. 3B, by days 2 and 3 postinfection, virus titers in the spleen were 10-fold higher in clone 13-infected mice than in Armstrong-infected ones. Quantitation of the number of infected cells by an infectious center assay showed that about 5 to 10 times as many spleen cells were infected by clone 13 as by Armstrong. Most of the infected cells were in the adherent cell fraction of the spleen, which is composed of macrophages. We could not detect any infection of lymphocytes (less than 1 in 10^6 cells) either by immunofluorescent staining or by infectious center assay. This *in vivo* observation is consistent with the *in vitro* studies showing that neither Armstrong nor clone 13 was able to infect lymphocytes efficiently (Fig. 1B). Results of immunostaining of spleen tissues from infected mice for viral antigen were in accord with the infectious center assay results (Fig. 5). Three days after infection, very few splenic cells were infected by Armstrong (Fig. 5A). In contrast, at this time there was substantial infection of the spleen by clone 13 (Fig. 5B); this infection was localized to cells of the marginal zone and white pulp, both of which contain macrophages that play a role in antigen presentation (Fig. 5B) (23). Infection of these splenic macrophages by clone 13 and alteration of their functions may provide an explanation for the immune suppression observed in mice infected by this variant.

The largest difference between these two strains in infectious virus production was seen in the lung. As shown in Fig. 3C, virus titers in lung tissue were up to 200-fold higher in clone 13-infected mice than in the Armstrong-infected ones. Although we did not identify the cell types infected in the lung, this *in vivo* growth difference is in accord with our *in vitro* results with alveolar macrophages. In primary cultures of these macrophages, clone 13 virus titers were 20-fold higher than Armstrong titers (Fig. 1D).

Our *in vivo* studies show that during the early stages of infection clone 13 replicates to a higher extent and within more macrophages than Armstrong does, which is in accord with our *in vitro* results (Fig. 1C and D). Better replication of clone 13 in macrophages was reflected in higher levels of virus in the serum of these mice (Fig. 3D). Early after infection (days 1 to 3), there was 10-fold more virus in the sera of clone 13-infected mice than in the Armstrong-infected ones. This initial high level of viremia allows clone 13 to disseminate and seed additional visceral organs (pancreas, kidneys, adrenal glands, and urogenital tract) as well as the bone marrow, thymus, and the central nervous system (brain and spinal cord). In contrast, because of its relatively poor initial replication and the resultant lower viremia, Armstrong is unable to disseminate to other sites; Armstrong infection remained localized within the primary sites of infection since virus was undetectable in the central nervous system and other visceral organs (4, 5, 22). Hence, the tremendous initial replicative advantage of clone 13 in macrophages results in more virus production and higher viremia, allowing this variant to disseminate and establish a more widespread infection. A consequence of dissemination is that it presents the immune system with a greater challenge since a greater and prolonged response is required to clear a widespread infection than a more localized one.

Genetic determinants of macrophage tropism. We next examined the genetic basis of clone 13 macrophage tropism. 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 (7, 32). The L RNA segment codes for a

FIG. 3. In vivo growth kinetics of Armstrong and clone 13 in various tissues. Six- to 8-week-old BALB/c ByJ mice were infected intravenously with 1.5×10^6 PFU of the indicated strain. At various times, virus titers in liver (A), spleen (B), lung (C), and serum (D) were determined by a plaque assay on Vero cells. Each point is the average of the results from two mice, with vertical bars indicating the range of titers. The first time point corresponds to 10 h postinfection. The dashed line indicates the level of detection by plaque assay.

