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Extreme Fitness Differences in Mammalian and Insect Hosts after Continuous Replication of Vesicular Stomatitis Virus in Sandfly Cells

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Continuous, persistent replication of a wild-type strain of vesicular stomatitis virus in cultured sandfly cells for 10 months profoundly decreased virus replicative fitness in mammalian cells and greatly increased fitness in sandfly cells. After persistent infection of sandfly cells, fitness was over 2,000,000-fold greater than that in mammalian cells, indicating extreme selective differences in the environmental conditions provided by insect and mammalian cells. The sandfly-adapted virus also showed extremely low fitness in mouse brain cells (comparable to that in mammalian cell cultures). It also showed an attenuated phenotype, requiring a nearly millionfold higher intracranial dose than that of its parent clone to kill mice. A single passage of this adapted virus in BHK-21 cells at 37°C restored fitness to near neutrality and also restored mouse neurovirulence. These results clearly illustrate the enormous capacity of RNA viruses to adapt to changing selective environments.

Numerous human and animal viral pathogens (arboviruses) are transmitted by insect vectors (19). Nearly all arboviruses utilize RNA genomes, probably because of the great mutability and genetic diversity accompanying RNA replication. Resulting RNA virus quasispecies (mutant swarms) facilitate rapid adaptation to changing host environments (7, 8, 12, 15, 16).

Vesicular stomatitis virus (VSV) causes disease in a variety of mammals (including humans), and clinical veterinary manifestations resemble those of foot-and-mouse disease (14). Epizootic outbreaks occur throughout the Americas, but most enzootic transmission appears to occur in Central America, particularly in Panama and Costa Rica (21, 22, 35). VSV replicates in phlebotomine sandflies, black flies, and mosquitoes and has been isolated from many other insects (3). Sandflies are considered important enzootic vectors, and females of several species transmit VSV vertically (transovarially) to offspring as well as horizontally to mammals during blood meals (4, 32, 33). Because transovarial transmission can proceed through several generations of sandflies, the virus can be maintained for rather long periods in insects without a need for horizontal transmission (4, 32, 33).

Prolonged replication in insect cells has been shown earlier to cause virus debilitation in mammalian hosts (20). Peleg (25) reported the gradual attenuation of Semliki Forest virus during serial virus transfers at 3- to 5-day intervals in mosquito (*Aedes aegypti*) cells, and similar results have been obtained for other RNA viruses (17, 29–31). It is therefore of both practical and theoretical importance to determine the effect on VSV biology of prolonged infection of sandfly cells. Of particular significance are possible changes in replication fitness in vertebrate and invertebrate cells and related virulence changes that might accompany persistent replication in sandfly cells. If such changes are due to the accumulation of multiple mutations deleterious for replication in a mammalian host species, such mutants might provide stable attenuation and potential vaccine strains (17, 20, 25, 27, 29–31). Replication in sandfly cells does not cause overt cytopathology, which thus allows the continuous production of significant numbers of viral particles for extended periods of time as the cells are passaged and continue to replicate.

To examine the process of RNA arbovirus adaptation to changing insect and mammalian host environments and the effect of such adaptation on vertebrate virulence, we tested fitness (W) in cell cultures and mouse brains as well as neurovirulence in mice with a VSV clonal population that had replicated continuously in a sandfly cell line for 10 months. After 10 months, this virus population showed extreme differences in fitness in insect and mammalian cells and an attenuated phenotype in mice.

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MATERIALS AND METHODS

Cells and viruses. We employed two different cell lines: a mammalian cell line (BHK-21) and an insect cell line (LL-5). LL-5 is a continuous sandfly cell line derived from *Lutzomyia longipalpis* (34). This cell line replicates as a mixture of epitheloid and fibroblastoid cells of various sizes and shapes and is susceptible to infection by a number of arthropod-borne viruses, including VSV. The viruses that we employed were the wild-type VSV Indiana serotype (Mudd-Summers strain) and clone U, a monoclonal antibody-resistant mutant (MARM) clone

