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DNA vaccines for targeting bacterial infections

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Abstract

DNA vaccination has been of great interest since its discovery in the 1990s due to its ability to elicit both humoral and cellular immune responses. DNA vaccines consist of a DNA plasmid containing a transgene that encodes the sequence of a target protein from a pathogen under the control of a eukaryotic promoter. This revolutionary technology has proven to be effective in animal models and four DNA vaccine products have recently been approved for veterinary use. Although few DNA vaccines against bacterial infections have been tested, the results are encouraging. Because of their versatility, safety and simplicity a wider range of organisms can be targeted by these vaccines, which shows their potential advantages to public health. This article describes the mechanism of action of DNA vaccines and their potential use for targeting bacterial infections. In addition, it provides an updated summary of the methods used to enhance immunogenicity from codon optimization and adjuvants to delivery techniques including electroporation and use of nanoparticles.

Keywords

bacterial vaccine; cellular and humoral immune responses; DNA vaccine; molecular adjuvants

Times have changed since Edward Jenner immunized James Phipps against smallpox in 1796 and created what years later became known as a vaccine [1]. From the first live-attenuated or killed vaccines to the era of DNA vaccines in the early 1990s, molecular biology and microbiology have aided medical research in the development of vaccines against infectious diseases, cancer, allergies and autoimmune diseases by inducing rapid and robust immune responses or by creating immune tolerance [2]. From the time of Jenner's first vaccine until the present day, there have been over 60 licensed vaccines in the USA. These vaccines come in many forms: killed microorganisms, live-attenuated microorganisms, subunits, conjugate vaccines or toxoids. Although there are no US FDA-approved DNA vaccines for use in humans, they are the newest vaccine platform currently in development and have already had success in veterinary medicine [3–8].

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DNA vaccines are ‘genetic vaccines’ that involve the intramuscular or subcutaneous injection of a DNA plasmid containing a transgene that encodes the sequence of a target protein from a pathogen under the control of a eukaryotic promoter. The first studies that suggested that injection of a DNA plasmid *in vivo* could lead to the production of the plasmid-encoded protein were conducted by Ito [9] who showed that injection of naked papillomavirus DNA could induce tumors in rabbits. In addition, Atanasiu *et al.* achieved comparable results after the inoculation of hamsters with polyomavirus [10] while other groups reported that utilizing similar techniques could induce rat cardiac myocytes to express recombinant β -galactosidase [11–13]. However, the first evidence in the literature of immunologic use of DNA was provided by Tang *et al.* in 1992 [14]. Their studies found that injection of human growth hormone (hGH) DNA into the skin of a mouse using a gene gun was able to raise both hGH- and human α 1-antitrypsin (hAAT)-specific antibodies, suggesting that DNA may be a suitable route for induction of an immune response against pathogenic infection. That year, at the annual vaccine meeting at the Cold Spring Harbor Laboratory (NY, USA), Liu *et al.* [15], Robinson *et al.* [16] and Weiner *et al.* [17] gave rise to a new era for vaccination by describing the use of DNA in immunizations against influenza and HIV-1. Subsequently, many others described similar results following DNA immunization against rabies virus [18], bovine herpesvirus 1 [19] and hepatitis B surface antigen [20].

This article provides a detailed review of the use of DNA vaccines and their potential use for targeting bacterial infections, and also summarizes the significant efforts that have been made to improve their potency in order to achieve better outcomes in future clinical trials. The purpose of this article is to provide an update of new achievements in the fields of immunology and molecular biology in tailoring an effective, protective and therapeutic immune response through the use of these vaccines and to analyze future prospects for this technology.

DNA vaccine construction

A DNA vaccine plasmid can be divided into two main structures: the plasmid backbone and the transcriptional unit (Figure 1). The transcriptional unit contains the promoter and an insert or gene encoding the antigen of interest followed by a transcript termination/polyadenylation sequence.

Backbone plasmid

The plasmid backbone contains an origin of replication for amplification of the plasmid in bacteria, as well as an antibiotic resistance gene, conferring resistance to kanamycin for example, to enable a selective growth of the plasmid in bacteria [21]. Commercially available vectors approved for clinical use, such as pVax1, contain an *Escherichia coli* origin of replication such as pUC or pBR322. Replication of the plasmid in bacteria produces many copies of the plasmid in a relatively short period of time. Additionally, a mammalian origin of replication may be added for replication in mammalian cells, which can help to prolong the persistence and expression of the transgene.