large protein, L (molecular size, 250 kDa), that is believed to be the viral polymerase and also contains a second open reading frame, designated Z, that encodes a 10- to 12-kDa protein that may play a role in regulation of transcription and/or replication of viral RNA (12, 27, 28, 30). The S segment codes for the three major structural proteins: the internal nucleocapsid protein (NP, 63 kDa) and the two surface glycoproteins GP-1 (43 kDa) and GP-2 (36 kDa) that are derived from a common precursor polypeptide, GP-C. Complete sequence analysis of the Armstrong and clone 13 genomes revealed four nucleotide differences: two in the L segment and two in the S segment (Fig. 6). In the L segment, both mutations were in the polymerase gene (Fig. 6A): the one at nucleotide 5952 is silent, whereas the mutation from A to C at position 3959 results in an amino acid change from K to Q at residue 1079 of the polymerase protein. There were no mutations in the Z gene, the intergenic region, or the non-coding regions at the 5' and 3' ends of the L segment. In the S segment both mutations were in the glycoprotein gene (Fig. 6B). The one at position 1298 is silent, whereas the change from U to C at nucleotide 855 results in an amino acid change

from F to L at residue 260 of the glycoprotein. Our sequence is in accord with the results of Salvato et al. (27–29), except for one nucleotide difference in the polymerase gene (31a). However, our genetic analysis is not affected by this discrepancy since both Armstrong and clone 13 share the same sequence at this position.

To identify the genetic determinants of clone 13 macrophage tropism, we constructed reassortants between Armstrong and clone 13 and confirmed their identities by sequencing (1, 22). The two reassortants used were C406a1 and T800. C406a1 has the L segment of clone 13 and the S segment of Armstrong, and its genotype is designated as 13/Arm. T800 (Arm/13) has the L segment of Armstrong and the S segment of clone 13. Macrophages were infected with these two reassortants and the two parental viruses (Armstrong and clone 13) to assess the contributions of the polymerase and glycoprotein mutations. Figure 7 shows that in resident peritoneal macrophages clone 13 (13/13) replicated much better than reassortant T800 (Arm/13). These two viruses (clone 13 and T800) are identical in all genes except for the single amino acid mutation in the polymerase. As shown in Fig. 7, this change (K→Q) is

FIG. 4. In vivo infection of Kupffer cells by clone 13 strain of LCMV. Liver sections from a BALB/c ByJ mouse 3 days after infection with clone 13 were double stained for LCMV antigen and for the macrophage-specific marker F4/80. Panel A shows presence of LCMV antigen as detected by rhodamine, and panel B shows staining for macrophage marker as detected by fluorescein.

the major determinant of virus yield in macrophages. This conclusion is further supported by comparison of a second pair of viruses that only differ in the polymerase mutation: reassortant C406a1 (13/Arm) had much higher yields of infectious virus than Armstrong (Arm/Arm) (Fig. 7). A similar contribution of the polymerase mutation to virus yield was also observed in alveolar and bone marrow-derived macrophages (data not shown). Analysis of steady-state RNA levels was in accord with the growth data; comparison of two viruses that differed only in the polymerase gene showed that the K→Q mutation of clone 13 enhances viral RNA replication in

macrophages (data not shown). The genetic and sequence analysis together establish unequivocally that the single amino acid change (K→Q) in the viral polymerase specifically enhances the growth of clone 13 as well as the intracellular levels of viral RNA in macrophages.

The importance of residue 1079 in the polymerase for macrophage tropism is further strengthened by analysis of phenotypic revertants isolated from clone 13-infected mice as well as by analysis of other macrophage-tropic LCMV variants derived from Armstrong. We have previously shown the emergence of revertants in the central nervous systems of mice

FIG. 5. Infection of spleen tissue by Armstrong and clone 13. Immunostaining of spleen sections from BALB/c ByJ mice 3 days after infection with either Armstrong (A) or clone 13 (B) for LCMV antigen. Note the infection (black staining areas) of follicles with clone 13 (panel B), especially the involvement of the marginal zone and white pulp. Levels of infectious LCMV ($\log_{10}$ PFU) in these samples were as follows: Armstrong, 6.7; clone 13, 7.6. Magnification, $\times 40$.