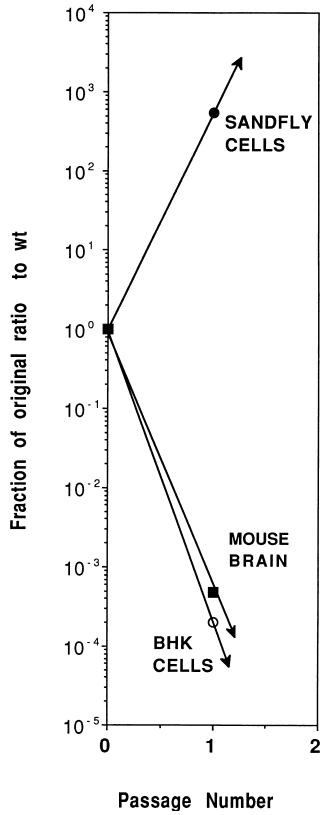

FIG. 1. Fitness vectors of MARM U (10Mo/S-A) virus in sandfly cells, BHK-21 cells, and mouse brains. wt, wild type.

that was plaque picked directly from the wild type in the presence of I1 monoclonal antibody. Compared with the wild type, MARM U is neutral (W = 1) in BHK-21 cells, LL-5 cells, and mouse brain cells, and it has been used as a genetically marked surrogate wild type. As controls, we also employed other very-low-fitness VSV clones (F, A21, and B25) which were obtained after repeated plaque-to-plaque (genetic bottleneck) passages in BHK-21 cells (see Results). A21 and F but not B25 are also MARM populations.

Establishment of the persistently infected LL-5 cell line. We infected LL-5 cells with neutral MARM U at 28°C and a high multiplicity and observed very minimal and temporary cytopathology after 2 to 3 days, which was followed by normal cell replication. During continuous replication, cells were diluted and transferred about twice weekly (when confluent), and there was persistent shedding of infectious virus at 28°C. After 10 months, the virus was amplified by a single passage in uninfected LL-5 sandfly cells and stored in multiple aliquots at −85°C. Methods for fitness determinations have been previously described in detail (1, 2, 9–11, 16, 24). Briefly, genetically marked MARM virus and wild-type neutral reference virus are mixed and seeded on a cell monolayer (BHK-21 or LL-5) or injected into mouse brains, where they are allowed to compete. Original mixtures and viral yields are plaque assayed with and without I1 monoclonal antibody in the overlay medium. Ratios of the wild type to MARM are calculated, and changes in those ratios are used to determine fitness values (1, 2, 9–11, 16, 24).

Fitness determinations for MARM A21 and F populations were carried out in competition with the wild type. For fitness assays of mutant B25, MARM U was used as the surrogate wild-type internal control.

Virulence tests. VSV neurovirulence was tested in mice. Young adult female white Swiss Webster outbred and virus-screened mice from Harlan were used. Virus (20 μl) was injected intracranially (unless otherwise indicated), and the mice were observed for pathological signs. Moribund mice were euthanized with CO_2 gas. Challenge virus was injected intracranially on day 11 (after the sandfly-adapted or bottlenecked virus had been cleared).

RESULTS

The 10-month-old, sandfly-adapted VSV (MARM U [10Mo/S-A]) formed small (1 to 2 mm) plaques on BHK-21 cells after 2 days at 37°C and caused no obvious cytopathology in sandfly cells. However, when we examined the viral replicative abilities in cell cultures (Fig. 1), we observed that MARM U (10Mo/S-A) had undergone a dramatic loss of fitness in BHK-21 cells at 37°C (W = 0.00024 ± 0.00009) and a dramatic gain in fitness in LL-5 cells at 28°C (W = 504 ± 32). This represents a 2,100,000-fold greater fitness in sandfly cells than in mammalian cells.

