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Review

The delicate balance in genetically engineering live vaccines☆

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ABSTRACT

Contemporary vaccine development relies less on empirical methods of vaccine construction, and now employs a powerful array of precise engineering strategies to construct immunogenic live vaccines. In this review, we will survey various engineering techniques used to create attenuated vaccines, with an emphasis on recent advances and insights. We will further explore the adaptation of attenuated strains to create multivalent vaccine platforms for immunization against multiple unrelated pathogens. These carrier vaccines are engineered to deliver sufficient levels of protective antigens to appropriate lymphoid inductive sites to elicit both carrier-specific and foreign antigen-specific immunity. Although many of these technologies were originally developed for use in *Salmonella* vaccines, application of the essential logic of these approaches will be extended to development of other enteric vaccines where possible. A central theme driving our discussion will stress that the ultimate success of an engineered vaccine rests on achieving the proper balance between attenuation and immunogenicity. Achieving this balance will avoid over-activation of inflammatory responses, which results in unacceptable reactogenicity, but will retain sufficient metabolic fitness to enable the live vaccine to reach deep tissue inductive sites and trigger protective immunity. The breadth of examples presented herein will clearly demonstrate that genetic engineering offers the potential for rapidly propelling vaccine development forward into novel applications and therapies which will significantly expand the role of vaccines in public health.

1. Introduction

The recent explosion in the availability of genomic sequences for a wide variety of pathogenic organisms, coupled with a rapid advance in powerful genetic engineering technologies, now offers the opportunity of efficiently developing highly immunogenic and protective vaccines against a wide variety of diseases. The pathogens against which these vaccines are developed may be of viral, bacterial, parasitic, or fungal origin, and the resulting vaccines can be engineered either for animal or human vaccination. In this review, we will focus on the engineering of live bacterial vaccines, and we will use the genus *Salmonella* to illustrate engineering strategies, which can in principle be applied to a variety of bacterial pathogens for which relevant molecular biology and pathogenicity data are available. A central theme of this review will be the importance of metabolic fitness and its impact on the immunogenicity and protective efficacy of live vaccines. The application of engineering technologies to pathogens without careful consideration of the balance between attenuation and immune responses can yield vaccine candidates that have excellent safety characteristics but have lost the capacity to reach immunological effector sites and consequently fail to induce protective immunity. Strategies that have been recently developed to address this critical balance between safety and immunogenicity will be emphasized within this context of metabolic fitness.

2. Engineering of bacteria intended as homologous vaccines

2.1. Attenuating strategies targeting virulence and metabolism

It is relatively easy to weaken pathogens and engineer safe candidate attenuated vaccines. Given that these pathogens are exquisitely adapted to grow and replicate within their hosts, engineering disruptions in their intricate balance of metabolic and

Abbreviations: GFP, green fluorescent protein; LD50, 50% lethal dose; RDAP, regulated delayed attenuation phenotype; RDAS, regulated delayed antigen synthesis.

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virulence mechanisms will certainly not require an inordinate amount of technical prowess to create attenuated strains. However, assuring safety while still achieving the immunogenicity and protective efficacy required with live vaccines has proven to be a much more challenging proposition for vaccine development. In cases where virulence factors such as toxins have been clearly defined, engineering deletions of such toxins has proven to be quite successful in creating effective vaccines. Complete deletion of virulence genes, rather than introduction of inactivating point mutations, is required to ensure that the likelihood of reversion of the vaccine candidate back to a wildtype pathogen is very low; to further reduce the possibility of reversion, introduction of one or more additional attenuating deletions is usually carried out as well. This early strategy for vaccine design was successfully applied by Tacket et al. almost a decade ago in the construction of an attenuated live cholera vaccine [1]. To accomplish this, the wildtype *Vibrio cholerae* classical Inaba strain 569B was engineered for removal of both the catalytic subunit of cholera enterotoxin, as well as deletion of a putative hemolysin virulence factor. When tested in volunteers, this vaccine was found to be safe and highly immunogenic, with a protective efficacy of 91% against moderate to severe diarrhea and 80% against any diarrhea, after challenge with 10^5 colony forming units (CFUs) of fully virulent *V. cholerae*.

In the case of *Salmonella* vaccines, attenuation of wildtype strains has focused both on deletion of virulence factors as well as disruption of metabolic pathways, and the two serovars of *Salmonella* with which most vaccine constructions have been carried out are *Salmonella enterica* serovars Typhimurium and Typhi. *Salmonella typhimurium* typically causes a self-limiting gastroenteritis in humans while *Salmonella typhi* is the etiologic agent of typhoid fever. In both serovars, virulence factors have been found to be chromosomally encoded within clusters called *Salmonella* Pathogenicity Islands (SPIs) which play critical roles in the manifestation of disease [2]. Much attention has been devoted in particular to two distinct pathogenicity islands that encode type III secretion systems (T3SS) that inject virulence proteins called effectors into target eukaryotic cells, disrupting normal host cellular functions and facilitating *Salmonella* invasion and systemic disease [3–5]. The SPI-1 T3SS externally targets eukaryotic host cells and injects effectors that trigger actin rearrangements to enhance uptake of *Salmonella*. Then using the SPI-2 T3SS, internalized *Salmonella* are able to inject additional effector proteins into the cytoplasm essential for bacterial intracellular survival and replication [6].

