Whole-virus Vaccine Development by Continuous Culture on a Complementing Host

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We have evaluated an adaptive strategy for generating whole-virus vaccines using a bacteriophage model. Wildtype phage T7 was cultivated in a two-stage continuous stirred-tank reactor (CSTR) utilizing a recombinant *E. coli* host that constitutively expressed T7 RNA polymerase, an essential enzyme of the early viral metabolism. Over the course of 180 generations a diversity of phage variants emerged, outgrew the wildtype, and were subsequently eclipsed by yet fitter variants, based on host-ranges, restriction patterns, and one-step growth responses of isolated clones. The fittest variant, which required complementation by the recombinant host in order to grow, deleted at least 12 percent of its genome and replicated twice as fast as the wildtype. Moreover, this variant was immunogenically indistinguishable from the wildtype, based on cross-reactivities of antisera raised against both. These results suggest the feasibility of the proposed strategy for the development of safe whole-virus vaccines.

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Given the plethora of discoveries from phage research that have been paradigmatic for the development of modern virology, molecular biology, and biotechnology[1], it is surprising that phage have not in recent years been more widely studied. Research on phage established the respective roles of the virus and host during an infection, showing the role of the virus as an information bearing instructive entity and the host as a replication machine[2]. The classic 'blender' experiments of Hershey and Chase provided the final proof that DNA, not protein is the genetic material of genes[3]. Moreover, phage research established that the order of amino acids in a protein is a linear mapping of its gene[4] and led to the discovery of restriction enzymes[5], enabling the birth of recombinant DNA and the biotechnology industries.

More recent phage research has played less obvious, but important, roles in defining new paradigms, particularly by clarifying the molecular-level mechanisms that drive the adaptation of viruses. The low fidelity of replication exhibited by the replicase from the single-stranded RNA phage, Q_β, has been found to enable the diversification of phage RNA populations during *in vitro*[6] and *in vivo*[7] laboratory passages . These studies inspired the quasi-species model of Eigen and co-workers[8–10], a physicochemical model that demonstrates how macromolecular information originates by way of specific, though error-prone, autocatalytic reactions, which give rise to stationary population distributions, termed "quasi-species." Experimental phage work and the Eigen model have established a rigorous framework for understanding the evolution of other viruses. Low fidelities of replication by the replicase of phage Q_β[11] foreshadowed, by more than a decade, measurements of low fidelities on the reverse-transcriptase of HIV-1 (ref. 12). Moreover, the heterogeneous nature of virus populations anticipated by the quasi-species model has been verified experimentally in recent years for HIV-1 (ref. 13), influenza A[14], foot-and-mouth-disease virus[15], vesicular stomatitis virus[16], and others[17].

Here we propose and test a strategy for vaccine development using phage as a model system. Attenuated 'live' vaccines have traditionally been developed by passage of a virulent virus strain until various ill-defined indicators for non-virulence such as cytopathogenicity, reduced infectivity, syncytium formation, serologic response[18], plaque size[19], or temperature sensitivity[20] have suggested the suitability of the vaccine for human testing. Since the molecular mechanisms of attenuation for different viral systems are not firmly established[21–23], we propose a procedure for attenuation and, ultimately, viral inactivation, for which the mechanism is predictable and verifiable. Here, we continuously culture phage T7 on BL21(DE2), a host that constitutively expresses the T7 RNA polymerase, an essential early enzyme[24]; earlier tests of a continuous phage culture in growing plaques showed that phage variants requiring the host-provided polymerase for growth could result[25]. By employing a two-stage continuous-stirred-tank reactor[26,27] rather than a growing plaque, we were able to continuously feed fresh host to the replicating phage over the course of the experiment.

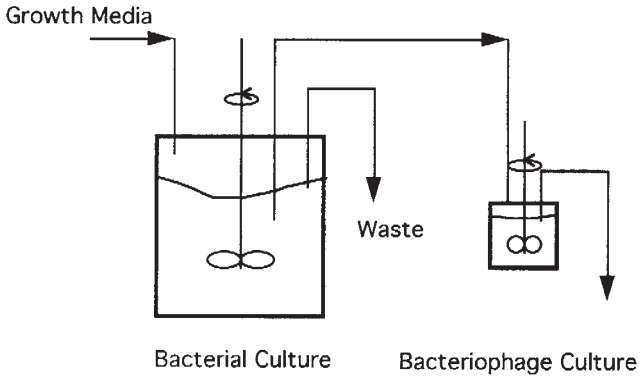

Figure 1. Two-stage flow reactor for continuous culture of phage.

