

# Arenavirus Diversity and Evolution: Quasispecies In Vivo

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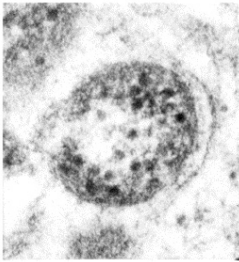
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**Abstract** Arenaviruses exist as viral quasispecies due to the high mutation rates of the low-fidelity viral RNA-dependent RNA polymerase (RdRp). This genomic heterogeneity is advantageous to the population, allowing for adaptation to rapidly changing environments that present varying types and degrees of selective pressure. The significant variation in biological properties observed among lymphocytic choriomeningitis virus (LCMV) strains, the prototypic arenavirus, indicates to what extent a quasispecies dynamics may play a role in arenavirus adaptability and pathogenesis. Several aspects of arenavirus variability and its contribution to pathogenesis will be discussed.

## 1 Genetic Variability of Arenaviruses

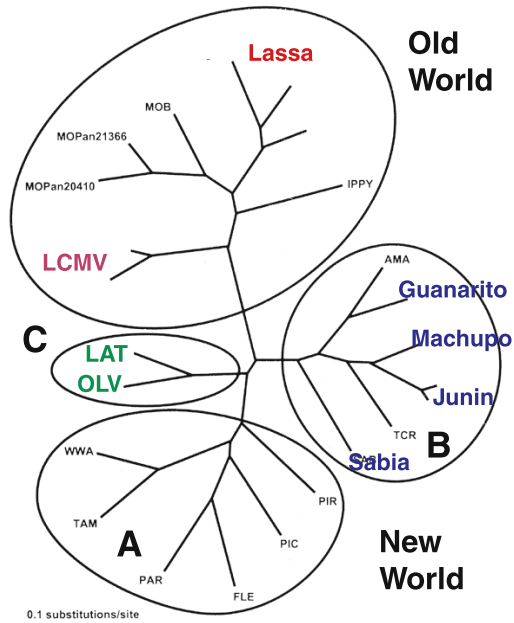
The family Arenaviridae comprises two distinct complexes: the LCMV-Lassa complex, which includes the Old World arenaviruses, and the Tacaribe virus (TACV) complex, which includes all known New World arenaviruses (Fig. 1). Early sequence analysis of arenaviruses revealed a significant degree of genetic stability with amino acid sequence homologies of 90%–95% among different strains of the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV), whereas significantly higher levels of genetic diversity (37%–56%) were observed for homologous proteins of different arenavirus species (Fulhorst et al. 1996; Southern 1996). More recently, genetic studies with a variety of arenavirus field isolates, including Lassa (Bowen et al. 2000), Junin (Garcia et al. 2000), Guanarito (GTO) (Weaver et al. 2000), Pirital (PIR) (Fulhorst et al. 1999), and Whitewater Arroyo (Fulhorst et al. 2001) have revealed a high degree of genetic variation among geographical and temporal isolates of the same virus species. Notably, a remarkably high level of genetic

### Arenaviruses



LCMV  
Lassa virus

Junin  
Guanarito  
Machupo  
Sabia



**Fig. 1** Phylogenetic organization of Arenaviridae and electron micrograph of the prototypic Arenavirus LCMV

divergence (26% and 16% at the nt and amino acid level, respectively) has been documented among PIR isolates within very small geographic regions (Weaver et al. 2001)

The substantial degree of inter- and intra-species genetic variation among arenaviruses appear to have important biological correlates, as suggested by the significant variation in biological properties observed among LCMV strains. In addition, early studies recognized the influence of the passage history on the virus biological properties (Hotchin et al. 1971; Hotchin et al. 1975; Hotchin and Sikora 1973). Thus, early mouse brain passages of LCMV were associated with immunologic tolerance and low mortality in newborn mice. In contrast, late mouse brain passages of LCMV lacked the tolerance-inducing capacity and caused high mortality. Likewise, neonatal infection of certain mouse strains with LCMV strains Armstrong (ARM) and E-350 induced growth hormone deficiency syndrome (GHDS) associated with severe hypoglycemia, which frequently resulted in the death of the infected mice. In contrast, infection of the same mouse strains with LCMV strains WE and Traub did not cause GHDS. These striking phenotypic differences were associated with the ability of LCMV ARM and E-350, but not WE and Traub, to replicate at high levels in the GH-producing cells in the anterior pituitary (Oldstone et al. 1985). Moreover, LCMV variants with distinct phenotypes are often selected in different organs of infected mice. Thus, most isolates from the central nervous system (CNS) of mice persistently infected since birth with ARM tend to produce acute infection in adult mice, whereas isolates from the spleen of the same mice exhibit frequently an immunosuppressive phenotype that favors the establishment of persistence (Ahmed and Oldstone 1988). Such dramatic phenotypic differences among genetically closely related LCMV isolates indicate that a few amino acid replacements in LCMV proteins could suffice to produce important alterations in the virus biological properties. This, in turn, raises the question as to what extent quasispecies dynamics may play a role in arenavirus adaptability and pathogenesis.

