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Variants Able To Cause Growth Hormone Deficiency Syndrome Are Present within the Disease-nil WE Strain of Lymphocytic Choriomeningitis Virus†

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Persistent infection of C3H/St mice with lymphocytic choriomeningitis virus (LCMV) strain Armstrong leads to disordered growth and hypoglycemia. Both host and viral determinants contribute to this growth hormone (GH) deficiency syndrome (GHDS). Development of the GHDS correlates with the virus's ability to replicate in the GH-producing cells and cause reduced levels of GH synthesis. LCMV strain WE infects few GH-producing cells and does not cause GHDS in C3H/St mice. We show here that clonal variants isolated from the GHDS-nil WE population are able to replicate at high levels in GH-producing cells and cause GHDS in C3H/St mice. These variants are stably maintained, but phenotypically silent, within the GHDS-nil WE population.

Viruses can establish persistent infections in the absence of cytolysis and inflammation, the classic hallmarks of virus infection (1, 8). Such nonlytic persistent infections may interfere with specialized cell functions, disturbing host homeostasis, and cause disease (8, 15). We have documented that C3H/St mice infected at birth with lymphocytic choriomeningitis virus (LCMV) develop a persistent infection that is associated with a growth hormone (GH) deficiency syndrome (GHDS) (18). This syndrome is manifested as retarded growth and marked hypoglycemia which is most often fatal (18). These symptoms are associated with a high viral load in the GH-producing cells of the anterior pituitary, but neither tissue nor cellular structural damage is observed even though GH mRNA and protein levels are significantly diminished in pituitary glands of infected mice (17, 18, 29). This virally induced reduction in GH synthesis is due in part to altered activity of the GH transactivator GHF1 (Pit-1) caused by LCMV infection of GH-producing cells (7).

Both host genetics and viral determinants contribute to the development of GHDS (16, 28). LCMV, the prototype of the arenaviruses, has a bisegmented negative-strand RNA genome (4). The large (L) RNA encodes the putative viral polymerase and a small polypeptide (Z) of unknown function (22, 23), while the small (S) RNA encodes the viral glycoprotein (GP) and nucleoprotein (NP) (20, 26). Genetic studies using reassortant viruses between strains of LCMV which do or do not cause GHDS mapped the ability to cause this disorder to the S RNA (19). However, the numerous amino acid substitutions within the S RNA between LCMV strains differing in their abilities to cause GHDS prevented more detailed analysis of the viral determinants responsible for the disease (26).

The isolation and characterization of viral variants which are genetically closely related but phenotypically distinguishable in regard to the trait under study provide valuable information about the relationship between sequence and function of the viral determinants contributing to the viral phenotype under study. Because of extremely high mutation frequencies per genome site, even cloned populations of RNA viruses consist not of a single genome species but rather of heterogeneous mixtures of genetically closely related genomes (quasispecies) (10–12). Thus, we reasoned that clonal analysis of the GHDS-nil WE parental population, designated WE, might uncover variants differing in their abilities to cause this disorder despite having very similar genome sequences. Here, we describe the isolation and biological characterization of clonal variants isolated from within the same GHDS-nil WE parental population which do or do not cause this disorder. WE clonal variants that caused GHDS were able to replicate at high levels in the GH-producing cells of the anterior pituitary, an ability which was associated with decreased steady-state levels of GH mRNA and protein.

To examine whether viral variants able to cause GHDS were present within the GHDS-nil WE parental population of LCMV, we isolated 61 virus clones from this population by plaque purification. These clones were then tested for the ability to cause GHDS upon inoculation into neonatal C3H/St mice. Mice infected with WE parental population grew similarly to mock-infected controls in terms of body weight and body length and had an equivalent rate of survival (Fig. 1). The majority of clones isolated from the WE parental population (58 of 61) displayed a phenotype similar to that of WE clone 54 (WE c54) (19); they neither markedly impaired the development of nor caused early death in infected C3H/St mice (Fig. 1). In contrast, 3 of the 61 clones, represented by WE c2.5, caused marked growth retardation upon intracerebral inoculation into C3H/St neonates compared with mock- and WE-infected mice (Fig. 1). In addition, WE c2.5-infected animals all died within 30 days postinfection, with the majority (70%) dying by day 15 (Fig. 1). Therefore, variants with the ability to cause marked growth retardation and rapid death, the hallmarks of GHDS, were present at a proportion of approximately 1 in 20 (5%) within the GHDS-nil WE parental population.