Next, we examined whether this profound loss of fitness in vertebrate (BHK-21) cells also occurred in neural cells in vivo by intracerebral infection of young adult mice. This virus replicated in the brain, but as can be seen from Fig. 1, the sandfly-adapted virus had an extremely low fitness in mouse brains (W = 0.00048 ± 0.00002). Therefore, we tested the neurovirulence of this sandfly-adapted virus in mice. Even small amounts of wild-type VSV injected intracranially killed nearly all the mice (Table 1). The same was true for the surrogate wild-type (genetically marked MARM U) parent of the sandfly-adapted virus. In striking contrast, MARM U (10Mo/S-A) exhibited no neurovirulence at intracranially injected doses of up to 11,000 PFU. Even when we injected nearly 1,000,000 PFU, only two of seven mice showed neurological signs and died (Table 1), indicating extreme attenuation of the sandfly-adapted virus for the mouse brain. After the mice which survived the wild-type virus intracranial challenge were euthanized, no infectious virus could be detected in brain homogenates of the tested mice.

It should be noted that direct intracerebral injection of massive virus doses is a very stringent test for VSV neurovirulence. We examined other very-low-fitness VSV clones derived from repeated genetic bottlenecks in BHK-21 cells (A21 clone, W = 0.0023 ± 0.0002; B25 clone, W = 0.017 ± 0.007; and F clone, W = 0.00004 ± 0.00001). A21 and F clones exhibited reduced mouse neurovirulence, but neither was as attenuated as the sandfly-adapted MARM U (10Mo-S/A) virus (data not shown). This indicates that persistent insect cell infection might be a useful approach for the preparation of some live attenuated virus vaccines, as was suggested in the earlier studies of Peleg (25), Singh (29), Hurlbut (17), and Taylor and Marshall (30, 31).

Mice previously infected intracranially with various doses of sandfly-adapted virus exhibited strong immunity after direct intracerebral challenge with wild-type VSV (Table 2, experiments 1 to 4). The exceptions were two of five mice that had

TABLE 1. Intracerebral neurovirulence tests with mice infected with wild-type or sandfly-adapted VSV Indiana

Virus and expt no.	Amt of virus injected intracranially (PFU)	No. died/no. injected	Days until dead (or moribund)
Wild-type virus			
1	6	7/7	3–6
2	22	6/7	3–4
3	450	5/5	2–4
Surrogate wild-type MARM U[a]			
1	45	7/8	3–5
2	53	8/8	3–5
MARM U (10Mo/S-A)[b]			
1	525	0/8	
2	7,300	0/8	
3	11,000	0/2	
4	950,000	2/7	5

[a] Statistical analysis (analysis of variance) showed that the wild-type and MARM U viruses are comparably neurovirulent ($P = 0.8593$).

[b] Statistical analysis (analysis of variance) showed that MARM U (10Mo/S-A) is significantly less virulent than MARM U ($P = 0.0016$).

TABLE 2. Intracranial challenge with wild-type VSV of mice previously infected with sandfly-adapted virus

Expt no.	Immunizing route of infection	Immunizing dose of sandfly virus (PFU)	Intracranial challenge dose of wild-type virus (PFU)	No. died/no. challenged
1	Intracranial	525	65	0/8
2	Intracranial	11,000	20	0/2
3	Intracranial	950,000	207	2/5
4	Intracranial	7,300	230	0/8
5	Intraperitoneal	19,000,000	20	0/7
6	Intramuscular	4,700,000	207	1/4
Total				3/38[a]

[a] An analysis of variance, a comparison of these data with data in Table 1, showed that the protection of mice after immunization is statistically significant ($P = 0.0001$).

survived prior intracranial infection with massive inocula of sandfly-adapted virus. The original massive inoculum might possibly have caused immune suppression. Experiments 5 and 6 (Table 2) showed that intraperitoneal and intramuscular injection of massive amounts of sandfly-adapted virus (up to 19,000,000 PFU) provided excellent immunity against a subsequent wild-type VSV challenge. Such massive inocula were employed to show that the virus was safe when given extraneurally (no signs of infection were observed, except in one mouse that received an intramuscular dose of nearly 5,000,000 PFU).