## 1.1

### **Arenavirus Error-Prone Replication: Mutation, Recombination, and Reassortment**

The lack of proofreading–repair activities in viral RNA-dependent RNA polymerases (RdRp) is a key determinant of the observed high mutation rates for riboviruses. Thus, mutation rates during RNA genome replication are in the range of  $10^{-3}$ – $10^{-5}$  substitutions per nucleotide copied (Drake and Holland 1999; Holland 1992). This means that ribovirus genomes are highly unlikely to undergo two rounds of template copying without introducing an incorrect

nucleotide into the product. As with other RdRp, the LCMV L polymerase does not contain a recognizable proofreading exonuclease domain. However, it cannot be excluded that the Arenavirus replicase complex could include some cellular subunit with a capacity for error correction.

In addition to mutation, both RNA recombination and genome reassortment can contribute to the genetic variability of arenaviruses. Recombination is particularly relevant for some positive-strand RNA viruses including picornaviruses and coronaviruses. Recombination can facilitate the generation of divergent genome combinations to produce large evolutionary jumps, as well as the rescue of fit genomes from debilitated mutated parents. In contrast, recombination in negative-strand (NS) RNA viruses, including arenaviruses, occurs at frequencies several orders of magnitude lower than in most positive-strand RNA viruses. Moreover, experimental evidence from both laboratory setting experiments and field molecular epidemiology indicates that recombination between two NS ribonucleoproteins (RNPs) is a very rare event, suggesting that perhaps only intramolecular recombination plays a significant role in the evolution of NS RNA viruses. Nevertheless, natural evidence for genetic recombination between two TACV complex members has been reported as the origin whitewater arroyo virus, the most recently discovered arenavirus (Charrel et al. 2001; Fulhorst et al. 1996).

The segmented nature of the arenavirus genome facilitates the generation of reassortant viruses during co-infection of the same cell by two distinguishable parental viruses. In cell culture, reassortant viruses can be generated very efficiently. In some cases, their frequency is close to the theoretical predicted maximum of 50%. However, even among genetically closely related viruses, some particular combinations appear to be excluded. Hence, some specific reassortants are never recovered (Teng et al. 1996a). It is more difficult, however, to assess the contribution of segment reassortment to Arenavirus biology and evolution in the field. As with recombinant viruses, the generation of such reassortants would require co-infection of the same rodent host with two different arenaviruses. Dual infection of the same host may be highly restricted due to homotypic viral interference, which can be readily demonstrated with several arenaviruses including LCMV (Welsh and Pfau 1972) and TV (Gimenez and Compans 1980). This phenomenon is not strictly strain-specific, as illustrated by the existence of interference among different pairs of LCMV strains. Heterotypic interference between arenaviruses has been occasionally reported, and its degree appears to correlate with the genetic relationship of the viruses (Damonte et al. 1983; Welsh and Pfau 1972). This superinfection exclusion phenomenon could contribute to explaining the observed population partitioning in the field, resulting in the maintenance of independent evolutionary lineage of the

same strain within a small geographic range (Weaver et al. 2001). Nonetheless, it is plausible that in some instances arenavirus reassortants occur and they can display pathogenic potential, which is not manifested by any of the two parental viruses (Riviere 1987; Riviere et al. 1986; Riviere and Oldstone 1986).

## 1.2

### **Quasispecies Swarms and Adaptive Value of Arenavirus Mutant Spectra**

RNA viruses, even clonal populations, are organized as dynamic mutant distributions termed viral quasispecies (Domingo et al. 2001a; Domingo and Holland 1997). In each infected cell, replicating genomes compete for whatever resources might be limiting (nucleotide substrates, ribosomes, membrane sites, etc.) and needed to complete the virus life cycle. RNAs replicating in two separate cells do not enter into competition and could be regarded as separate quasispecies in separate compartments. However, virus progenies from different cells or tissues may eventually meet in a common compartment (e.g., a new organ such as the brain, or the blood stream), where they may then compete. Error-prone replication might contribute to spatial and temporal heterogeneities in RNA genome populations. Thus for LCMV, selection of organ-specific variants has been extensively documented (Ahmed and Oldstone 1988; Ahmed et al. 1984a).