Parts of the transcriptional unit: promoter, transgene & polyA tail

Promoter—Viral promoters such as cytomegalovirus (CMV), Rous sarcoma virus (RSV), simian virus (SV) 40 and long terminal repeat (LTR) are often utilized to drive the expression of the transgene/antigen in a vast variety of mammal cells types. CMV is perhaps one of the most commonly used and strongest of the transgene promoters known to date. Regardless of its widespread use, the CMV promoter has its limitations. For example, studies have suggested that various cytokines may differentially regulate the CMV promoter and have shown that IFN- γ and IL-10 may downregulate the CMV promoter’s activity while IL-4, IL-1 β and TNF- α may upregulate its activity [22]. Given these findings, the use of nonviral promoters is currently the

topic of intensive investigation. Despite the relatively weak activity of these promoters as compared with their viral counterparts, research into the use of nonviral promoters, such as that of the MHC class II promoter, are currently being investigated as possible alternatives [23,24]. Chimeric and other synthetic promoters are also being considered in the field [25–27].

Transgene—The possibility of encoding multiple proteins in a single construct is an important advantage that DNA vaccines have over other platforms, because not only can more than one antigen be added, but the sequences encoding adjuvant can also be added to enhance the vaccine potency. A hepatitis C virus vaccine with a transgene encoding a total of five proteins under a single promoter was reported for the immunization of chimpanzees [28]. Furthermore, data suggest that the selection of immunogenic antigens for incorporation into the construct as well as the optimization of codon usage may be crucial for vaccine success.

Polyadenylation sequence (poly[A])—The polyadenylation sequence is an essential aspect of gene expression, playing an important role in mRNA stability and translation [29]. Most vectors contain the SV40 or the bovine growth hormone polyadenylation signal. Studies have revealed that the latter is up to two-times more efficient and is therefore most commonly used [30].

Vaccine platforms currently in use

Currently, vaccines can be classified into five types: live-attenuated, inactivated microorganisms, subunit vaccines (subunits, polysaccharides and protein conjugates), toxoids and genetic based [31]. Attenuated vaccines are avirulent organisms typically due to culture or passage in adverse conditions. Pathogens can be weakened for the purpose of human vaccination in multiple ways, such as repeated serial passage to lessen the organism's virulence. Although weakened, these pathogens can still infect and multiply in human host cells, providing continuous antigenic stimulation, and eliciting both cellular and humoral immunity similar to those elicited by the original pathogen. *Bacillus Calmette-Guérin* (BCG), Sabin, measles, mumps and rubella vaccines are the best examples of this vaccine platform. However, with every benefit there lies a risk, and in this case, there is risk of reversion into a virulent strain. For this reason, they cannot be administered to immunocompromised patients. Killed vaccines, on the other hand, consist of inactivated virulent pathogens by chemical or γ -rays. There is no risk of mutation or reversion in this platform; however, they only induce humoral immune responses and multiple boosters are required for protection. Examples of inactivated vaccines in common use today are the typhoid and Salk poliomyelitis vaccines. Subunit vaccines are purified antigenic proteins from the pathogen such as flagella, capsule or a surface protein. Although they can be produced at large scales, they are poorly immunogenic, only induce humoral immune responses and require a booster vaccine every few years. Subunit vaccines have also been used to generate humoral immune responses to the polysaccharide capsules of bacteria. In this case, the polysaccharide antigen can be conjugated with a carrier protein to increase its immunogenicity. Examples of this platform could include the *Haemophilus influenza B* (Hib) and *Bordetella pertussis* vaccines. Lastly, toxoids vaccines are based on inactivated bacterial toxins, which successfully induce neutralizing antibodies against these virulent factors. Some major disadvantages can be the need for boosters, development of adverse reactions and the possible manufacturing issues in the detoxification process [3, 32]. The widely used vaccine against *Clostridium tetani* toxin is one example.