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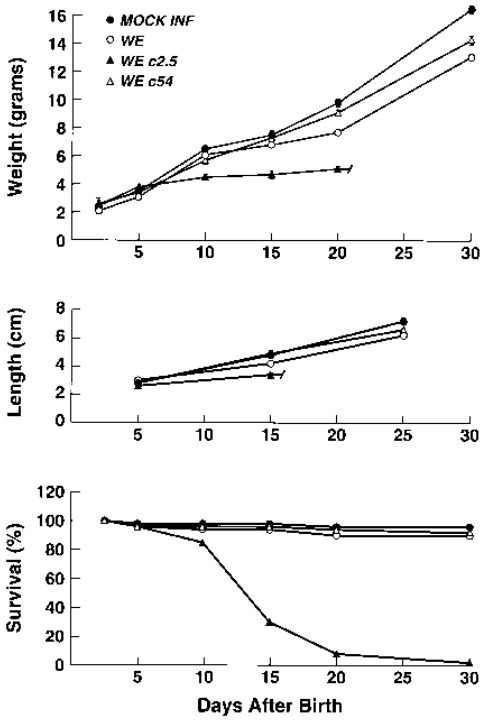

FIG. 1. Body weight, length, and survival of C3H/St mice persistently infected with the LCMV WE parental population and WE c54 and WE c2.5 clonal variants. Values correspond to the means and standard errors of 30, 25, 35, and 30 mice for mock-infected (MOCK INF), and WE-, WE c2.5-, and WE c54-infected mice, respectively. Body weight and length measurements and blood collection to determine glucose levels were taken at approximately the same hour of the day (5:00 p.m.) to minimize differences due to circadian rhythms.

Another characteristic of LCMV-induced GHDS is severe hypoglycemia, which likely is responsible for the death of the infected animals (18). We measured the blood glucose levels of C3H/St mice infected with the prototypic virus clones WE c54 and WE c2.5. WE c54, which did not significantly affect the development of infected mice (19) (Fig. 1), also did not cause hypoglycemia, similar to infection with the WE parental strain (Table 1). However, WE c2.5, which caused marked growth retardation and death, induced a dramatic and significant ($P < 0.0001$, Student's t test) decrease in blood glucose levels compared with both WE- and mock-infected controls (Table 1). Thus, WE c54 and WE c2.5 differ in the ability to cause hypoglycemia in neonatal C3H/St mice. This difference was unlikely due to a difference in overall levels of infection, since serum virus titers were similar in infected mice from all groups (Table 1).

TABLE 1. Serum glucose levels and virus titers in 15-day-old C3H/St mice infected since birth with LCMV WE parental population and variants WE c54 and WE c2.5[a]

Infection	Blood glucose (mg/dl)	Serum viremia (PFU/ml, 10^5)	Virus titer (PFU/g)	
			Brain	Liver
Mock				
WE	121 ± 25	8.0 ± 3.0	(7.0 ± 2.0) ± 10^5	(4.0 ± 2.0) ± 10^5
WE c54	134 ± 19	7.5 ± 4.0	(1.0 ± 0.8) ± 10^6	(6.0 ± 2.0) ± 10^3
WE c2.5	34 ± 10	2.1 ± 0.7	(8.0 ± 3.0) ± 10^5	(7.5 ± 2.5) ± 10^5

[a] Values of serum glucose levels represent averages ± standard deviations for eight infected mice. Virus titers in serum, brain, and liver correspond to averages ± standard deviations obtained with five infected mice per group. Virus titers were determined by plaque assay on Vero cells (16). Blood glucose levels were determined by using Chem Strip bG (Boehringer Mannheim, Indianapolis, Ind.) and read in an AccuChek II monitor (Boehringer Mannheim).