Viral quasispecies contain a plethora of variants, which allows these quasispecies to display different phenotypes in response to environmental demands. The influence of mutant distributions on the biology of RNA viruses is supported by demonstration of the presence and selection of variants in quasispecies evolving *in vivo* as well as evidence that mutant spectrum complexity may have a predictive value with regard to the response to antiviral treatments and to shifts from acute into chronic phases of viral disease. The presence of biologically significant variants within quasispecies is well documented by the presence of HIV variants resistant to viral inhibitors in patients who have not been subjected to therapy with the inhibitors, and some of these variants are likely to contribute to viral persistence and pathogenesis (Domingo 2003a, 2003b, Domingo et al. 2001a, 2001b; Domingo and Holland 1997). Although a great majority of mutations harbored in mutant swarms of RNA virus populations may not have either short- or long-term selective value, the evolution of viral quasispecies can, however, be adaptive and influence viral pathogenesis (Domingo 2001a, 2003a; Domingo and Holland 1997).

Increasing evidence indicates that the complexity of mutant spectra influences the virus pathogenic potential, response to treatments and its ability to establish chronicity (Domingo 2003a, 2003b; Domingo et al. 2001b). Moreover,

it seems plausible that parameters that can be quantified in viral quasispecies could be used as surrogate markers to anticipate the virus responses to treatment and its potential for chronicity (Farci et al. 2000; Pawlotsky et al. 1998).

### 1.3

#### **Mutation Frequencies in Arenavirus Populations**

No systematic measurements of mutant spectrum complexity have been reported for arenavirus populations. However, CTL escape mutants were readily selected in T cell receptor (TCR) transgenic mice infected with high doses ( $10^6$  PFU) of LCMV (Pircher et al. 1990). Escape mutants included single amino acid replacements in T cell epitopes. CTLs from infected, transgenic mice lysed target cells coated with peptides representing the wild-type T cell epitopes, but not cells coated with variant peptides. Observations in cell culture also suggest high frequencies of CTL escape mutants. The latter were readily selected when B6-SV40 primary fibroblasts were infected, under CTL selective pressure, at a multiplicity of infection (MOI) of 1 or  $10^{-3}$ , but not  $10^{-5}$ , suggesting that escape mutants either pre-existed in the LCMV population or occurred early in the course of one replication round in the infected cells (Aebischer et al. 1991). Although CTLs play a key role in the control of LCMV infection, a neutralizing antibody response may be involved in the control of viremia and viral clearance in the absence of  $CD8^+$  T cells (Ciurea et al. 2001). High doses of LCMV-WE led to an enhanced antibody response, and control of virus replication in  $CD8^{-/-}$  mice early on during infection. Nonetheless, neutralizing-resistant LCMV mutants emerged after weeks of CTL absence. The mutants included amino acid substitutions within GP1, and manifested a strong tendency to persist in mice. In addition to amino acid replacements, silent mutations (synonymous, not leading to amino acid substitutions) were found at frequencies of  $3 \times 10^{-4}$  substitutions per nucleotide, which would argue for considerable complexity of LCMV in these infected mice.

Recent studies exploring the use of lethal mutagenesis to combat arenavirus infections have provided more direct estimates of the error frequency associated with RNA synthesis mediated by the Arenavirus polymerase (Grande-Perez et al. 2002; Ruiz-Jarabo et al. 2003). The observed mutation frequencies of  $2.6 \times 10^{-4}$  to  $5.5 \times 10^{-4}$  for the NP, GP, and L regions analyzed are consistent with previous findings on genetic heterogeneity of LCMV (Sevilla et al. 2002) and other arenaviruses (Bowen et al. 1996, 1997, 2000; Charrel et al. 2003; Fulhorst et al. 2001; Weaver et al. 2000, 2001), and also within the range commonly observed for riboviruses (Domingo et al. 2001a).

