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REVIEW

Pathogenic Bacteria as Vaccine Vectors: Teaching Old Bugs New Tricks

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As our scientific knowledge of bacteria grows, so does our ability to manipulate these bacteria to protect rather than infect mammalian hosts from a diverse group of diseases. The old axiom that the best way to protect from a disease is to get infected in the first place is not feasible in the face of the diverse group of pathogens that infect humans. Therefore, reprogramming bacteria to protect against diverse bacterial, viral, and parasitic diseases as well as cancer is a new reality in the field of vaccines.

INTRODUCTION

Vaccines remain the most effective tool to prevent infectious diseases and also have been evaluated as a therapeutic tool to treat diseases such as cancer. A hallmark of a good vaccine is the ability to induce long-term protective immunity against a particular pathogen. The immune system is capable of recalling encounters with pathogens and can still mount a protective response decades after the initial contact [1]. This response to pathogens can be used beneficially to design a vaccine vector capable of eliciting the desired long-term immune response. Bacterial vaccine vectors offer multiple advantages: (1) there are several well-characterized virulence attenuating mutations; (2) the quantity and *in vivo* location of antigen expression can be regulated; (3) multiple vaccine delivery routes are possible; and (4) they are potent innate and adaptive immune system stimulators. These bacterial vaccine vectors can be used to impart protection against self-antigens as well as heterologous antigens. For example, attenuated *Salmonella* Typhimurium vaccine vectors have been used to generate protective immune responses in mice and in some

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†Abbreviations: DAP, diaminopimelic acid; GALT, gut-associated lymphoid tissue; PRRs, pattern recognition receptors; DCs, dendritic cells; T3SS, type III secretion system.

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cases humans against viral (e.g., LCMV, SIV, influenza), bacterial (e.g., *Listeria monocytogenes*, *Streptococcus pneumonia*), and protozoal (*Plasmodium falciparum*) pathogens, as well as cancer [2-8]. The versatility and immunogenicity of this platform make it an excellent vaccine vector.

VIRULENCE ATTENUATED BACTERIAL VECTORS

Historically, virulence attenuation of bacterial vaccine vectors was derived by chemical mutagenesis and repeated laboratory passaging of virulent bacterial isolates. Two modern examples of licensed live attenuated bacterial vaccines derived in this manner are *Salmonella enterica* serovar Typhi Ty21a and *Mycobacterium bovis* BCG [9,10]. Nowadays, attenuated vaccine vectors are constructed using recombinant DNA technology based on current understanding of bacterial virulence. Several virulence attenuated strains of pathogenic bacteria have been evaluated as vaccine vectors, including strains of *Salmonella* spp., *L. monocytogenes*, *Vibrio cholera*, *Shigella* spp., *Yersinia enterocolitica*, *Bacilius anthracis*, *Mycobacterium bovis* BCG, and *Bordetella pertussis* [11,12].

Virulence attenuated mutants must balance decreased reactogenicity with maximal immunogenicity. Therefore, several different virulence mutations have been studied, alone and in combination, to determine suitable virulence attenuated bacterial vectors for diverse antigens. A well-characterized class of virulence attenuating mutations is gene deletions that affect virulence gene regulation. One example of this class of virulence attenuated bacterial vectors are *Salmonella* spp. strains that contain deletions in the *phoP* and/or *phoQ* genes [13]. These genes are part of a global virulence regulatory system in *Salmonella* and comprise a two-component regulatory system for phosphate sensing. These mutants have been demonstrated to be non-reactogenic and immunogenic in the context of an oral *Salmonella* Typhi vaccine tested in humans [14]. Auxotrophs are another important class of virulence attenuated bacterial vectors. Auxotrophic mutants, which require a metabolite not available in vertebrate tissues, generally undergo limited replication once delivered to the host and are cleared from the host within days to weeks. Auxotrophs that contain a deletion in a gene or genes that are part of the aromatic amino acid (*aro*) biosynthetic pathway have been demonstrated to be attenuated as well as immunogenic in several bacterial strains, including *Salmonella* spp., *Bordetella* spp., *S. flexneri*, *L. monocytogenes*, and *Y. enterocolitica* [15-19]. Both of these classes of virulence attenuated mutants make promising bacterial vaccine vector candidates.