DNA vaccines: mechanism of action

Although the mechanism of immune induction by which genetic vaccines confer protection still remains unclear, the slow rise of the immune response following DNA immunization suggests that it follows a complex pathway that can mimic natural viral infection. However, it

is believed that once the DNA plasmid is administered to the skin, subcutaneum, vein, nasal cavity or muscle, the plasmid vector enters the cell, translocates to the nucleus and the transcription of the immunogene is initiated using the host's cellular machinery. The two major types of cells that become transfected through this method are myocytes and antigen-presenting cells (APCs) but there are studies in fish that show other cells taking up DNA such as hepatocytes or keratinocytes [33]. Following transfection, the host cell's replication machinery translates the transgene into protein, the peptides of which are subsequently presented by MHC-I to the immune system. Additionally, antigenic protein can be secreted in and around the surrounding tissue either by active secretion of the protein or its release following apoptosis of a transfected cell. The shed antigenic protein can then be engulfed and presented by APCs on both MHC-I and MHC-II, after which, the antigen-loaded APCs can migrate to the corresponding draining lymph node to activate naive T cells. Costimulatory signals help to trigger an immune response and the recruitment of more T cells [34]. At the lymph node, the T cells' secreted cytokines and shed antigens activate B cells and induce antibody production. The initial wave of T- and B-cell activation is small, but once these cells migrate back to the vaccine-transfected tissue, they can undergo restimulation. At this point, CD8⁺ T cells can lyse transfected myocytes presenting antigenic peptide on their MHC-I molecules, causing the release of more antigen. Furthermore, CD4⁺ T cells can activate immature dendritic cells (DCs) that later drain to the lymph node and take on a whole new cohort of B and T cells [35], repeating the cycle of activation again. Therefore, both humoral and cellular immune responses are primed for their next challenge. A summary of each vaccine platform and its mechanism of action is provided in Figure 2.

Why DNA vaccines may be better

Genetic vaccines have numerous advantages over traditional vaccines in terms of safety, ease of manufacturing and stability. Possibly the best current vaccine platform that induces both humoral and cellular immune responses is the use of live-attenuated pathogens; however, this also poses important safety concerns with the risk of reversion of the pathogen to a more virulent form [36,37]. DNA vaccines can also induce both long-lasting cellular and humoral immune responses but do not revert into virulence, and therefore raise fewer safety concerns. In addition, clinical trials with DNA vaccines have shown fewer incidences of systemic adverse effects such as redness, transient pain, swelling, fever and headache [38]. It was also reported that they pose no risk of integration into the genome, an earlier concern about this technique development [34]. Furthermore, DNA vaccines have the advantage of being used therapeutically [39]. Since they are unable to induce antivector immunity, as in the case of recombinant vaccines, they theoretically have unlimited boosting potential. This could be especially useful in the developing field of cancer vaccines, which rely on repeated boosting of T-cell responses to tumor antigens.

An important factor for preserving the potency of a vaccine is maintaining proper storage conditions to ensure the stability of its contents. Cold storage is a must for ensuring the survival of live vaccines and preserving the original structures of subunit vaccines in order for them to remain antigenically effective. This is a difficult and continuing problem in tropical countries and endemic areas. An advantage of DNA vaccines is that they are highly stable and do not require refrigeration. Therefore, DNA vaccines may be more practical for use in developing nations where the cold chain is more likely to be compromised.

In addition, current vaccines have complicated biology and structure, making modifications difficult to accomplish. For example, it takes approximately 8 months for the entire influenza virus production process to be completed, meaning companies have to predict almost 1 year in advance the strains that are to be included in the vaccine. Sometimes these predictions are incorrect, leading to a higher number of influenza infections than expected for that particular

year. Owing to the simple structure of DNA vaccines, modifications of the transgene can be made in a short period of time so that it reflects a specific pathogen's strain. Since DNA vaccines can also be easily replicated and amplified in bacteria, this permits fast, cheap and large-scale production within a short time.

Despite these obvious advantages, the lack of potency of first-generation DNA vaccines has been questioned due to lackluster results in humans as compared with those in mice [40]. Thus, investigators have been diligently working on new ways to improve the potency of the technique by developing novel adjuvants, codon optimization schemes and innovative methods of delivery that will make them more suitable and immunogenic for human vaccination [32].