We next determined how rapidly the MARM U (10Mo/S-A) virus would regain fitness and mouse neurovirulence following replication in mammalian cells. A single low-multiplicity passage in BHK-21 cells at 37°C restored mouse neurovirulence (75% mortality at an intracranial dose of 114 PFU) (Table 3, experiment 1). In experiment 2 (Table 3), all mice died following the injection of 94 PFU of virus that had undergone six additional passages in BHK-21 cells at 37°C. These mouse neurovirulence results correlated with MARM U (10Mo/S-A) virus fitness changes observed following passages in BHK-21 cells (Fig. 2). Fitness increased over 1,000-fold after only one passage in these mammalian cells at 37°C (values for three independent replicates were $W = 0.32 \pm 0.04$, $W = 0.32 \pm 0.05$, and $W = 0.44 \pm 0.07$) (Fig. 2). Fitness increased nearly 10,000-fold after seven BHK-21 cell passages ($W = 2.0 \pm 0.2$) (Fig. 2).

Finally, we determined whether persistent infection in sandfly cells could convert VSV to a low-neurovirulence strain within a time much shorter than 10 months. Following only 3 months of persistent infection in sandfly cells, VSV still retained its high neurovirulence for mice (data not shown).

TABLE 3. Recovery of neurovirulence by sandfly-adapted virus following back adaptation to mammalian (BHK-21) cells

Expt no.	No. of back-adaptation passages in BHK-21 cells[a]	Amt of virus injected intracranially (PFU)	No. died/no. injected[b]	Days until death
1	1	114	3/4	4–7
2	7	94	6/6	3–6

[a] MARM U (10Mo/S-A) VSV was back adapted to mammalian cells by either one passage or seven passages in BHK-21 cells at 37°C and a low multiplicity of infection (0.01).

[b] An analysis of variance of the results indicated that the recovery of virulence was significant in both experiments and was comparable to those for the MARM U and wild-type viruses ($P > 0.3$).

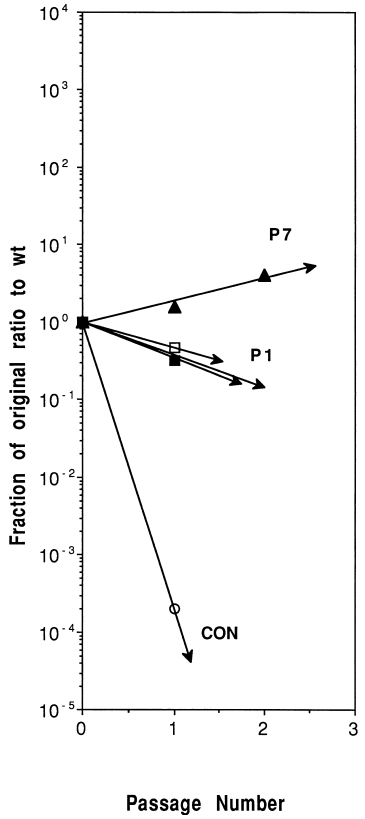

FIG. 2. Fitness recovery of MARM U (10Mo/S-A) upon passage in BHK-21 cells. CON represents the fitness vector of the MARM U (10Mo/S-A) population. P1 represents the fitness vectors of viral populations after one passage in BHK-21 cells. Results for three independent replications are shown. P7 represents the fitness vector of a viral population after seven passages in BHK-21 cells. wt, wild type.

DISCUSSION

The results outlined above emphasize that insect and mammalian cells can be very different selective environments. Because the genetically marked virus (MARM U) used to initiate the persistent infection in sandfly cells was a clone from a plaque, our results also demonstrate positive Darwinian selection for variants with increased fitness in sandfly cells. Such an increase in fitness is likely to be the composite result of adaptation to several parameters provided by persistence in growing, surviving insect cells: intracellular replication in different cell types, a different set of host cell factors, different temperatures, etc. Our results provide evidence that fitness changes can be enormous, but their molecular basis remains unknown. The extreme differences in fitness in insect and mammalian cells also imply that such terms as "neutral," "high fitness," and "low fitness" have meaning only in the context of a specific environment. Our cell culture and mouse neurovirulence studies with VSV are very far removed from the real world of VSV interactions in nature. Neurovirulence in mice inoculated intracranially does not, of course, reflect the natural pathogenesis of VSV disease transmitted by phlebotomine insects to bovine and other hosts. Virus maintenance in nature by repeated transovarial transmission in sandflies and the occurrence of annual outbreaks of disease in vertebrates (21, 22, 35) strongly suggest that such attenuation does not usually occur in the VSV natural cycle.