Some concerns with using live attenuated bacterial vectors are the possibility of pathogenic reversion of the vector once administered and pre-existing immunity to the vector. One way to circumvent potential pathogenic reversion is to introduce multiple virulence attenuating mutations into the bacterial vector. In addition, these mutations should be capable of attenuation independently. Therefore, the risk of pathogenic reversion as a result of recombination events or horizontal gene transfer is virtually eliminated. Another risk with using pathogenic bacteria as vaccine vectors is complications that can arise due to pre-existing immunity. Prior exposure to the bacterial vector has been demonstrated to decrease efficacy of the vaccine [20]. Thus, different bacterial species or serotypes can be prepared as vaccine vectors depending on the prior exposures of the population to be vaccinated as well as whether the vaccine must be administered in multiple doses. By taking these limitations into account during the initial vaccine development, an effective virulence attenuated bacterial vector can be designed to virtually any disease.

ANTIGEN EXPRESSION IN BACTERIAL VECTORS

Heterologous antigens can be expressed either from chromosomally integrated antigen cassettes or plasmid-based antigen expression systems in bacterial vaccine vectors.

Chromosomal expression of antigens offers several advantages, including genetic stability and the ability to integrate and express multiple antigen genes. One substantial disadvantage of chromosomal integration is that generally one copy of the antigen gene will be expressed per bacterial cell; therefore, sufficient levels of the antigen may not be reached to confer protection. One way to circumvent this limitation is to express the antigen from a plasmid. The quantity and location of antigen expression can be regulated by using a plasmid-based system. The amount of antigen expressed can be controlled by using either high-copy or low-copy plasmid backbones as well as inducible systems that produce large quantities of antigen upon addition of the induction agent, such as arabinose [21,22]. Furthermore, the location of antigen expression can be controlled *in vivo* to give the maximal antigen dosage depending on subcellular localization. Constant antigen synthesis can result in decreased bacterial vector fitness and decreased immunogenicity; therefore, using *in vivo* inducible promoters to control antigen expression in a plasmid can improve immune responses to the bacterial vaccine vector. One example of *in vivo* inducible promoters is the promoter for the *Salmonella* Typhimurium gene *pagC*, which has been shown to have high *in vivo* expression, while *in vitro* it is poorly expressed [23]. The model antigen OVA, when expressed from the *pagC* promoter, was shown to elicit potent cellular immune responses, compared to a promoter that was not induced *in vivo*. Other promoters have been studied that are induced in anaerobic conditions or low-iron conditions [24,25]. Both of these conditions are found in host tissues, and antigens expressed from these promoters have had variable success in inducing protective immune responses.

Concerns about plasmid stability are a challenge with using a plasmid-based expression system in a bacterial vaccine vector. Although maintenance of plasmids traditionally has been achieved though using antibiotic resistance markers in bacteria, safety concerns preclude the use of antibiotic selection with vaccine vectors. Therefore, antibiotic-free plasmid selection methodologies have been developed for the use in vaccine vectors [26]. In *Salmonella*, a balanced-lethal plasmid system has been developed that is based on a gene, *asd*, required for the synthesis of diaminopimelic acid (DAP†), an essential component of the bacterial cell wall [27]. In *Salmonella* vectors in which *asd* has been deleted from the chromosome, complementation with a plasmid carrying an intact *asd* gene, as well as the vaccine antigen, allows for the survival of the bacteria in DAP-free environments such as host tissues. This balanced-lethal expression system allows for the stable expression of vaccine antigens from a plasmid in a bacterial vaccine vector.

DELIVERY OF LIVE-ATTENUATED VACCINE VECTORS

Most pathogens are restricted by mucosal membranes and have evolved elegant mechanisms to either transit the mucosal barrier or infect the cells that form the mucosal membranes. Therefore, vaccines that can elicit a protective immune response directly at the mucosal barrier are important to protect the host from subsequent infection. Several different vaccine vectors have been developed to deliver antigens mucosally, including viral particles, live-attenuated viral vectors, liposomes, microspheres, ISCOMs, transgenic plants, mucosal adjuvants, and live-attenuated bacterial vectors [11,28]. Of all these options, the live-attenuated bacterial vectors are perhaps the best characterized. These vectors can be delivered mucosally via the oral, intranasal, rectal, vaginal, or inhalation route and have been shown to not only stimulate the mucosal immune response but also a systemic immune response [29].