Enhancing immunogenicity

Codon optimization—Codon optimization involves specific alteration of the gene sequence to maximize gene expression in a foreign host cell, based upon the commonly available levels of tRNA in that cell. In this way, optimized codons will utilize tDNAs that are more abundant in the cell, which can result in higher rates of protein translation, as described by several authors [41–44]. Furthermore, mRNA optimization is also essential for higher gene expression. Large numbers of C-G sequences in the mRNA can inhibit protein translation from increased formation of secondary structures. The presence of cryptic splice sites is able to cause anomalous gene expression, and the existence of *cis*-acting elements on the RNA molecule dictates specific transport and translation patterns of localized mRNAs [45]. Graf *et al.* obtained a significant immune response in mice after immunizing with an optimized synthetic HIV-1 *Pr55gag* gene over the wild-type control owing to increased mRNA stability [46]. Both codon and mRNA optimization improved the production of complex macromolecules and led to higher yields of antibodies [47].

Untranslated regions—Regulation of gene expression by elements located 5' and 3' to the encoded genes are critical regulators of vaccine gene expression. A modification of the Kozak consensus sequence or its insertion upstream of the gene as well as the addition of double stop codons can guarantee increased *in vitro* expression and mRNA stability [48].

Cytosine–phosphate–guanine—Unmethylated cytosine–phosphate–guanine (CpG) motifs, termed GACGTT in mice and GTCGTT in humans, are responsible for the activation of the Toll-like receptor (TLR)9 through dimerization. Thus, they play a key role in innate and acquired immunity by stimulating B lymphocyte conventional and plasmacytoid DCs, macrophages, natural killer (NK) cells and NKT cells [49,50]. The receptor can discriminate between host and foreign DNA based on these sequences. However, they are largely absent in human cells. Its adjuvant property and safety in the vaccine platforms against infectious diseases in different animal models has been clearly reviewed by Klinman *et al.* [51,52]. CpGs have been used in DNA vaccine platforms on several occasions; namely, the inclusion of CPG 7909 into the hepatitis B vaccine prior to the inoculation of HIV-seropositive patients was capable of enhancing antigen production and T-helper cell responses to the vaccine antigen [53]. There is also uncertainty regarding the influence of non-CpG elements on TLR9, and its other binding tendencies are currently being studied [54].

Delivery option: the key for success?

Next-generation DNA vaccines require an appropriate delivery technology, as the chosen method and the route of administration can play a key role in the magnitude and quality of the triggered immune response. Therefore, determining the ways in which DNA vaccines prime the immune response is critical for engineering the type of immune response induced. This means that while dermal delivery will first elicit a humoral response with the production of

IgA and IgG₁, intramuscular injection will prime a cellular response with the activation of CTLs and the production of IgG_{2a} antibody [55].

Traditionally, the skin has been a popular approach for vaccination owing to its accessibility, size and cell population that comprises of Langerhans cells, APCs and migrating lymphocyte cells. Amid the cutaneous techniques the following can be mentioned:

- Subcutaneous or intradermal injection: although it targets skin fibroblasts and keratinocytes, this is a technique that requires refinement. DNA tattooing is a modern intradermal version to cross the so-called 'simian barrier' by obtaining encouraging results in both murine and nonhuman primates in the case of HIV-1 immunization [56];
- Topical: DermaVir: transfects Langerhans cells via a dermal patch that consists of a nanoparticle that carries an HIV-1 antigen-encoding plasmid DNA [57];
- Painting DNA: consists of stripping a few layers of the skin in order to achieve a more successful transfection. Okuda *et al.* reported the use of fast adhesive glue for this purpose but they suggested multiple administrations of adhesive tape in combination with sodium dodecyl sulphate or urea cream (or both) for clinical application [58];
- Assisted by a vesicular system: involves the application of a vaccine to intact skin facilitated by a carrier (liposomes, niosomes, ethosomes and transfersomes) [59];
- Particle-mediated epidermal delivery or gene gun: Langerhans cells and keratinocytes become directly transfected by the bombardment of gold particles coated with DNA from a needle-free device. It has several advantages that make it capable for self-administration and useful in current clinical trials. To specify, it requires less DNA, and coated particles are stable and do not need cold chain, thus avoiding needle-stick injuries and disposable sharp issues [60].

However, intramuscular injection has proven to be not only practical but also more effective in transfecting myocytes than infiltrating APCs, and elicits long-lasting immunity. Early studies have preferred special routes for specific purposes like: vaginal mucosa [61], intranasal [62] or oral administration [63].