## 2

### **Contribution of Arenavirus Genetic Variability to Persistence and Disease**

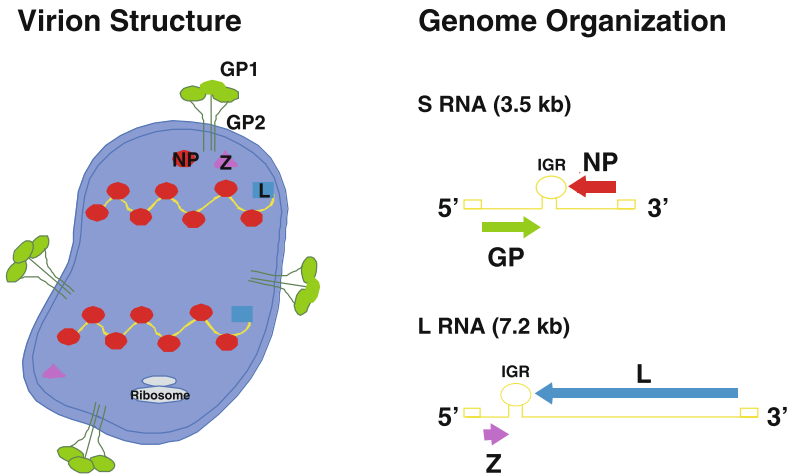
#### 2.1

##### **Overview of LCMV Biology**

The prototypic Arenavirus LCMV has been used extensively as a model system for the study of viral persistence and pathogenesis. LCMV can infect its natural host, the mouse, either acutely or persistently. The outcome of the infection depends on both host and viral determinants, including the genetics, age, and immune status of the host, as well as route and dose of the virus inoculum (Buchmeier et al. 2001; Buchmeier and Zajac 1999). Investigations using this viral model have been central to defining basic virologic and immunologic concepts including the major histocompatibility complex (MHC) restriction of T cell recognition, tolerance, immunological memory, immune-mediated pathology, as well as the strategies by which viruses evade the host immune responses (Buchmeier et al. 2001; Buchmeier and Zajac 1999). Studies of LCMV virus–host interaction have also uncovered the ability of noncytolytic persistent viruses to induce disease by disrupting specialized functions of infected cells, revealing a new way by which viruses do harm in the absence of the classic hallmarks of cytolysis and inflammation (de la Torre and Oldstone 1996, 1989, 1991, 1998, 2002; Oldstone et al. 1982).

LCMV is an enveloped virus with a bisegmented negative strand (NS) RNA genome (Buchmeier et al. 2001; Meyer et al. 2002) (Fig. 2). Each of the two-genome segments, designated L (~7.2 kb) and S (~3.4 kb), expresses two viral gene products using an ambisense coding strategy. The S RNA directs the synthesis of the nucleoprotein, NP, (ca 63 kDa) and the GP-C enveloped glycoprotein precursor. NP, encoded in antigenome polarity, is the most abundant viral protein and encapsidates viral genomes and antigenomic replicative intermediates. GP-C, encoded in genome polarity, is post-translationally cleaved by the cellular subtilase S1P into mature viral glycoproteins, GP-1 (40–46 kDa) and GP-2 (35 kDa) (Beyer et al. 2003; Pinschewer et al. 2003). Noncovalently associated GP1/GP2 complexes make up the spikes on the virion envelope, and mediate virus interaction with the host cell receptor (Cao et al. 1998; Kunz et al. 2002). The L segment codes for the virus RNA-dependent RNA polymerase (RdRp) (L, ~200 kDa) (Salvato et al. 1989) and a small (11 kDa) RING finger protein called Z (Salvato 1993; Salvato et al. 1992; Salvato and Shimomaye 1989), which functions as the arenavirus counterpart of the matrix protein found in many NS RNA viruses (Perez et al. 2003). Additional roles of Z in the Arenavirus life cycle have been proposed based on its interaction with several host cell proteins and its ability to inhibit RNA synthesis mediated

## Lymphocytic choriomeningitis virus (LCMV)



**Fig. 2** Schematic representation of the Arenavirus genome organization and virion structure

by the LCMV polymerase (Borden et al. 1998a, 1998b, Campbell Dwyer et al. 2000; Cornu and de la Torre 2001, 2002).

Closely related variants of the same LCMV strain can display remarkable phenotypic differences *in vivo*, including those related to disease manifestations. We will next discuss two different LCMV–host interactions that illustrate how the generation and selection of specific variants during the natural course of LCMV infection can significantly influence the outcome of the infection.

### 2.2

#### Selection of LCMV Variants *In Vivo*: Role in Viral Pathogenesis

When immunocompetent adult mice are injected with LCMV, they generate a marked immune response to eliminate the infectious agent. Virus clearance is mediated by major histocompatibility complex (MHC) class-I restricted CD8<sup>+</sup> antiviral cytotoxic T lymphocytes (CTL) (Borrow and Oldstone 1997; Buchmeier and Zajac 1999; Oldstone 2002). In contrast, mice infected neonatally or in uterus with LCMV become persistently infected for life. This persistent infection is due to the infection of thymic cells and the specific removal (negative selection) of lymphocytes with potential responsiveness to LCMV (Borrow and Oldstone 1997; Buchmeier and Zajac 1999; Oldstone 2002). This



long-term persistent infection allows the generation of viral variants that have specific growth advantages in certain tissues and can display phenotypic differences *in vivo*, including those related to disease manifestations.