Results

Phage culture in a flow-through reactor. The continuous culture of phage requires a constant source of receptive host bacteria. Although phage have traditionally been propagated over multiple generations by serial passage[7], their continuous propagation can be facilitated by employing a two-stage continuous reactor cascade (Fig. 1), as proposed by Husimi[26]. Here, a continuous culture of host *E. coli* in the first stage constantly supplied fresh host to the second stage for the continuous propagation of phage. The phage concentration in the second stage oscillated immediately following the inoculation of phage (Fig. 2A, inset) due to the lag–burst nature of the

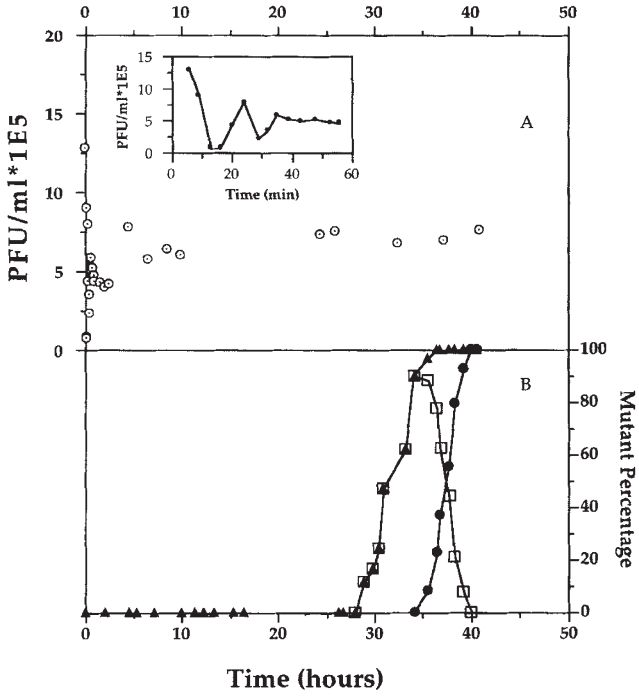

Figure 2. Continuous bacteriophage culture over 40 hours. (A) Maintenance of infectious titer. (B) Emergence of phage mutants. ▲ Total mutant; □ (kin-) mutant; ● (poly-) mutant.

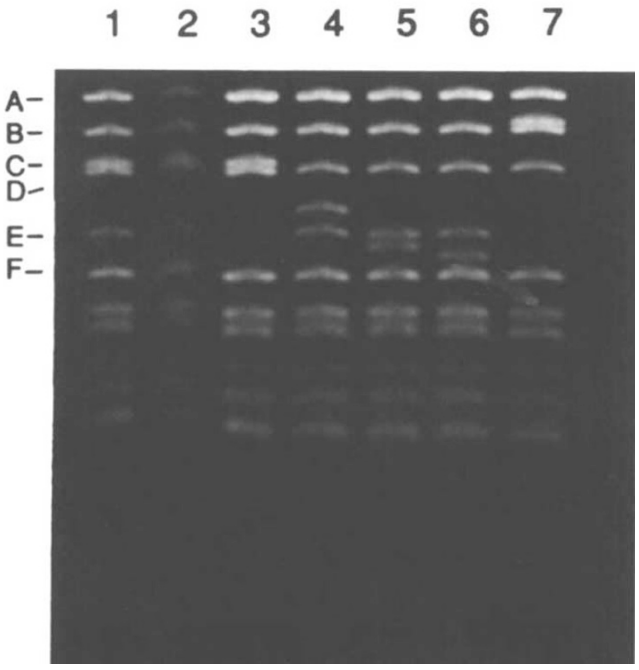

Figure 3. Hae II digestion patterns of emerging phage strains. Detailed descriptions are provided in the text. Lane 1: WT, original phage stock; Lane 2: WT, isolated at 36.4 hr; Lane 3: (kin-), 28.4 hr; Lane 4: (poly-), 35.7 hr; Lane 5: (poly-), 39.3 hr; Lane 6: (poly-), 43.7 hr; Lane 7: (poly-), 43.7 hr.

phage replication[28] and the constant dilution experienced by the phage population. The oscillations were damped over the course of several generations, a phenomenon that has been observed[29]. After the initial transients passed, the phage concentration was maintained at about 7×10^5 PFU/ml for more than 40 hours (Fig. 2A).