In addition, both chemical and physical augmentation of transfection has been widely studied. The prima donna of these technologies is the innovative electroporation or electropermeabilization method. The application of short, high voltage pulses for millisecond duration on a tissue can disrupt the cell membrane and generate pores that can persist for hours. This allows the entry of macromolecules such as drugs, peptides and naked DNA vaccines in the cytoplasm. To sum up, although the major mechanism in which electroporation enhances the vaccine potency is increasing gene transfection, it also increases the distribution of plasmid DNA throughout the tissue [64]. Furthermore, it generates a local danger signal due to the production of endogenous cytokines, consequently recruiting immune cells to the site of administration involving T-cell migration [65]. The mechanism is not as easy as passing a thread through the eye of a needle because there are certain parameters regarding pulse length, voltage, number of pulses, equipment and formulation that have to be optimized to obtain superior effects [66–68]. Nevertheless, there is no doubt that it has demonstrated its potential in several preclinical and clinical studies including: SARS-CoV [69], hepatitis C virus [70], HIV-1 [71,72] and Chikungunya virus [44]. Some authors have commented on the disadvantages of electroporation [73] and others have also explained that the complexity of the electroporator machine design may mean it receives less attention for human application [74]. Instead, we believe it is a field worth pursuing in the search for advanced strategies, such as the development of patches that contain electrodes, due to its promising outcomes. Another electrically driven method widely studied in the pharmacology field is iontophoresis. This

involves a low electrical-driven force to promote the movement of ions to the stratum corneum without changing the natural skin barrier [74]. Iontophoresis is used to optimize intradermal delivery of DNA vaccines and, therefore, optimize transfection [75]. Furthermore, investigations by the private sector have led to a number of other novel technologies that may be applicable to vaccination such as sonophoresis [76], chemical permeation enhancers [77] and microneedles [78]. Six different physical methods of gene transfer, including ultrasound, magnetically and electrically mediated ones, were analyzed in terms of mechanisms, advantages and disadvantages by Villemejeane and Mir [79]. They concluded the choice of one over the other depended on the proposed therapeutic application because they were all useful to deliver genes into tissues but they had preferential targets and limitations. Among these other techniques, Donnelly *et al.* explained that DNA vaccines can be formulated in or on phagocytosable microparticles like polylactide-co-glycolide, which is broadly used in drug delivery, so that they can exclusively target APCs [80]. The multiple vesicular systems carriers such as liposomes, niosomes, ethosomes and transfersomes are also under extensive study, with the latter being a useful option [81]. Furthermore, intracellular bacteria can be a cost-effective delivery method; these live-attenuated bacterial delivery vaccines have proven efficacious against infectious diseases and cancer. Some examples include: *Salmonella typhi* [82], *Listeria monocytogenes* [83,84], *Shigella flexneri* [85], *Yersinia enterocolitica* [86] and invasive *E. coli* [87]. Finally, nanotechnology that is complexed with DNA is currently being explored for the facilitation of receptor-mediated vaccine internalization. This technology is presently applied to drug delivery but is being explored for the delivery of diagnostic agents, gene therapy and, in this case, DNA vaccines [88]. All of these methods pursue the same goal to enhance DNA transfection and increase the potency of DNA vaccines.

Molecular adjuvants

In accordance with many laboratories, the coinjection of immunomodulatory plasmids that encode cytokines, chemokines or costimulatory molecules is a promising strategy to improve the efficacy of DNA vaccines. The aim is to expand the APC pool or to enhance its potency with these adjuvants without the adverse effects of administering purified cytokine proteins. However, among the cytokines, there are plenty of interleukins (ILs), interferons (IFNs), colony-stimulating factors and tumor necrosis factors (TNFs) that can be coadministered with the engineered vaccine. Each of them is useful for a different aspect of the immune response, thus providing flexibility in modulating the type of immune response desired. This method also noticeably increased the efficacy of prime–boost strategies by helping to compensate for weak outcomes due to antivector immunity of recombinant vaccines [89].