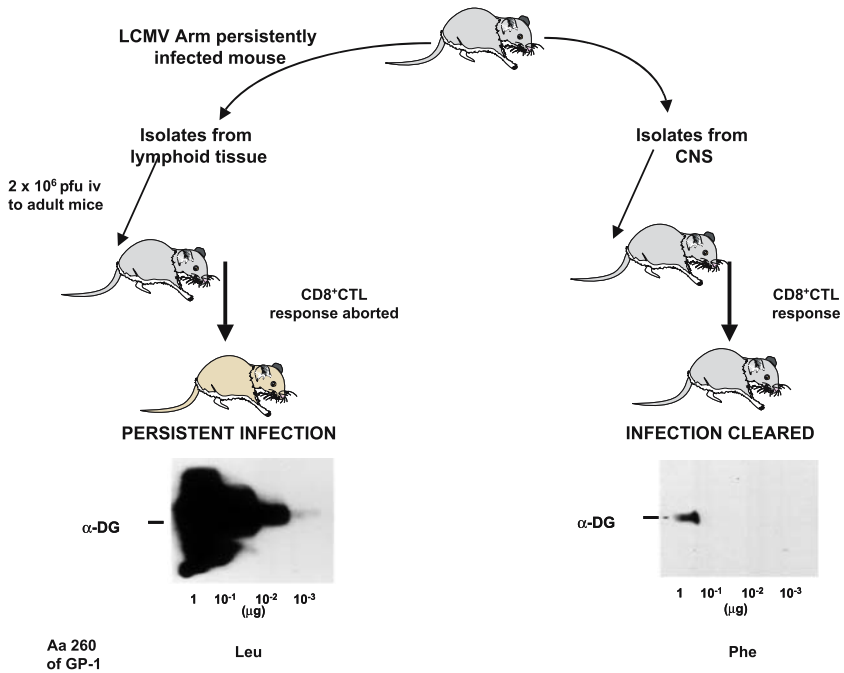
Several members of the arenavirus family use  $\alpha$ -dystroglycan ( $\alpha$ -DG) as a cellular receptor, including the Old World arenaviruses LCMV, Lassa fever virus (LFV), and Mobala as well as Clade C New World arenaviruses (Kunz et al. 2002). This receptor,  $\alpha$ -DG, is a ubiquitously expressed, highly versatile cell surface receptor that provides a molecular link between the extracellular matrix (ECM) and the actin-based cytoskeleton and plays a critical role in cell-mediated assembly of basement membranes. Differences in binding properties to  $\alpha$ -DG have been documented among arenaviruses, and such differences have been linked to differences in virus-host interactions, including tropism and pathogenesis, which is discussed below.

### 2.2.1

#### **Selection of Immunosuppressive Variants in LCMV Persistently Infected Mice**

In the course of persistent infections of mice with LCMV, distinct viral variants can be isolated from the brain and lymphoid tissue (Ahmed et al. 1984b). These viral variants have different biological properties that correlate with the type of tissue from which they are isolated. One type of variant predominates in the CNS (Evans et al. 1994), whereas the other type predominates in lymphocytes and macrophages of the immune system (Fig. 3) (Ahmed and Oldstone 1988; Ahmed et al. 1984a). Most of the CNS isolates are similar to the parental Armstrong (Arm) strain used to infect the mice and induce a potent virus-specific CTL responses in adult mice, in which the infection is cleared within 2 weeks. In contrast, the majority of the isolates derived from the lymphoid tissue cause chronic infections in adult mice associated with absence of LCMV-specific CTL response, absence of CTL response to other viruses, and absence of antibody responses to soluble and foreign antigens (Ahmed and Oldstone 1988; Ahmed et al. 1984a). The prototypic member of the immunosuppressive variants is clone 13 (Cl 13), which was derived from the spleen of a mouse persistently infected from birth with the nonsuppressive LCMV Arm isolate. Sequence analysis revealed only two amino acid changes in Cl 13 compared with the parental Arm, F260L in the viral glycoprotein-1 (GP1) (Salvato et al. 1988), and K1079Q in the viral polymerase encoded by the L gene (Salvato et al. 1991). Injection of mice with reassortant viruses between Cl13 and Arm showed that only reassortants containing the S segment (GP1) that came from Arm exhibited high levels of LCMV-specific CTL and the infection was cleared in 2 weeks (Matloubian et al. 1993). These findings indicate that the immunosuppressive potential of Cl 13 maps to the S segment, with strong

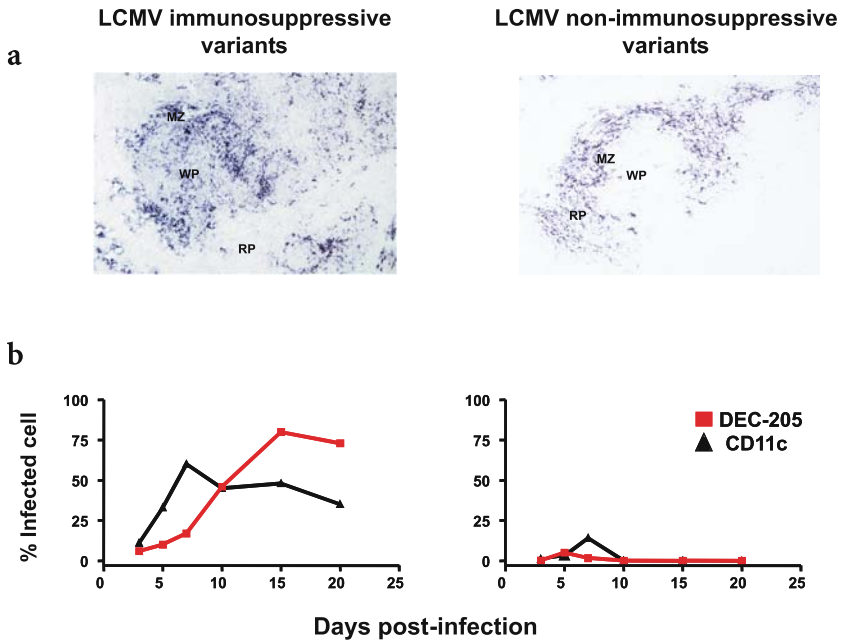
evidence for an association of amino acid substitution F260L in GP1 with the immunosuppressive phenotype (Fig. 3). The emergence of cell-specific viral variants can be explained by the fact that when a viral infection occurs in the whole animal, the various organs and cell types present in the body provide a rich milieu for the selection of viral variants. During long-term persistence in carrier mice with continuous virus replication, and given the high mutation rate of RNA viruses, LCMV variants that have a growth advantage in certain cell types are likely to emerge. In addition, the contribution of the selective