Emergence of phage mutants. After 25 hours, mutant phage appeared and by 36 hours displaced the wildtype (Fig. 2B). Since eleven hours elapsed between the first detection of the mutants and their outgrowth, a lower bound of 44 phage generations were required for displacement of the wildtype, assuming four phage generations per hour. The displacement of the wildtype was accomplished by the interplay of at least two mutants. The first was designated (kin-) because its limited host range suggested that it was kinase defective, and the second was (poly-) because it required host complementation of the T7 RNA polymerase in order to grow. Between 35 and 36 hours, the second mutant was detectable against a population dominated by the (kin-) phenotype, but still containing detectable wildtype.

Genetic characterization of phage mutants. The emergent genealogy was revealed by comparing Hae II restriction patterns of 26 mutants isolated over the course of the selection process, between 28 and 44 hours. The correspondence between restriction fragments and genes is known[30]. From clones of the 26 isolates, five digestion patterns were found (Fig. 3). Only one digestion pattern corresponded with the (kin-) mutant (Fig. 3, lane 3), whose deletion involved fragment E. The remaining four digestion patterns were (poly-) mutants, deleted in fragment C. Mutant 26 contained the largest deletion, affecting both fragments C and E (Fig. 3, lane 7). The size of this deletion was calculated to be at least 12 percent of the wildtype T7 genome. It is evident that a heterogeneous population contributed to the displacement of the wildtype T7 following the appearance of the initial (kin-) mutant; the patterns suggest that the (poly-) mutants did not descend from the (kin-) mutant, because the early (poly-) mutants contain fragment E, which the (kin-) mutant deleted. The reason for the displacement of the wildtype by the mutants was apparent from one-step growth cultures, where it was found that all the isolated mutants exhibited significantly greater infection yields than wildtype T7 (data not shown). Notably, Mutant 26 produced a two-fold higher yield than wildtype T7. Recent computer simulations of the intracellular kinetics of T7 replication suggest that the larger burst size can directly be attributed to a shift towards earlier transcription of phage proteins by the deletion mutants, relative to the wildtype (in preparation).

Immunogenicity of phage mutant. Mutant 26 was immunogenically indistinguishable from wildtype T7, based on titrations of antisera activity (Fig. 4). In the left panel of Figure 4 antiserum raised against wildtype T7 completely neutralized both wildtype T7 and Mutant 26 at a 10^5-fold dilution, but was inactive at a 10^6-fold dilution; at intermediate dilutions the survival fractions of the wildtype and mutant were indistinguishable from each other. Similar results were found for the antiserum raised against Mutant 26 (Fig. 4, right panel). Neutralization curves for phage M13, an unrelated phage serving as a control, were at the background level, as expected. Mutant 26 was unable to infect normal cells, due to a deletion in the gene encoding its RNA polymerase, an essential enzyme of the normal phage replication process. Together, these results indicate that the evolutionary strategy that gave rise to Mutant 26 may be feasible as an approach for the generation of safe intact-virion vaccines.

Discussion

Our results indicate that *trans* complementation of an

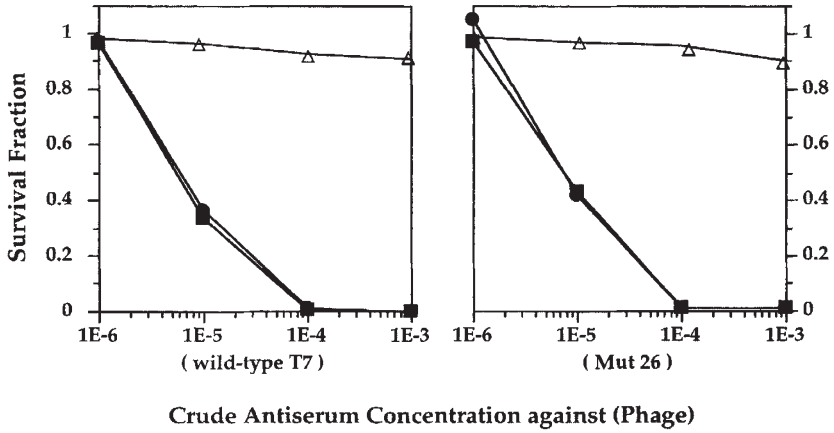

Figure 4. Neutralizing curves for antisera against wildtype T7 and Mutant 26. Concentration units are relative to undiluted antisera, and survival fraction refers to the fraction of phage that resist neutralization. ● Wildtype T7; ■ Mut 26; △ Phage M13 (control).

essential viral function, supplied over the course of multiple virus generations, can promote the emergence of mutant viruses, defective in the function. The mutants, which are unable to replicate on natural hosts, are immunogenically indistinguishable from the wildtype virus, suggesting their suitability as safe, whole-virion vaccines.