Selection similar to that occurring in our persistent sandfly

infection might occur rarely in nature whenever arthropod-borne viruses remain isolated in the insect selective environment during many months or years of transovarial transmission (4, 32, 33). Very-long-term persistent replication in insects might even very rarely generate viral populations completely unable to infect vertebrate hosts. This may have occurred in nature with the sigma virus, a rhabdovirus which is transovarially transmitted in some lines of *Drosophila* fruit flies without causing apparent disease. Sigma virus cannot replicate in vertebrate cells, in contrast to related rhabdoviruses, such as VSV, which can multiply in both insect and vertebrate hosts. An alternative explanation is that sigma virus never was a vertebrate virus and has rarely been exposed to vertebrates. Dengue virus has also been transmitted transovarially in the laboratory (26), and infected male mosquitoes have been recovered from wild larvae in nature (18).

Earlier studies showed that a virus phenotype can sometimes be greatly altered by adaptation of arboviruses to insect cell culture replication (20). Both the Chikungunya and Semliki Forest viruses lost mouse virulence following prolonged, persistent infection of cultured insect cells but retained immunogenicity (25, 29). One strain of Ross River virus lost mouse neurovirulence after only five serial passages in whole mosquitoes and yet retained wild-type virulence when passaged alternately in mice and mosquitoes (30, 31). Loss of mouse neurovirulence during replication in insect cells may sometimes occur only after long-term cultivation. Thus, we observed that 3 months were not enough to cause the loss of neurovirulence for VSV. We have not yet determined the time frame or variability of such fitness losses. Analogous results were obtained after one to six passages of Venezuelan equine encephalitis virus in *Aedes aegypti* (27), seven passages of West Nile virus in *Culex univittatus* cells, and eight passages of Japanese encephalitis virus in *Culex quinquefasciatus* cells (17).

Prolonged adaptation to insect cells might sometimes be a useful method to attenuate certain arthropod-borne (and perhaps other) RNA viruses for live virus vaccines, as was suggested by Peleg (25). In particular, such viruses as dengue virus, for which animal disease models are lacking, would be possible candidates. Fitness assays, as employed here, can provide quantitative guidelines for selecting a proper degree of attenuation. The recovery of fitness of BHK-21 after only one passage underscores the importance of the proper handling of vaccine seed stocks and emphasizes the great adaptability of RNA viruses. With regard to the possible applicability of these observations for the design of new live virus vaccine strains, the fact that many RNA viruses have a broad host range must be considered. VSV hosts include cattle, swine, horses, humans, and rats as well as several laboratory animals, such as mice and guinea pigs. A low fitness trait quantitated in cell cultures or in the nervous systems of mice may not hold for other host species. Adequate testing would be necessary to ensure that each specific host species is protected. Furthermore, fitness gains in RNA virus quasispecies can be very rapid (23), and the load of deleterious mutations in any live virus vaccine would have to be sufficiently high to render rapid reversion to virulence unlikely. This would require a statistical evaluation of the safety and efficacy of any candidate vaccine strains.

The evolution of virulence is a poorly understood but very important topic (12, 13). The structure of RNA virus populations as complex mixtures of variants or quasispecies often makes it extremely difficult to achieve effective control by vaccination, in particular by the use of synthetic peptides or subunit vaccines (5, 6, 28). Because it is important to stimulate the immune system with a variety of T-cell and B-cell epitopes, this can often be best achieved with attenuated viruses showing partial replicative competence in their host organisms. Several insect-adapted RNA viruses (e.g., the Semliki Forest, Chikungunya, and Ross River viruses) have been shown to behave as attenuated strains (25, 29–31). In this work, we have demonstrated that VSV displayed similar properties, further suggesting that this might sometimes be a useful method for arbovirus vaccine production.

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I.S.N. and D.K.C. contributed equally to this work.

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