**Fig. 3** Selection of viral variants from mice persistently infected at birth with LCMV Arm. Variants isolated from a variety of immune cells (lymphoid tissue), when inoculated intravenously into adult immunocompetent mice, fails to generate a sufficient primary day-7 CTL response to clear viral infection resulting in a persistent viral infection (immunosuppressive variants). In contrast, those variants isolated from the CNS, upon inoculation of adult immunocompetent mice, generate a robust CTL response and clear the virus infection (nonimmunosuppressive). The immunosuppressive variants bind at high affinity to  $\alpha$ -DG immobilized on membranes while the nonimmunosuppressive variants bind at low affinity to  $\alpha$ -DG. The difference in GP amino acid residues is a single amino acid at position 260, having a Leu the immunosuppressive variants as opposed to the nonimmunosuppressive with a Phe at that site

pressure of the host immune system has to be taken into consideration. In order to evaluate all these components, the selection of a large number of LCMV variants in vivo has been documented (Sevilla et al. 2000). The vast majority of viruses isolated from lymphocytes and macrophages of CD4 knock out (ko), perforin ko, and TNF- $\alpha$  ko mice persistently infected with Arm showed an immunosuppressive phenotype when inoculated i.v. into adult immunocompetent mice. In contrast, all the CNS viral isolates from the same mice exhibited a nonimmunosuppressive phenotype. Selection of immunosuppressive and nonimmunosuppressive variants occurred at similar frequencies in normal and immunocompromised mice, indicating that it is highly unlikely that the host immune response represents a main driving force in this selection process. Sequence analysis of a large number of these variants consistently showed an amino acid exchange F260L or F260I in GP1 of the immunosuppressive viruses. As the selection of such viral variants is influenced in a critical way by the host tissue, it may involve, among other factors, virus-receptor interactions (Sevilla et al. 2000). In fact, these viruses showed a 200- to 500-fold enhanced binding to  $\alpha$ -DG and a high dependence on  $\alpha$ -DG for the infection of cells (Kunz et al. 2002). The analysis of the anatomic distribution of immunosuppressive variants in the spleen of adult immunocompetent mice 3 days after infection showed a different tropism for both group of variants (Fig. 4) (Sevilla et al. 2000). Cl13-like viruses localized exclusively in cells of the marginal zone and white pulp of spleen, whereas Arm-like viruses localized primarily in cells within the red pulp. This distinct distribution in spleen indicated that different subsets of cells were infected by the immunosuppressive and the nonimmunosuppressive viruses. The identification of cells in the spleen infected by LCMV revealed two subsets mainly infected by immunosuppressive viruses, CD11c- and DEC-205 positive dendritic cells (Fig. 4) (Sevilla et al. 2000). The major cell population expressing  $\alpha$ -DG in spleen is CD11c<sup>+</sup> cells. All these data together suggest that those viral variants with increased binding receptor affinity might be selected within the replicating quasispecies to bind to CD11c<sup>+</sup> and DEC-205<sup>+</sup> cells. The selective pressure is likely based on the interaction of the virus in initiating the dysfunction of the host antigen presentation, aborting the necessary host immune responses required to terminate the viral infection (Sevilla et al. 2004).