FIG. 6. Sequence differences between Armstrong and clone 13. The complete genomes of Armstrong and clone 13 were sequenced by the primer extension method as described in Materials and Methods. (A) The L segments (~7.2 kb) of these two viruses differ by only two nucleotide changes, both in the polymerase gene. The mutation at position 3953 results in a K→Q change at amino acid 1079 of the polymerase, whereas the mutation at position 5952 is silent. (B) The S segments (~3.4 kb) of these two viruses differ by only two nucleotides, both in the glycoprotein gene. The mutation at position 855 results in an amino acid change from F→L at residue 260 of the glycoprotein, whereas the mutation at position 1298 is silent. There are no nucleotide changes in the Z and nucleoprotein genes, the intergenic regions, or the noncoding regions at the 5' and 3' ends of both segments.

persistently infected with clone 13 (3). In contrast to clone 13, these revertants cause an acute infection in adult mice. The growth of two independently (different mice) derived revertants, 1a-1 and 15a-1, in macrophages is shown in Table 1. Neither revertant replicated as well as clone 13 in macrophages; for both revertants the amount of infectious virus released in the supernatant was 8- to 20-fold lower than that of clone 13. Partial sequence determination of the polymerase gene showed that at residue 1079 of this protein both of these revertants have changed from Q (clone 13 sequence) to K (Armstrong sequence) (Table 1). Hence, reversion at this site back to lysine (Q→K) in these isolates resulted in the loss of macrophage tropism. Analysis of an independently derived macrophage-tropic isolate, clone 28b, showed that a change from K to an amino acid other than Q at residue 1079 of polymerase could also affect replication in macrophages. Clone 28b was isolated from the spleen of an LCMV carrier mouse infected at birth with Armstrong. Table 1 shows that clone 28b replicated as well as clone 13 in macrophages *in vitro*; levels of infectious virus released into the supernatant of clone 28b-infected macrophages were >10-fold higher than those in the supernatant of parental Armstrong-infected macrophages.

FIG. 7. Single amino acid change in the polymerase (K→Q at position 1079) determines virus yield in macrophages. Primary cultures of resident peritoneal macrophages were infected with Armstrong, clone 13, and the two reassortants, C406a1 and T800, and the amount of infectious virus released in the supernatant was quantitated by a plaque assay. The following notation is used to indicate the genotype: Arm/Arm, virus with both L and S segments of Armstrong; 13/13, virus with both L and S segments of clone 13; 13/Arm, reassortant with the L segment of clone 13 and the S segment of Armstrong; Arm/13, reassortant with the L segment of Armstrong and the S segment of clone 13. Clone 13 (13/13) and reassortant T800 (Arm/13) differ by only a single amino acid in the polymerase. The same difference is also true for Armstrong (Arm/Arm) and reassortant C406a1 (13/Arm).

Furthermore, adult mice infected by this variant had high levels of viremia even a month after infection. Partial sequence analysis of clone 28b showed that this variant has a mutation from K→N at position 1079 of the polymerase. It is worth noting that in two macrophage-tropic isolates derived from Armstrong (clones 13 and 28b) the same amino acid in the polymerase has been changed from K to two very similar amino acids, Q and N. In summary, the results presented in Table 1 emphasize the importance of this position in the polymerase for enhanced replication in macrophages. Furthermore, the data presented in this table also show the correlation between macrophage tropism and ability to persist in adult mice. Mice that are infected with the two variants that grew well in macrophages (clones 13 and 28b) had high levels of viremia 30 days postinfection. In contrast, the variants that grew poorly in macrophages (Armstrong and revertants 1a-1 and 15a-1) were cleared from serum within 2 weeks after infection. These results stress the importance of macrophage tropism for *in vivo* viral persistence.

Immunofluorescence analysis of infection of macrophages by the two strains (Armstrong and clone 13) and their reassortants showed that the mutation in the viral glycoprotein was the determinant of the percentage of macrophages infected by LCMV (Fig. 8). Twice as many macrophages were infected by clone 13 (13/13) as by reassortant C406a1 (13/Arm); these two viruses differ only in one amino acid in the glycoprotein. The difference in the percentage of macrophages infected was present initially at 24 h postinfection and diminished by 72 h. These results show that the single amino acid change (F→L) in the viral glycoprotein increases the ability of clone 13 to infect

TABLE 1. Importance of amino acid 1079 of the polymerase for growth in macrophages and persistence in adult mice