We have shown that the ability of LCMV to induce GHDS correlates with its ability to infect the GH-producing cells in the anterior lobe of the pituitary (16, 19). To examine whether this correlation occurred with the WE clones, pituitaries isolated from infected C3H/St mice were analyzed by two-color immunofluorescence for expression of viral antigens and GH. WE clones that did not cause GHDS, like WE c54, replicated poorly in the GH-producing cells in the pituitaries of infected mice (Fig. 2), with less than 15% of GH-positive cells staining positive for LCMV antigens. In contrast, a high viral load was observed in pituitary glands isolated from WE c2.5-infected mice. Greater than 95% of the GH-producing cells in these pituitaries expressed LCMV antigens (Fig. 2). This level is equivalent to that seen in LCMV Armstrong 53b-infected animals that also develop GHDS (18).

LCMV replication in GH-producing cells produces decreases in the amounts of both steady-state mRNA and protein synthesis of GH (13, 18, 29). Therefore, we analyzed the levels of GH mRNA and protein in the pituitaries of mice infected with the WE clones. We used an RNase protection assay (RPA) to examine the levels of GH and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNAs in the pituitaries of infected mice. GH RNA levels were similar among mock-, WE-, and WE c54-infected mice (Fig. 3A, lanes 1 to 3). However, significantly lower levels of GH RNA were seen with pituitary RNA prepared from WE c2.5-infected mice (Fig. 3A, lane 4). Quantitation of the RPA showed that GH RNA levels were approximately fivefold lower in WE c2.5-infected mice than in mock-infected controls (Fig. 3B). In contrast, GAPDH RNA levels were similar between mock-infected and mice infected with the original WE parental population or WE c54 and WE c2.5 clones (Fig. 3B). The decrease in GH RNA in WE c2.5-infected mice was reflected in the amount of GH protein synthesized. Figure 3C shows Western blots (immunoblots) of pituitary extracts for GH and prolactin (PL). Again, WE or WE c54 infection did not affect the amount of GH in pituitary glands (Fig. 3C, lanes 2 and 3), while WE c2.5 infection markedly decreased GH protein levels in pituitary glands (Fig. 3C, lane 4). This effect was specific for GH, since the levels of PL were comparable between the different samples (Fig. 3C). As with other strains of LCMV that cause GHDS (17, 18), WE c2.5 did not infect PL cells in the pituitary glands of C3H/St mice. Densitometric quantitation of the Western blot revealed approximately a 2.5-fold decrease in the amount of pituitary GH due to WE c2.5 infection compared with controls. Quantitative determination by radioimmunoassay of the amount of GH present in pituitaries of mock- and virus-infected mice were consistent with Western blot results (Fig. 3D). Thus, the levels of GH mRNA and protein in the pituitary are reduced significantly by infection of neonatal C3H/St mice with WE c2.5, whereas infection with WE or WE c54 had little effect on pituitary GH synthesis.

Several gene products synthesized by the liver and brain play a central role in the regulation of GH synthesis and glycemia. Moreover, the liver is the organ mainly responsible for production of glucose. Therefore, differences in the ability to replicate in these tissues could contribute to the phenotypic differences between WE variants regarding the GHDS. Both WE variants that caused and those that did not cause GHDS were able to replicate at high levels in the brains of C3H/St mice, as determined by Northern (RNA) blot hybridization (not shown). Consistent with this finding, brains isolated from these mice had similar amounts of infectious virus, as indicated by