The differential tropism of immunosuppressive (high-affinity binders) and nonimmunosuppressive (low-affinity binders) LCMV variants may relate to their ability to compete with host-derived ligands of  $\alpha$ -DG. Variants with high-affinity receptor binding may out-compete host-derived ligands of  $\alpha$ -DG, such as Arm, and may be blocked by host-derived ligands. Given the highly complex tissue-specific binding pattern of dystroglycan, the ability of



**Fig. 4a,b** Differential tropism of LCMV immunosuppressive and nonimmunosuppressive variants in vivo. **a** Viral nucleic acid sequences of these variants located the immunosuppressive to the white pulp of spleen and the nonimmunosuppressive to the red pulp. **b** Replication of immunosuppressive variants in CD11c<sup>+</sup> and DEC-205<sup>+</sup> dendritic cells (up to 80% of such cells are infected) is shown. By comparison, <12% of such cells are infected by LCMV nonimmunosuppressive variants

a viral variant to compete for dystroglycan binding with host-derived ligands may be crucial for the infection of a particular cell type such as dendritic cells. Both in vivo and in vitro competition assays between Arm and Cl13 in neurons, but Cl13 has a higher fitness than Arm during replication in liver and spleen (Evans et al. 1994; King et al. 1992). This illustrates the relationship between virus–host molecular interactions and the selection of biologically relevant viral variants.

### 2.2.2

#### Contribution of LCMV Variants to Virally Induced Growth Hormone Deficiency Syndrome

LCMV induced growth hormone (GH) deficiency syndrome (GHDS) is manifested by marked growth retardation and the development of severe hypoglycemia, which frequently leads to the death of the infected mice (Oldstone

et al. 1982, 1984). Both viral and host genetic determinants contribute to GHDS (Oldstone 2002; Oldstone et al. 1982, 1984, 1985; Riviere et al. 1985a, Tishon and Oldstone 1990). The development of GHDS is associated with high viral load in the GH-producing cells of the anterior pituitary, despite an absence of noticeable tissue structural damage or inflammation, yet levels of GH, both mRNA and protein are significantly diminished (Klavinskis and Oldstone 1986, 1989; Oldstone et al. 1985; Valsamakis et al. 1987). Studies using a pituitary-derived somatotroph cell line, PC, indicated that LCMV-induced reduction in GH synthesis is likely due to the altered activity of the GH transcriptional transactivator GHF1 (Pit-1) in the infected cells (de la Torre and Oldstone 1992, 1996). This, in turn, results in lower levels of GH promoter activity and the subsequent decrease in mRNA synthesis and protein production.

Studies using reassortant viruses between strains of LCMV, which do (ARM), or do not (WE) cause GHSD, mapped the ability to cause this disorder to the S RNA (Riviere et al. 1985b). As predicted, clonal analysis of the parental GHDS-nil WE population showed that the majority (58/61) of the clones examined behaved as the parental WE clonal population and did not cause GHDS (Buesa-Gomez et al. 1996). Notably, however, three of the 61 clones isolated from the WE parental population did cause the characteristic GHDS (Buesa-Gomez et al. 1996). These results provided evidence that variants with the ability to cause GHDS (GHDS<sup>+</sup>) may constitute up to 5% of the RNA species within a GHDS-nil replicating WE parental population. WE variants that caused GHDS replicated at high levels in the GH-producing cells of the anterior pituitary and caused reduced levels of GH synthesis in the infected mice. In contrast, the majority of the WE clonal isolates had the same GHDS-nil phenotype as the parental WE and replicated poorly in the GH-producing cells (Buesa-Gomez et al. 1996).

Clonal isolates from the same parental virus population are expected to be genetically very closely related. Therefore, the molecular characterization of GHDS-nil (e.g., Wec54) and GHDS<sup>+</sup> (e.g., Wec2.5) WE variants facilitated the investigation of the precise viral determinants required for the development of GHDS. The biological characterization of reassortants between Wec54 or Wec2.5 variants and ARM (GHDS<sup>+</sup>), confirmed that the virus's ability to cause GHDS was associated with the S RNA (Teng et al. 1996a). Sequence analysis of the S RNA of Wec54 and Wec2.5 showed that the genetic basis for their phenotypic differences is a single amino acid substitution S to F at position 153 in the GP1 (Teng et al. 1996a). With the exception of their different tropism for GH-producing cells, both Wec54 and Wec2.5 have the same tissue and cell distribution in GHDS susceptible infected mice. Therefore, the single amino acid substitution S153F in GP-1 appears to confer Wec2.5-like variants with

the ability to recognize a virus receptor that is specifically expressed in the GH-producing cells. PC cells did not exhibit the restriction for LCMV entry observed *in vivo*. Both GHDS (+) and (-) LCMV variants were able to replicate in PC cells and both caused a similar decrease in GH mRNA steady-state levels (Teng et al. 1996a). These findings suggest that in addition to GP-1, involved in receptor recognition and virus entry into the GH target cells, other viral determinants also contribute to LCMV-mediated impairment of GH synthesis.

Interestingly, WEc54 and WEc2.5 also exhibited distinct CTL<sup>-</sup>P<sup>+</sup> and CTL<sup>+</sup>P<sup>-</sup> phenotypes, respectively. As with Cl 13; The CTL<sup>-</sup>P<sup>+</sup> phenotype displayed by WEc54 correlated with the virus's ability to target dendritic cells in the spleen. It is then intriguing that the same mutation S153F in GP-1 extends the tropism of WE to the GH-producing cells, but abrogates its capability to target dendritic cells. The mechanisms underlying the association of a single amino acid at position 153 in GP-1 with two different and seemingly unrelated phenotypes, namely, the ability to cause GHDS and immunosuppression, remain to be determined. Nevertheless, these findings illustrate how only minor changes in the virus genome may have a great impact in the biology of the infected host.