LCMV strain ^a	Amino acid at residue 1079 of polymerase	LCMV titer in macrophages ^b (log ₁₀ PFU/ml) at day postinfection:			LCMV titer in serum ^c (log ₁₀ PFU/ml) at day postinfection:		
		1	2	3	8	15	30
Armstrong	K	4.2	4.6	5.0	<1.7	<1.7	<1.7
Clone 13	Q	5.2	5.9	5.8	5.9	5.4	4.4
Clone 28b	N	5.3	5.8	5.7	6.2	5.4	4.4
Revertant 1a-1	K	4.9	5.0	4.3	1.9	<1.7	<1.7
Revertant 15a-1	K	4.3	5.1	4.8	2.0	<1.7	<1.7

^a Clone 28b was isolated from the spleen of a 12-week-old BALB/c LCMV carrier mouse infected at birth with Armstrong. Revertants 1a-1 and 15a-1 were isolated from brain tissues of two individual BALB/c mice persistently infected with clone 13.

^b Cultures of resident peritoneal macrophages were infected with the indicated LCMV strain at a multiplicity of infection of 0.25, and the amount of infectious LCMV released in the supernatant was determined by plaque assay.

^c Adult BALB/c mice were infected intravenously with 10⁶ PFU of the indicated virus. Blood samples were obtained from the eyes of mice at the indicated times, and the titer of infectious LCMV in the serum was determined by plaque assay on Vero cells. The data shown are the averages of two to four mice per group.

macrophages. In summary, genetic and sequence analyses show that both mutations in clone 13 contribute to its macrophage tropism: the polymerase mutation determines the yield of infectious virus, whereas the glycoprotein change affects the number of infected macrophages.

DISCUSSION

This study provides a cellular and molecular basis for the persistence of LCMV variant clone 13 in adult mice. In this study, we showed that both in vitro and in vivo, clone 13 replicates better in macrophages than its parental strain Armstrong. This difference in replication is specific for macrophages since both strains grow equally well in fibroblasts and neither infects lymphocytes efficiently. Furthermore, we documented that single amino acid mutations in the viral polymerase and glycoprotein are responsible for this macrophage tropism.

Our previous studies have shown that the same two mutations that enhance replication in macrophages are also necessary for clone 13 persistence in adult mice, thus giving support to the notion that the macrophage-tropic phenotype of clone 13 is the basis for its ability to establish a chronic infection (1, 5, 22). Studies with several other viral systems, such as human immunodeficiency virus, have documented the important role of viral surface glycoproteins in determining cell tropism (9, 26, 34). We found that a single amino acid mutation (F→L at amino acid 260) in the LCMV glycoprotein affects an early step in infection, such as the efficiency of viral attachment or penetration. However, the surprising finding of our study is that a single amino acid change (K→Q at amino acid 1079) in the viral polymerase is the major determinant of infectious virus production in macrophages. This finding emphasizes the fact that genes other than those in the envelope glycoprotein can be important determinants of cell tropism. The contribution of the polymerase to cell tropism is strengthened by two additional observations. (i) A reversion at residue 1079 in the polymerase back to lysine (Q→K) results in loss of macrophage tropism, and (ii) a different mutation (K→N) at the same residue conferred macrophage tropism to another independently derived LCMV isolate, clone 28b.

These results show that mutation of K (parental Armstrong) to either Q (clone 13) or N (clone 28b) results in enhanced replication in macrophages. The exact mechanism by which a mutation at this residue in the polymerase specifically affects replication in macrophages is currently not known. Since this mutation affects virus yield in macrophages and not in fibroblasts, one possibility is that the amino acid residue at this

position is important for, or facilitates, interaction of the LCMV polymerase with a macrophage-specific factor(s), resulting in enhanced replication of viral RNA. It is worth noting that in both cases there is a mutation from an amino acid (K) that is positively charged at physiological pH to an amino acid (Q or N) which is neutral at this pH. Hence, the charge of the residue at position 1079 of the polymerase may determine its function as well as its interaction with macrophage-specific factors. Studies are in progress to investigate this possibility.

What are the in vivo biological consequences of macrophage tropism? Enhanced replication in these crucial cells of the immune system can influence at least two important aspects of virus-host interactions: (i) the extent of viral dissemination and (ii) the magnitude of the host's immune response.