Bacterial vaccine vectors can overcome the obstacles faced by antigens alone at mucosal surfaces. These obstacles include enzymatic degradation, low pH, and poor absorption by mucosal cells. Since enteric pathogens such as *Salmonella* spp., *Shigella* spp., and *Yersinia enterocolitica* can serve as bacterial vectors, the methods that these bacteria use to infect the intestinal tract can

be exploited to deliver antigen to the mucosal immune cells in the intestinal tract. For example, *Salmonella* has been shown to target M cells during intestinal infection that overlay the gut-associated lymphoid tissue (GALT) [30]. The GALT is an inductive site for immune responses and a key player in the stimulation of mucosal immunity. Additionally, *Salmonella* is known to transverse the enterocytes of the intestinal tract and access the reticuloendothelial system which can lead to systemic immune responses as well [31]. Therefore, live bacterial vectors make excellent vehicles for the delivery of antigens at mucosal surfaces.

BACTERIAL VECTORS AS POTENT IMMUNE SYSTEM STIMULATORS

The innate immune system can recognize microbes directly through pattern recognition receptors (PRRs) expressed on innate immune cells such as dendritic cells (DCs), macrophages, neutrophils, mast cells, endothelial cells, and fibroblasts. Although it has been empirically shown that the stimulation of the innate immune response is key to mounting a protective adaptive immune response, only recently have the mechanisms begun to be elucidated [32]. Bacterial vaccine vectors express many different molecular patterns that can be detected by innate immune cells like DCs and translated to the adaptive immune system cells to modulate the type of immune response (Th1 or Th2 biased), strength, and persistence.

The type of bacterial vector used as a vaccine delivery vector plays a key role in the kind of adaptive immune response elicited. The intracellular lifestyle of the bacterial vector (cytoplasmic versus membrane-bound) determines whether antigens are delivered to the MHC class I or class II pathway. For some diseases, including those due to viral and bacterial pathogens as well as cancer, the mounting of a protective immune response requires the delivery of antigens to the MHC class I pathway so that protective cytotoxic $CD8^+$ T cells are generated [33,34]. Both *L. monocytogenes* and *Shigella* spp. vectors directly access the cytoplasmic compartment during intracellular infection and can deliver antigens directly to the MHC class I pathway while other bacterial vectors such as *Salmonella* spp. or *M. bovis* BCG remain localized in a membrane-bound compartment and inefficiently deliver antigens to the MHC class I pathway [35,36]. In the case of *Salmonella* spp., this deficiency can be overcome by secretion of antigens through the type III secretion system (T3SS) [3]. The T3SS is basically a bacterial nanosyringe that can be used to deliver proteins directly into the cytosol of both antigen presenting cells and non-phagocytic cells. By fusing the secretion signal and chaperone binding domain of a T3SS secreted effector protein to an antigenic peptide, virtually any antigen can be delivered to the MHC class I pathway by *Salmonella* [37]. The delivery of antigens through the T3SS in *Salmonella* has been shown to elicit protective cytotoxic $CD8^+$ T cells in mice to various viral, bacterial, and parasitic diseases as well as cancer [38,39].

CONCLUSION

The use of live-attenuated bacterial vaccine vectors offers the potential of an orally delivered vaccine that is capable of eliciting protective mucosal and systemic immune responses. A range of heterologous antigens expressed in these vectors have been shown to confer protection against disease in mice and humans in some cases [40,41]. While much research is being done in the field of live-attenuated bacterial vaccine vectors, currently there are no licensed vaccines that utilize this approach, although several formations are in clinical trials. Furthermore, as research continues to elucidate the key components that are part of the balance between reactogenicity and immunogenicity, even better vectors can be developed.

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