An intriguing question is how GHDS<sup>+</sup> WE variants are stably maintained, although phenotypically silent, within the GHDS-nil WE population. Likewise, it would be important to determine whether changes in the virus population dynamics within the infected host influence disease progression. The single S153F substitution in GP1 associated with the phenotypic difference between WEc54 (GHDS-nil) and WEc2.5 (GHDS<sup>+</sup>) was caused by a single nucleotide substitution G to A. As a consequence of this nucleotide change, cDNAs derived from WEc54 and WEc2.5 differ by the presence or absence, respectively, of a EcoRI site, which provided the basis for a sensitive RT-PCR and restriction enzyme cleavage assay to analyze the dynamics of these WE variants during the natural course of infection (Teng et al. 1996b). Results from these studies indicated that neonatal C3H/St mice infected with a mixture of WEc54 (GHDS-nil) and WEc2.5 (GHDS<sup>+</sup>) developed GHDS only when WEc2.5 represented a fraction higher than 0.1 in an inoculum of 1,000 PFU (Teng et al. 1996b). Importantly, mice infected with less than 10 PFU of WEc2.5 developed GHDS, indicating that the amount of WEc2.5 (10 PFU), corresponding to a fraction of 0.1 in the inoculum of the mix virus population, should have been sufficient to induce GHDS. Interestingly, WEc2.5 was not entirely out-competed by a large excess of WEc54 and was stably maintained at low levels, but phenotypically silent, in the infected mouse. As predicted, WEc54 and WEc2.5 also exhibited quasispecies structures but with sequence distributions that differed between them and with respect to the parental WE population (Teng et al. 1996b). About 50% (14/32) of the analyzed clones

derived from the EcoRI-resistant PCR band obtained from the RNA of cells infected with the parental WE population had the G to A change resulting in the S153F GP-1 mutation present in Wec2.5. In contrast, when the same analysis was applied to the Wec54 quasispecies, none of the 28 clones analyzed had the Wec2.5 genotype (Teng et al. 1996b). These findings illustrate how differences in the sequence distributions between two quasispecies with very similar, or identical, consensus sequences can significantly influence the virus's impact on the infected host.

Direct fitness measurements of Wec54 and Wec2.5 variants have not been performed. However, one-step growth studies in cultured cells showed that Wec2.5 appears to replicate slightly faster and to higher titers than Wec54. The question then is why Wec2.5 species do not raise to dominance over Wec54 species within the WE population. In this respect, studies with VSV have shown that virus clones with relatively high fitness could be hidden and suppressed by viral quasispecies containing a large excess of variants with lower average fitness (de la Torre and Holland 1990). These findings would suggest that the phenotypic manifestations, including disease symptoms, of a virus infection can be significantly influenced by the levels at which particular variants are present within the population. Hence, pathogenic variants, including those having high fitness, can be maintained at low levels, and thereby phenotypically silent, within a nonpathogenic viral population. Upon bottleneck transmission events, levels of a previously phenotypically suppressed variant might reach the required threshold for its phenotypic manifestation, which can drastically alter the outcome of the infection in the newly infected host or tissue.

### **3 Perspectives**

It is now well established that most, if not all, RNA viruses have the genetic structure of quasispecies. Increasing amounts of information obtained from different viral systems are contributing to the elucidation of the complex dynamics of viral quasispecies. This is important to understand not only virus evolution, but also viral pathogenesis. Such population dynamics may often confound efforts to correlate phenotypic manifestations of virus-induced disease with single genome variants of a defined sequence. Viral systems, like LCMV, for which both cell culture systems and suitable small laboratory animal models are available, provide us with ideal tools for the investigation of the impact of viral quasispecies on the biology of the infected host. The findings on LCMV–host interactions we have discussed here would support

the view that persistent viral infections can be viewed as a succession of invasions modulated by the response of the host. The manifestations of these chronic infections will differ not only because of the genetic, physiological and immunological differences among the infected hosts, but also because each host is likely to face a unique array of quasispecies challenges during infection. These features of viral infections are mostly overlooked because disease symptoms are often, but not always, similar for a given type of virus. It is worth noting that variants, present within a given viral quasispecies, and with the potential of exerting a very different impact on the biology of the infected host can, however, exhibit indistinguishable biochemical and immunological properties. Hence, the use of classic biochemical and genetic assays that define features of the consensus population sequence may fail to detect the presence of variants capable of triggering different disease symptoms. This, in turn, might lead clinicians to disregard the contribution of relevant infectious agents to certain disease manifestations.

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