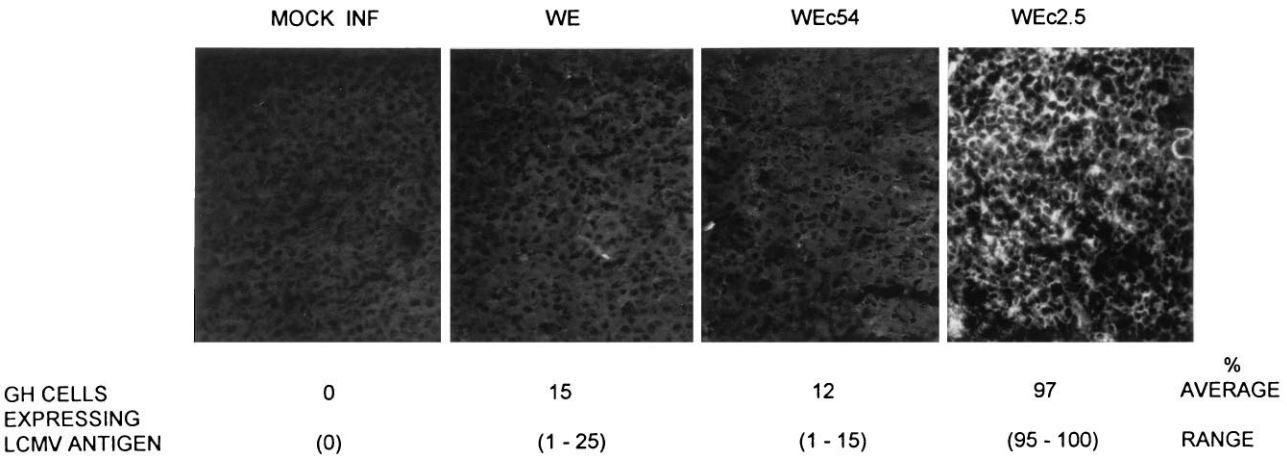

FIG. 2. Immunofluorescence analysis of pituitary glands from infected mice. Photomicrographs show representative fields of pituitaries stained for viral antigen by using a guinea pig antiserum to LCMV followed by a fluorescein-coupled secondary antibody. Double labeling was performed with an anti-GH antibody followed by a Texas red-coupled secondary to determine the percentage of GH-producing cells infected by LCMV. MOCK INF, mock infected.

the similar titers determined by plaque assay (Table 1). In contrast, WE c54 was unable to replicate in the livers of C3H/St mice to the same high levels as WE c2.5, and viral titers in the livers of the WE c54-infected mice were also significantly lower than titers in the livers of WE- or WE c2.5-infected animals (Table 1). However, infection of C3H/St mice with the WE parental population did not lead to the development of GHDS despite high levels of viral replication in the livers of infected mice (Table 1). This finding led us to investigate whether variants like WE c2.5 were preferentially selected in the livers of C3H/St mice infected with the WE parental population. For this purpose, we examined whether liver homogenates from WE-infected C3H/St mice could induce GHDS. C3H/St mice inoculated at birth with liver homogenate from WE-infected C3H/St mice developed GHDS symptoms and expressed high levels of viral antigen in the anterior pituitary (Fig. 4A), which was associated with reduced levels of GH mRNA synthesis (Fig. 4B). In contrast, C3H/St mice inoculated at birth with brain homogenates of WE-infected C3H/St mice, or with brain or liver homogenates prepared from mock-infected C3H/St mice, did not develop GHDS.

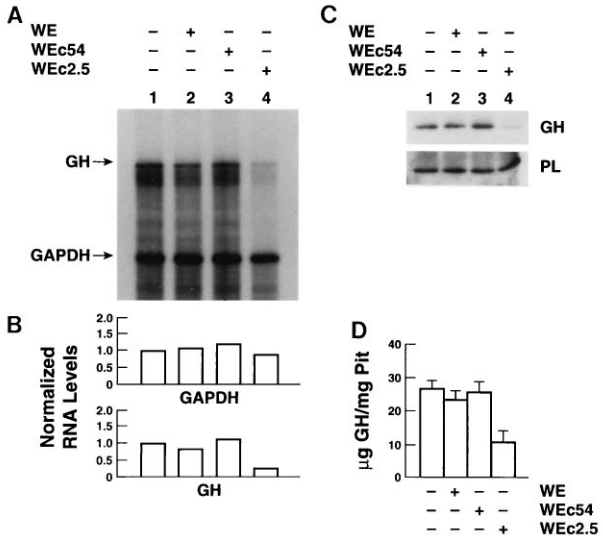

FIG. 3. Pituitary steady-state levels of GH mRNA and protein in infected mice. (A) Steady-state levels of GH mRNA. Total RNA (5) isolated from pituitaries of mock-, WE-, WE c54-, and WE c2.5-infected C3H/St mice was analyzed by RPA using specific antisense ^{32}P-RNA probes for GH and GAPDH. The RPA was conducted as described previously (27). Protected ^{32}P-RNA samples were analyzed by electrophoresis in denaturing polyacrylamide gels. (B) Quantitation of RNA levels from panel A. Levels of protected ^{32}P-RNA samples were directly quantitated on dried gels by using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Values were normalized to those for mock-infected C3H/St mice (lane 1). (C) Western blot of pituitary whole protein extracts from mock- and virus-infected mice. Pituitaries were collected and whole protein extracts were prepared as described above. Equivalent amounts of protein samples were separated by SDS-PAGE (14) and analyzed by Western blotting using specific antibodies for GH and PL. (D) Quantitation of GH levels. Pituitary (Pit) GH levels in mock- and virus-infected C3H/St mice were determined by radioimmunoassay as described previously (24).