For example, pretreatment with a plasmid encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) has increased the recruitment of DCs to the immunization site and expanded professional APCs in the lymph node [90], and Laddy and Weiner stated their lack of clinical use according to clinical trial results [91]. By contrast, recent work in Phase I/II clinical trials involving the previous application of GM-CSF DNA followed by a multi-peptide vaccine in melanoma patients was able to elicit a polyfunctional CD8⁺ T-cell response [92]. These data are in accordance with those found in mice where murine GM-CSF amplified HIV antigen-specific T-cell proliferation and increased the proportion of antigen-specific polyfunctional memory CD8⁺ T cells. This technique enhances not only the magnitude of the response but also the quality [93].

Plasmid IL-2 and DNA vaccination were combined in order to increase humoral and cellular responses in the latest animal models for pathogens such as *Mycobacterium tuberculosis* [94, 95], infectious bronchitis virus [96] and human papillomavirus [97]. Barouch *et al.* also reported that the fusion of IL-2 and Ig protein was even more efficient in augmenting cellular responses because of increased half-life and avidity [98]. Years later, they published that this

effect was more likely due to enhanced initial priming of memory T lymphocytes than due to chronic cytokine expression [99]. Furthermore, IL-12 and IL-15 adjuvants significantly increase the antigen-specific Th1 response and maintain LT memory cells. Recently, their implication in HIV-1 vaccine development has been tested in rhesus macaques showing that IL-12 alone or along with IL-15 elevated cell-mediated and humoral immune responses [100]. In brief, even better results could be obtained after combining IL-12 and electroporation [72]. Similar to IL-12, IL-18 plays an important role in Th1 response due to the induction of synthesis of IFN- γ by NK and T cells. Wei *et al.* [101] selected this adjuvant for their vaccine against *Schistosoma japonicum*, leading to a notable antibody response as well as a splenocyte proliferative response. Furthermore, IL-8 and RANTES were identified to enhance antigen-specific Th1-type CD4⁺ T-cell response after herpes simplex virus (HSV) vaccination, resulting in better survival rates and less severe herpetic lesions [102]. Finally, IFN- γ was found to be effective in immunocompromised mice after malaria and influenza immunization [103].

Given that DCs play a central role in priming the immune response, upregulating their activation by the combination of plasmids encoding the chemokine macrophage inflammatory protein-1 α (MIP-1 α) and the DC-specific growth factor fms-like tyrosine kinase 3 ligand (Flt3L) is a promising approach. Flt3 generates expansion, maturation and chemoattraction that synergically work in the injection site [104]. The use of MIP-1 α showed a dramatic augmentation of antibody production and has proven to be the most potent CD8⁺ activator [105]. It is important, however, to understand the synergic activity some molecules have. When GM-CSF is administered alone, macrophages are recruited to the immunization site and a CD4⁺ response is triggered. In the case of MIP-1 α administration, DCs are recruited to induce a CD8⁺ T-cell response. Interestingly, when these plasmids are coadministered, both responses are observed, suggesting an additive effect [106].

An additional adjuvant alternative may be the modification of intra- and extracellular trafficking. This can amplify the CD8⁺ response by targeting the antigens for degradation by the ubiquitin (Ub)/proteasome pathway, allowing for efficient processing and presentation through the MHC class I pathway [107]. This method could also be used to amplify the CD4⁺ response by lysosomal or endosomal targeting [108] or could generate a higher antibody production by plasmids encoding cytotoxic T lymphocyte-associated antigen 4 or L-selectin [109]. Furthermore, immunogenicity of plasmid DNA may be due in part to the production of type 1 IFNs following the cellular detection of intracellular dsDNA by signaling molecules including STING and TBK1, which complex and traffic to endosomal compartments to associate with exocyst components including Sec5 [110]. Thus, the data regarding the use of plasmid-based molecular adjuvants are promising and prolific, and such data suggest that these techniques could help to augment the previously weak responses conferred by first-generation DNA vaccines. However, there is still a lot to learn regarding the complex behavior of specific adjuvants and their use in combination with next-generation DNA vaccines.

What to expect from the prime–boost approach?

Instead of improving vaccine immunogenicity by increasing the number of doses, researchers suggest the use of a mixed vaccine by priming first with a formulation and then boosting with a different one. As remarked by Plotkin, this heterologous prime–boost strategy is a way of increasing the DNA vaccine potency, particularly the antibody production, either by employing naked DNA followed by the same gene carried by a vector or two different vectors carrying the same genes [111]. However, he remarks that as vectors in recombinant vaccines induce immunity against themselves, using viruses that a population is naturally exposed to will be ineffective. A similar case is seen when boosting with viral recombinants like vaccinia virus, MVA and E1-deleted adenovirus, but the priming with DNA vaccines may counterbalance this interference [35].

