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Stability of RNA virus attenuation approaches

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ABSTRACT

The greatest risk from live-attenuated vaccines is reversion to virulence. Particular concerns arise for RNA viruses, which exhibit high mutation frequencies. We examined the stability of 3 attenuation strategies for the alphavirus, Venezuelan equine encephalitis virus (VEEV): a traditional, point mutation-dependent attenuation approach exemplified by TC-83; a rationally designed, targeted-mutation approach represented by V3526; and a chimeric vaccine, SIN/TC/ZPC. Our findings suggest that the chimeric strain combines the initial attenuation of TC-83 with the greater phenotypic stability of V3526, highlighting the importance of the both initial attenuation and stability for live-attenuated vaccines.

1. Introduction

Despite their dramatic impact on the reduction of infectious diseases, vaccines are viewed skeptically by some, and modern vaccines are subject to ever-tightening safety requirements. Many successful vaccines are attenuated strains that undergo limited replication in the vaccinee. These live-attenuated vaccines offer advantages over inactivated agents, subunit or virus-like particles that do not replicate in the host, including a typically low cost of manufacture and the induction of rapid and long-lived humoral and cell-mediated immunity after a single dose. Vaccines for polio, smallpox, and yellow fever are a few examples of highly successful, live-attenuated viral vaccines. However, these vaccines have a low but significant risk for reactogenicity and can sometimes produce life-threatening disease [1–5]. Because of their reactogenicity, current regulatory standards would probably prevent their licensure today.

Today, successful live-attenuated vaccines must carefully balance robust immunogenicity and safety. In many cases, the more immunogenic a vaccine, the more likely it will result in adverse effects. A major challenge to the generation of safe, live-attenuated vaccines to protect against RNA viral diseases is the inherent instability of the RNA genome as a result of the high error rate of RNA-dependent RNA polymerases [6]. In general, RNA viruses have mutation rates ranging from 10^{-3} to 10^{-5} substitutions per nucleotide copied [7–9]. This allows rapid adaptation and evolution when RNA viruses are subjected to changing selective pressures. These high mutation frequencies result in heterogenous mutant populations of viral genomes, even from clonal sources such as plaque harvests or cDNA [10]. This presents a major challenge for the generation of safe, live-attenuated vaccines. RNA viral vaccine genetic instability is underscored by studies of type 3 oral polio vaccinees (OPV), which commonly reveal the presence of virulent mutants [11–13]. It is therefore essential to evaluate the genomic stability of a new vaccine to ensure the genomic heterogeneity generated by replication does not impact the stable attenuation of the population.

Traditional approaches for generating live-attenuated RNA viral vaccines have relied principally on serial passage through cell cultures or animals. Examples include the yellow fever 17D, OPV, and the TC-83 Venezuelan equine encephalitis (VEE) vaccine. Genetic characterizations have revealed that attenuation of these vaccine strains results from only a few point mutations. Because of the inherent instability of the RNA genome, attenuating point mutations are likely to revert in vivo, or the virus may acquire a compensatory point mutation(s) that restores virulence (pseudoreversion). An example is TC-83, produced via 83 passages of the virulent Trinidad donkey strain (TrD) of VEEV in guinea pig heart cells (4). TC-83 attenuation is attributed to only two mutations: position three of the 5′ untranslated region (UTR) and a nonsynonymous mutation that changes amino acid 120 of the E2 glycoprotein [14]. This small number of attenuating mutations may contribute to

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the high rates of reactogenicity seen in human vaccinees [15–17]. Attenuation of TC-83 has also been shown experimentally to be unstable; as few as three serial intracranial (i.c.) passages in infant mice result in reversion to a neurovirulent phenotype characteristic of the parent TrD strain [18,19]. Of even more significance are rodent-virulent isolates made from human TC-83 vaccinees, indicating that reversion can occur in humans [20].

To overcome the instability and reactogenicity of TC-83, a new VEEV attenuation approach was developed for the V3526 vaccine. A full-length cDNA clone derived from the Trinidad donkey (TrD) strain was modified to contain two independently attenuating mutations: (1) a deletion of the four amino acid furin cleavage recognition site between envelope proteins E3 and E2; and (2) a nonsynonymous mutation encoding a Phe-to-Ser residue change at position 253 (F253S) of the E1 envelope glycoprotein [21]. The latter mutation is required to rescue viability of the cleavage site mutant, so its reversion should be prevented as long as the E3–E2 cleavage site deletion cannot be restored. Thus, this attenuation design of V3526 should be inherently more stable than that of TC-83. Previous studies examining neurovirulence of this vaccine candidate indicated it does not gain virulence following five serial i.c. passages in adult mice or five cell culture passages. Also, V3526 replicates poorly in adult mouse brains and causes less pathology when compared to TC-83 [20].