Our data demonstrate that clonal isolates of the same parental clonal virus population can differ markedly in their phenotypes in vivo. Because of the high mutation rates operating during RNA virus genome replication (10–12), variants are generated continuously during LCMV persistent infection in C3H/St mice. It might therefore seem paradoxical that a heterogeneous population such as the LCMV WE parental population can maintain a stable GHDS-nil phenotype during its replication in C3H/St mice. We need to consider that specific tissue and cell host factors may favor virus population stability, as well as selection of particular variants within the population (2, 12). In C3H/St mice infected with the WE parental population, most of the infected tissues may provide a rather constant environment where selection for fit sequences prevents dominance and phenotypic expression of WE c2.5-like sequences already present within the original WE parental population (10–12). A selective advantage for replication in liver may favor a preferential accumulation of variants like WE c2.5 in this tissue in WE-infected mice. However, this selection did not lead to GHDS, a finding consistent with the requirement of virus replication in GH-producing cells of the anterior pituitary for the development of GHDS (17, 18).

The molecular bases underlying WE c54 limited replication in the liver of C3H/St mice remain to be determined. Because of the complex dynamics of interactions among RNA species replicating within a viral quasispecies, the relative levels at which variants are present within the population may determine their fate in the infected host. Thus, variants of vastly superior fitness could not rise to dominate its diverse quasi-

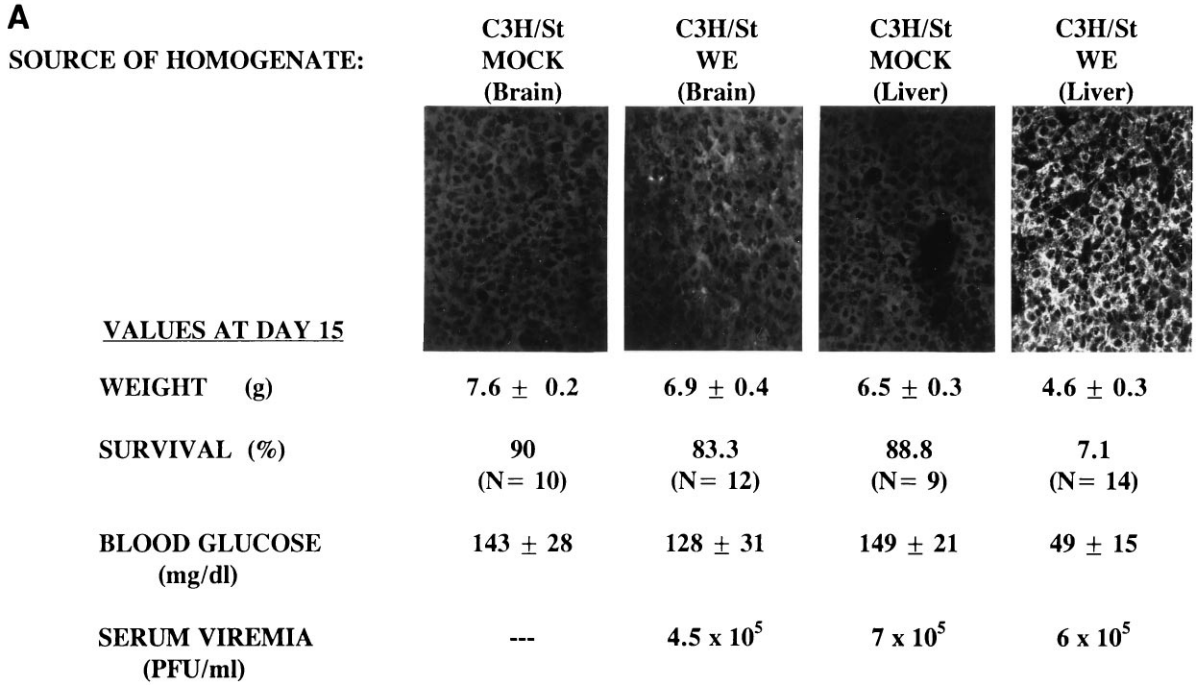

FIG. 4. Consequences of infection of C3H/St mice with brain and liver homogenates from WE-infected C3H/St mice. (A) Expression of viral antigen in pituitary, weight, survival, blood glucose, and serum viremia of C3H/St mice infected at birth with brain and liver homogenates from WE-infected C3H/St mice. (B) Pituitary steady-state level of GH mRNA. Experimental procedures were as described for Fig. 1 to 3.