Macrophages play a pivotal role during the early stages of viral infections, since they are among the first cells to encounter the invading virus. Hence, they form a cellular barrier, whereby the outcome of infection depends on the extent of viral replication within these cells. A virus that replicates well in macrophages can overcome the first barrier and spread not only to other cells within that tissue but also to other tissues. In our study, better replication of LCMV clone 13 in liver macrophages resulted in subsequent spread to hepatocytes. Furthermore, the initial successful infection and replication in macrophages resulted in higher viremia and provided a source of dissemination to other tissues. Productive infection of macrophages can lead not only to a higher viremia, but infected blood monocytes could also serve to disseminate virus to other tissues.

Infection of adult immunocompetent mice with clone 13 not only leads to a chronic infection but results in suppressed T-cell immunity (4, 33, 35). These mice have low levels of virus-specific cytotoxic T lymphocytes and are also impaired in generating T-cell responses to other antigens. This generalized immune suppression results in susceptibility to opportunistic infections (35). As a first step toward understanding the underlying mechanisms of this immune suppression, it was important to compare the infection of cells of the immune system (lymphocytes and macrophages) by this virus and its parental strain, Armstrong, which is not immunosuppressive. Our in vivo and in vitro results document that neither strain infects lymphocytes efficiently. However, a basic but crucial difference between clone 13 and Armstrong is the greater ability of the immunosuppressive variant (clone 13) to infect and replicate in macrophages. Macrophages play a central role in initiation and maintenance of T-cell responses by presenting virus-derived peptides with major histocompatibility complex (MHC) class I and II molecules as well as by providing other

FIG. 8. Contribution of the F→L mutation in the LCMV glycoprotein to infection of macrophages. Resident peritoneal macrophages were infected with the indicated LCMV strain or reassortant, and the percentage of macrophages that were infected was determined as described in Materials and Methods. (A) Representative experiment with each point being the average of duplicate samples. Designation of reassortant genotypes is described in the legend to Fig. 7. (B and C) Immunofluorescent staining of macrophages for LCMV antigen 24 h after infection with clone 13 (13/13) (B) or reassortant C406a1 (13/Arm) (C). Clone 13 (13/13) and reassortant C406a1 (13/Arm) differ by only a single amino acid in the glycoprotein. Clone 13 has an L at residue 260 of the glycoprotein, whereas C406a1 has an F at this position. The same difference is true for Armstrong (Arm/Arm; F at residue 260) and reassortant T800 (Arm/13; L at residue 260). Magnification, $\times 200$.

costimulatory factors, such as adhesion molecules and cytokines. Viral infections have been shown to affect expression of MHC and adhesion molecules as well as to alter cytokine production (14, 15, 31). Perturbation of any of these primary (antigen and MHC) or secondary (adhesion and costimulatory molecules and cytokines) signals by viral infection may lead to a suboptimal T-cell response or to suppression of an ongoing response.

In conclusion, macrophage tropism is a crucial first step for establishment of a chronic viral infection, since it not only allows the virus to overcome the initial barrier but also provides the virus with an opportunity to alter the initiation and magnitude of the impending immune response. Two factors that can affect the immune response are the number and types of macrophages infected by clone 13 *in vivo*. Substantial numbers of macrophages within lymphoid tissues are infected by this virus, resulting in the presence of a greater amount of viral antigen. This antigenic overload leads to chronic stimulation of LCMV-specific lymphocytes and may induce an anergic state in the overstimulated cells (19, 25). Furthermore, as noted before, during the early stages of infection clone 13-infected spleen macrophages were within the marginal zone and white pulp. These cells play a crucial role in antigen presentation and priming of the T-cell response (23). Infection of these cells by clone 13 may have a profound effect on their immunomodulatory functions. Experiments are in progress to more precisely identify the type of macrophages infected in lymphoid tissues and to measure their functional capacity. The results of our study have implications toward understanding how other viruses cause immune suppression. Two of the best-studied examples of immune suppression associated with a viral infection in humans are human immunodeficiency virus and measles virus, and it is worth noting that both of these viruses infect macrophages *in vivo* (11, 13, 17). Infection of these key cells of the immune system may be a critical determinant of virus-mediated immune suppression.

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