To date, studies on adenovirus, herpes simplex virus (HSV), and simian immunodeficiency virus (SIV), support the feasibility of the proposed strategy for generating animal vaccines. More than a decade ago, deletion mutants of adenovirus, created using recombinant techniques, were found to be unable to grow on normally susceptible human embryonic kidney cells, yet they readily propagated on the cells transformed by fragments of adenovirus DNA[31], indicating that the transformed cells were able to complement the deleted function. More recently, an HSV construct containing a deletion in the gene for its essential glycoprotein H could only be propagated on a cell line that supplied the glycoprotein in *trans*[32]. In addition, immunization of mice with this construct made them resistant to challenge with a high dose of wildtype virus[33]. More recent work showing that rhesus monkeys vaccinated with a mutant of SIV lacking a sequence presumably involved in promoting vigorous virus replication *in vivo*[34], were protected when challenged by pathogenic SIV[35].

Although these deletion mutants have already been created using the tools of molecular genetics, the strategy of generating deletion-mutant vaccines by continuous culture or serial passage may offer advantages by enabling the production of heterogeneous vaccines. This is indirectly supported by theoretical and experimental observations. Eigen's quasi-species theory indicates that the target of selection in natural populations of error-prone replicators, such as rapidly evolving viruses, is not a genetically homogeneous species, but a defined distribution of many closely related species[8–10], the so-called quasi-species[36]. This suggests that an animal's immune response to infection by a natural viral quasi-species may be more effective when primed by a vaccine that anticipates and reflects the heterogeneity of the quasi-species than by one that is genetically pure. It is noteworthy that in the presence of a high-titer of neutralizing monoclonal antibody (MAb), *in vitro* infection of MT4 cells by a cloned HIV strain selects plaques containing MAb-resistant virus[37], demonstrating the remarkable ability of HIV to mutate and escape a homogeneous anti-HIV environment. Finally, we speculate that the success of serial passage in generating effective live-attenuated vaccines against rubella, yellow fever, mumps, polio, measles, and smallpox[38] can in part be attributed to their heterogeneity and the resultant breadth of their elicited immune responses.

Experimental Protocol

Phage and host strains. Standard techniques were employed to handle and store bacteria and phage[28,39–41]. Bacteriophage T7, and *E. coli* BL21(DE2), BL21 and BR3, were provided by F.W. Studier. Strain BL21(DE2), which was used to seed the host reactor, constitutively expresses the T7 RNA polymerase to a level such that T7 deletion mutants lacking the RNA polymerase, indicated here as (poly-), are complemented. Host strains BL21(DE2) and BL21 can support the growth of kinase defective (kin-) mutants, but BR3 cannot.

Continuous culture. Two continuous stirred reactors in series were used for phage cultivation, as shown in Figure 1. The first reactor was inoculated with *E. coli* BL21(DE2) growing on M9 media. It was first run as a batch culture for 5–7 hours to reach an OD_{600} of 0.1 and was then switched to continuous culture at 0.05 to 0.08 hr^{-1} dilution rate. The bacteria culture was run continuously for at least 12 hours before feeding the second reactor and inoculating with phage. The dilution rate in the phage reactor was typically set in the range 0.5 to 1 hr^{-1}.

Double-layer plating assay. Three bacterial host strains, BL21(DE2), BL21 and BR3, were used to characterize mutant phages[42,43]. Based on their growth on each of these bacterial strains, three phenotypes can be identified: Wildtype phage (WT) grows on all strains; kinase-defective mutants (kin-) grow on BL21 and BL21(DE2) but not on BR3; and polymerase defective mutants (poly-), which may also be defective in kinase, grow only on BL21(DE2).

Restriction patterns. Phage DNA was prepared from phage lysates and separated by gel electrophoresis as previously described[25]. Agarose gels at 1% were loaded and run at 4 V/cm for 2 to 5 hours.

Antisera cross-reactivity. After being concentrated by PEG 8000/NaCl precipitation, phage stocks of wildtype and Mutant 26 were purified by CsCl gradient ultracentrifugation and membrane dialysis[44]. Rabbit antisera against the phage were raised by Cocalico Biologicals, Inc., Reamstown, PA. Following an initial inoculation, rabbits were boosted with purified phage stocks on the 14th, 21st and 49th days. Test antisera were collected on the 35th and 56th days. The neutralization activities of the 56th day antisera were determined by subjecting phage for three minutes to different dilutions of antisera and measuring phage titers by standard agar-layer plating. The survival fraction, defined as the ratio of final to initial phage titers, was determined for each antiserum dilution.

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