species progenitor population of lower mean fitness unless they reached a critical threshold level (6). WE and WE c54 quasispecies may represent two very different distributions of RNA species. WE c2.5-like sequences could be present within the WE c54 quasispecies at a frequency significantly lower than that in the original WE parental population, i.e., below the critical threshold level required for its selection in the liver of WE c54-infected C3H/St mice.

Given the quasispecies structure of RNA virus populations, all possible single and double genome site mutations are likely present within an infected host (11). Recently, we have obtained evidence that the ability of WE c2.5 to cause GHDS correlates with a single amino acid change in the viral glycoprotein (27). This finding, together with other well-documented examples (3, 9, 21, 25), illustrates that drastic phenotypic changes are frequently due to one or only a few substitutions in the viral genome. Therefore, a plethora of variants with the potential for causing different pathologies are likely to coexist within the same infected host. Specific tissue and cell type factors can contribute to the selection of these variants. Study of the mechanisms whereby these variants are maintained phenotypically silent within the population, and how under certain conditions they emerge to dominate, generating a new quasispecies with novel biological properties, will contribute to understand the pathogenesis of RNA viruses.

The first two authors contributed equally to this work.

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REFERENCES

1. **Ahmed, R., and J. G. Stevens.** 1990. Viral persistence, p. 241–265. *In* B. N. Fields, D. M. Knipe, et al. (ed.), Virology, 2nd ed. Raven Press, New York.
2. **Ahmed, R. A., A. Salmi, L. D. Butler, J. M. Chiller, and M. B. A. Oldstone.** 1984. Selection of genetic variants of LCMV in spleens of persistently infected mice. J. Exp. Med. **160:**521–540.
3. **Bae, Y.-S., and J.-W. Yoon.** 1993. Determination of diabetogenicity attributable to a single amino acid, ala^{776}, on the polyprotein of encephalomyocarditis virus. Diabetes **42:**435–443.
4. **Bishop, D. H. L.** 1990. Arenaviridae and their replication, p. 1231–1243. *In* B. N. Fields, D. M. Knipe, et al. (ed.), Virology, 2nd ed. Raven Press, New York.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium isothiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
6. **de la Torre, J. C., and J. J. Holland.** 1990. RNA virus quasispecies populations can suppress vastly superior mutant progeny. J. Virol. **64:**6278–6281.
7. **de la Torre, J. C., and M. B. A. Oldstone.** 1992. Selective disruption of growth hormone transcription machinery by viral infection. Proc. Natl. Acad. Sci. USA **89:**9939–9943.
8. **de la Torre, J. C., and M. B. A. Oldstone.** 1996. The anatomy of viral persistence: mechanisms of persistence and associated disease. Adv. Virus Res. **46:**313–345.
9. **Dietzschold, B., W. H. Wunner, T. J. Wiktor, A. D. Lopes, M. Lafon, C. L. Smith, and H. Koprowski.** 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proc. Natl. Acad. Sci. USA **80:**70–74.
10. **Duarte, E. A., I. S. Novella, S. C. Weaver, E. Domingo, S. Wain-Hobson,**