Another approach to alphavirus attenuation is to generate chimeric strains in which nonstructural genes and *cis*-acting RNA elements are derived from a relatively benign virus [e.g., Sindbis (SINV)], while the structural proteins are derived from the vaccine target virus. Multiple chimeric alphavirus vaccine candidates have been developed and shown to be safe, immunogenic, and protective in rodent models [22–25]. For one SIN/VEEV chimera, the capsid is derived from the TC-83 VEEV strain to enhance viral packaging and the remaining structural protein genes and *cis*-acting RNA elements are derived from enzootic VEEV strain ZPC738 (subtype ID) [26]. This strain, SIN/TC/ZPC (previously referred to as SIN/ZPC) is highly attenuated, immunogenic, protects against VEEV challenge, and generates no detectable viremia in mice or hamsters (Fig. 1) [27].

In this study we tested the hypothesis that the traditional, point mutation-dependent attenuation approach exemplified by TC-83 is less stable than the attenuation strategies represented by V3526 and SIN/TC/ZPC. To assess attenuation stability, we selected for reversion to virulence and compared the phenotypic and genetic stability of each attenuation approach.

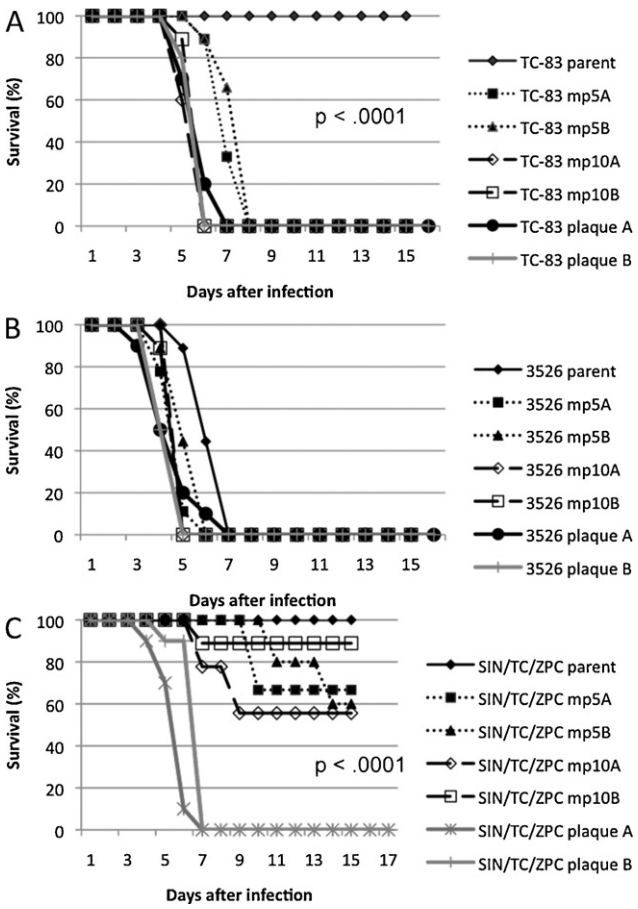

Fig. 1. Kaplan–Meier survival curves of consensus and plaque isolates from each vaccine strain before and after passage. (A) TC-83; (B) V3526; and (C) SIN/TC/ZPC.

2. Methods

2.1. Viruses

Each of the three viruses used in this study was derived from a cDNA clone and transcribed RNA was electroporated into BHK cells to generate infectious virus. The TC-83 [28], SIN/TC/ZPC [27], and V3526 clones [21] were described previously. The V3526 mutations were introduced into a V3000 clone of a VEEV TrD isolate that had previously been passed once in guinea pig brain and 14 times in embryonated eggs [21,29].

2.2. Selection for virulence

We subjected each VEEV strain to 10 serial, i.c. inoculations in 6-day-old outbred NIH Swiss mice (Harlan Laboratories, Indianapolis, IN). For each passage, approximately 50 μl of a 6 $\log_{10}$ plaque forming unit (pfu)/ml stock of virus was inoculated into five mice per replicate. Two parallel replicate passage series (A and B) of each strain were performed to assess variation in the results and to detect convergent mutations that might indicate positive selection. Following ca. 48 h of incubation, after which the mice were euthanized, their brains were harvested, triturated, and titrated by plaque assay on Vero cells. Virus isolated from one brain sample from each replicate was used as the inoculum for the next passage. During the passage series, we observed that mice in both the V3526 and TC-83 series began dying less than 48 h after infection. Therefore, during later passages we harvested at earlier time points to retrieve brains prior to death. Following 10 passages, the brain isolates were plaqued on Vero cell monolayers to isolate clonal populations for further study. A single clonal population for each replicate was randomly selected and subjected to further analysis. While there was some plaque size variation observed in some replicates of passages 2–5 for V3526 and SIN/TC/ZPC, no major plaque phenotype differences were observed in p10 of any of the strains. Thus, the plaque clonal isolates were derived from average size plaques for each p10 replicate.

2.3. Phenotypic virulence

The virulence of the parental strains, consensus viruses isolated from passages 5 (p5) and 10 (p10), and plaque clonal isolates was evaluated by inoculating 6-day-old mice NIH Swiss mice subcutaneously (s.c.) with 50 μl of a 6 $\log_{10}$ pfu/ml stock and assessing survival over 14 days. Subcutaneous infection was selected because intracranial infection results in rapid death with little opportunity to observe increased virulence.