In a study reported by Hindle et al. [7], attenuated vaccine candidates from both *S. typhimurium* (designated WT05) and *S. typhi* (designated M01ZH09) were engineered such that delivery of all SPI-2 effectors was disrupted by deletion of a critical structural protein *ssaV* involved in the assembly of the effector injectisome apparatus. This deletion mutation was accompanied by a further deletion in *aroC* involved in the aromatic amino acid biosynthesis pathway, creating candidate vaccine strains which were then compared in a Phase 1 dose-escalating clinical trial [7]. Both strains were shown to be safe, with negligible clinical symptoms and no vaccine organisms detected in the blood. The *S. typhi* M01ZH09 vaccine was shed from the majority of volunteers for 3 days. However, the attenuated *S. typhimurium* WT05 strain established an unacceptably persistent colonization of volunteers with shedding for up to 3 weeks, and this strain was not pursued further. When evaluated in Phase 2 clinical trials [8], single oral doses of M01ZH09 up to 1.7×10^{10} CFUs were found to be safe and immunogenic, with 97.4% of subjects responding to vaccination with either IgG or IgA responses to *S. typhi* LPS, and 92.1% of those receiving a dose of 7.5×10^9 CFUs having a positive *S. typhi* LPS-specific ELISPOT response.

2.2. Balancing safety and immunogenicity

Live vaccines that are insufficiently attenuated elicit unacceptable clinically defined adverse events in vaccines and are considered unacceptably reactogenic. As work with the attenuated M01ZH09 *S. typhi* vaccine illustrates, attenuation strategies targeting both virulence determinants and metabolic factors can be quite effective for constructing safe and immunogenic live vaccines that perform well in clinical trials. However, care must be taken to ensure that metabolic attenuation strategies do not result in the over-attenuation of vaccines, with subsequent loss of immunogenicity resulting from the crippling of metabolic fitness of the live vaccine. Genetic inactivation of too many critical genes, or inappropriate selection of targets, can result in vaccine candidates that fail to colonize a host sufficiently to engage innate and acquired immunity, and elicit durable protection. We have previously reviewed the results of clinical trials conducted with attenuated *S. typhi* candidate vaccines, and noticed a striking relationship between reactogenicity and immunogenicity [9], which we illustrate schematically in Fig. 1. Fully virulent strains, as well as vaccine candidates, which are insufficiently attenuated elicit unacceptable clinical symptoms (*i.e.* highly reactogenic) but also tend to be highly immunogenic (Fig. 1A). Vaccines that have been genetically engineered to minimize reactogenicity may become insufficiently immunogenic (Fig. 1B). Ideally, the most promising live vaccines that perform well in clinical trials will achieve a delicate balance between reactogenicity and immunogenicity (Fig. 1C).

This concept is clearly illustrated by a series of attenuated *S. typhi* candidate oral vaccines engineered from the wildtype strain CDC10-80, all carrying a deletion in the *aroA* gene critical to the aromatic amino acid biosynthesis pathway. When coupled with an additional mutation in *aroD*, the resulting Δ*aroA* Δ*aroD* strain proved to be insufficiently attenuated but highly immunogenic (Fig. 1A) [10]. When the triple deletion mutant Δ*aroA* Δ*aroD* Δ*htrA* was constructed (by further deletion of *htrA* encoding a heat-shock serine protease), safety improved at lower oral dosage levels but immunogenicity declined (Fig. 1B); at higher oral doses which improved immunogenicity, reactogenicity (*i.e.* fever and bacteremia) was unacceptably high (Fig. 1A) [10]. Combining Δ*aroA* with deletions in either *purA* (involved in the purine biosynthesis pathway), or *phoP*/*phoQ* (a two-component environmental regulatory system of virulence in *Salmonella*) dramatically reduced both reactogenicity and immunogenicity (Fig. 1B) [11,12]. It was only when the *phoP*/*phoQ* deletion mutation alone was introduced into a different parent strain of *S. typhi* (Ty2) that it became possible to balance reactogenicity with immunogenicity at high oral dosage levels (Fig. 1C) to induce vaccine-specific immunity [13]. These observations clearly illustrate that the engineering of an attenuated live bacterial vaccine requires a carefully crafted balance between attenuation and immunogenicity that is not always attainable by deliberate engineering and may sometimes only be achieved by trial and error, with clinical trials ultimately determining the fate of vaccines that animal models can only suggest as promising candidates.

Over-attenuation and the subsequent failure of engineered strains to reach appropriate immune inductive sites was encountered by Kong et al. [14] with efforts to construct attenuated strains of *S. typhimurium* by engineering modifications to lipopolysaccharide (LPS). LPS is the major component of the outer membrane of *Salmonella*, and is a key virulence determinant that confers protection against complement activation and killing by macrophages [15]. LPS is comprised of a lipid A membrane anchor, a core oligosaccharide, and the outer O-antigen which defines the various serovars of *Salmonella*. Since the enzymatic pathways involved in LPS synthesis are well characterized for *Salmonella* [16], Kong et al. undertook a systematic analysis of the effects of engineering

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