D. K. Clarke, A. Moya, S. F. Elena, J. C. de la Torre, and J. J. Holland. 1994. RNA virus quasispecies: significance for viral disease and epidemiology. Infect. Agents Dis. **3:**201–214.
11. **Eigen, M.** 1993. Viral quasispecies. Sci. Am. **269:**42–49.
12. **Holland, J. J., J. C. de la Torre, and D. A. Steinhauer.** 1992. RNA virus populations as quasispecies. Curr. Top. Microbiol. Immunol. **176:**1–20.
13. **Klavinskis, L. S., and M. B. A. Oldstone.** 1989. Lymphocytic choriomeningitis virus selectively alters differentiated but not housekeeping functions: block in expression of growth hormone gene is at the level of transcription initiation. Virology **168:**232–235.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
15. **Oldstone, M. B. A.** 1989. Viruses can cause disease in the absence of morphological evidence of cell injury: implication for uncovering new diseases in the future. J. Infect. Dis. **159:**384–389.
16. **Oldstone, M. B. A., R. Ahmed, M. J. Buchmeier, P. Blount, and A. Tishon.** 1985. Perturbation of differentiated functions during viral infection in vivo. I. Relationship of lymphocytic choriomeningitis virus and host strains to growth hormone deficiency. Virology **142:**158–174.
17. **Oldstone, M. B. A., M. Rodriguez, W. H. Daughaday, and P. W. Lampert.** 1984. Viral perturbation of endocrine function: disordered cell function leads to disturbed homeostasis and disease. Nature (London) **3–7:**278–281.
18. **Oldstone, M. B. A., Y. N. Sinha, P. Blount, A. Tishon, M. Rodriguez, R. von Wedel, and P. W. Lampert.** 1982. Virus-induced alterations in homeostasis: alterations in differentiated functions of infected cells in vivo. Science **218:** 1125–1127.
19. **Riviere, Y., R. Ahmed, P. Southern, and M. B. A. Oldstone.** 1985. Perturbation of differentiated functions during viral infection in vivo. II. Viral reassortants map growth hormone defect to the S RNA of the lymphocytic choriomeningitis virus genome. Virology **142:**175–182.
20. **Romanowski, V., Y. Matsura, and D. H. L. Bishop.** 1985. Complete sequence of the S RNA of lymphocytic choriomeningitis virus (WE strain) compared to that of Pichinde arenavirus. Virus Res. **3:**101–114.
21. **Salvato, M., P. Borrow, E. Shimomaye, and M. B. A. Oldstone.** 1991. Molecular basis of viral persistence: a single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with suppression of the antiviral cytotoxic T-lymphocyte response and establishment of persistence. J. Virol. **65:**1863–1869.
22. **Salvato, M., E. Shimomaye, and M. B. A. Oldstone.** 1989. The primary structure of the lymphocytic choriomeningitis L gene encodes a putative RNA polymerase. Virology **169:**377–384.
23. **Salvato, M. S., and E. M. Shimomaye.** 1989. The completed sequence of lymphocytic choriomeningitis virus reveals a unique RNA structure and a gene for a zinc finger protein. Virology **173:**1–10.
24. **Sinha, Y. N., F. Selby, V. Lewis, and W. Vanderlaan.** 1972. Studies on GH secretion in mice by a homologous radioimmunoassay for mouse GH. Endocrinology **91:**784–792.
25. **Sitbon, M., L. d'Auriol, H. Ellerbrok, C. Andre, J. Nishio, S. Perryman, F. Pozo, S. F. Hayes, K. Wehrly, P. Tambourin, F. Galibert, and B. Chesebro.** 1991. Substitution of leucine for isoleucine in a sequence highly conserved among retroviral envelope surface glycoproteins attenuates the lytic effect of the Friend murine leukemia virus. Proc. Natl. Acad. Sci. USA **88:**5932–5936.
26. **Southern, P. J., M. K. Singh, Y. Riviere, D. R. Jacoby, M. J. Buchmeier, and M. B. A. Oldstone.** 1987. Molecular characterization of the genomic S RNA segment from lymphocytic choriomeningitis virus. Virology **157:**145–155.
27. **Teng, M. N., P. Borrow, M. B. A. Oldstone, and J. C. de la Torre.** 1996. A single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with the ability to cause growth hormone deficiency syndrome. J. Virol. **70:**8438–8443.
28. **Tishon, A., and M. B. A. Oldstone.** 1990. Perturbation of differentiated functions during viral infection in vivo. In vivo relationship of host genes and lymphocytic choriomeningitis virus to growth hormone deficiency. Am. J. Pathol. **137:**965–969.
29. **Valsamakis, A., Y. Riviere, and M. B. A. Oldstone.** 1987. Perturbation of differentiated functions in vivo during persistent viral infection. III. Decreased growth hormone mRNA. Virology **156:**214–220.