Although studies were developed in both preclinical and clinical stages for human and veterinary applications, a fixed prime–boost regimen is still not available. Recent publications from the veterinary field demonstrated that combination of a gene gun-mediated naked DNA vector as priming and MVA as booster in mice is an attractive line of attack against porcine circovirus type 2 (PCV2) [112]. A vaccination scheme for highly pathogenic avian influenza H5N1 (AsHP H5N1) virus revealed that priming with a fowlpox-vectored vaccine followed by a boost with inactivated vaccine stimulated a broader protection than two administrations of a fowlpox recombinant (vFP-H5 Asia) [113]. In the case of human diseases, encouraging results in mice regarding the JEV pointed out that the pDNA-TEP prime– recombinant protein (rEP) provoked the development of strong humoral and cellular immune responses [114]. Moreover, in their eagerness to develop a universal influenza vaccine, Lo *et al.* reported that choosing DNA prime–rAd boost seems to be a more attractive alternative for providing robust heterosubtypic immunity than vaccination with cold-adapted virus [115]. Recently, prime–boost regimens have been applied to a large number of vaccines including HIV [116–120], avian influenza virus [121], malaria [122,123], anthrax [124], TB [125] and certain nosocomial infections [126]. This information demonstrates that incorporating a prime–boost regimen to a vaccine platform may yield better outcomes.

Vaccines against bacterial infection

New vaccines are needed to fight antibiotic resistance and to have better control over bacterial diseases [127]. A total of nine bacterial pathogens are targeted by licensed vaccines, according to the records of the FDA, and none of them are based on a gene vaccine (Table 1). Mortality and morbidity rates are still high, regardless of the usage of current bacterial vaccines, which suggest novel techniques are required. Despite a DNA vaccine's mode of action, it can be the prototype against any other tumor cell, or infectious or autoimmune disease. Although it is clear that genetic vaccines have several advantages over other types of vaccines, their application to bacterial infections has been scarcely documented. Reports on different ways to induce an immune response against bacteria have been described since the emergence of this technology. Studies of their use in fish targeting *Vibrio anguillarum* [128] and *Edwardsiella tarda* [129] show they could be as protective as DNA vaccines for viruses by provoking both specific and nonspecific immunities.

Depending on the pathogenicity of the bacterial infection, the host will typically mount the type of response required for eradication; intracellular bacteria are combated predominantly by the cellular response and extracellular bacteria by antibody production. Nevertheless, antibodies are indispensable to control most bacterial infections. However, the complexity of bacteria in general requires the development of antibodies against different structural proteins, toxins or capsule sugars. Understanding the relevance of the microbial antigenic epitopes in eliciting an effective response is key for progress in DNA vaccine development. As complete DNA sequences of bacteria have been decoded, researchers can now select the appropriate antigens and design the specific construct.

There are special concerns regarding the expression of bacterial proteins in eukaryotic cells, particularly because the host cell synthetic machinery has to express the bacterial antigen. This can result in difficulties in folding, transport and post-translational modifications, leading to unwanted effects [35]. Another challenge is to design vaccines against carbohydrate antigens, which are classified as thymus-independent antigens. They elicited a response that is poorly immunogenic in young children with IgM dominance, lack of isotype switching and memory response [130]. Another major problem is that capsular monosaccharides can link to each other in many different ways to form an epitope and that leads to a great serologic variability. To sum up, some capsular polysaccharides of certain pathogens have structural similarity to their host's endogenous carbohydrates, leading to possible autoimmune responses after

immunization [131]. However, the developments of peptides that mimic these antigens have opened the possibility to induce T-lymphocyte-dependent polysaccharide immunity. The mechanism of the mimicry is not completely understood, but these peptides can be identified either by phage display libraries or anti-idiotypic antibodies [132]. It was also published that these peptide immune responses have a predominance of IgG_{2a}, which is particularly important against encapsulated organisms because of its ability to fix complement and opsonize [133]. The first report on DNA vaccine that achieved a T-dependent response against a polysaccharide antigen was performed by Kieber-Emmons *et al.* [134]. Balb/c mice were immunized using a plasmid encoding designed peptide mimotopes of the neolactoseries antigen Lewis Y, which induced antibody response of the IgG_{2a} isotype. Other results showed that using anti-idiotypic immunoglobulins acting as surrogate images of the polysaccharide of *Neisseria meningitidis* could protect mice against lethal exposure to the pathogen. Their construct consisted of a multiepitope DNA vaccine encoding a peptide mimic of meningococcal serogroup C capsular poly-saccharide, a universal T-cell helper epitope and a secretary leader sequence [130]. Although this seems to be a proven alternative, several problems need to be solved. It was found that they have poor chemical stability, and thus lower antigenicity. In addition, smaller peptides can be degraded easily, resulting in an ineffective response. There are also technical issues regarding the production of anti-idiotypic monoclonal antibodies [135].

Bacterial pathogens are still responsible for increased mortality rates worldwide. Available vaccines are limited for a small number of bacterial organisms and most of them cannot confer full protection. DNA vaccine technology has been applied for different types of bacteria, which have an important role in human infections; examples are mentioned previously (Table 2).

Helicobacter pylori—*Helicobacter pylori* is a Gram-negative microaerophilic spirochete that has infected half of the world's population. It colonizes the antral region of the stomach and is capable of surviving the stomach's hostile environment due to its virulence factors. It was classified as a class I carcinogen, which means it is proven to be carcinogenic for humans. The high prevalence worldwide of *H. pylori* infection and its role in the pathogenesis of gastritis, peptic ulcer, MALTomas and adenocarcinomas make it an important target for DNA vaccination [136]. Although antibiotic therapy is the treatment of choice, resistant strains have recently been identified [137]. Studies demonstrated the efficacy of a plasmid encoding the neutrophil activating protein or the urease B protein to elicit a good immunogenic response in animal models [138,139]. It was shown that urease B vaccines could induce an upregulation of IL-10 and β -defensins and lower the bacterial load in the stomach [140]. Others concluded that the immune response against heat-shock protein was not only effective but also decreased the level of gastric mucosal inflammation [141].

Bacillus anthracis—*Bacillus anthracis* is a Gram-positive rod-shaped bacteria that forms spores. Its virulent factor consists of two toxins as a result of the combination of a protective antigen (PA) with either the lethal factor or edema factor, and it cause disease by contact, inhalation or ingestion of the spores [142]. It is a public health priority to develop vaccines against biothreats, such as anthrax. An efficient, cheap, stable and easy to transport vaccine platform such as DNA vaccines is needed for biodefense purposes. The current vaccine against anthrax (AVA) is based on bacterial supernatant absorbed (Table 1) and has proven to be reactogenic with a complicated schedule to follow. On the contrary, DNA vaccines do not present any of these disadvantages and can induce anti-PA immunity largely associated with anthrax protection [143]. The first attempts to induce protection against lethal toxin challenge were performed by Gu *et al.* [144]. They obtained neutralizing antibodies in addition to Th1 and Th2 cytokine-secreting cells corresponding to the production of IgG_{2a} and IgG₁. Recent assays have shown that vaccination with an optimized DNA encoding anthrax protective antigen, followed by electroporation, induces a more rapid neutralizing anti-PA IgG response in mice, rats and rabbits [145].

Tuberculosis: *Mycobacterium tuberculosis* is an aerobic intracellular bacterium responsible for approximately 1.7 million deaths each year worldwide. Undoubtedly, it is one of the major pathogens that researchers are most eager to target. The increasing number of cases seen each year and the lower efficacy of the BCG vaccine in controlling pulmonary infection are the reasons why novel vaccine alternatives should be tested. Multidrug-resistant strains and coinfection with HIV-1 are the greatest challenges for novel vaccine techniques.

Nucleic acid vaccines are good candidates for the prophylaxis of intracellular pathogens like mycobacterium due to the Th1 response mediated by CD4⁺ and CD8⁺ that they induce. TB DNA vaccines for both therapeutic and protective purposes have proven to restore the Th1/Th2 balance, resulting in a significant reduction of the pathology in the animal [50]. Different antigens given alone in recombinant and DNA vaccines have been under investigation, but to date, no vaccine alone tested in clinical trials has proven to be more effective against *Mycobacterium* than BCG. The Ag85 family has by far been the most studied encoded antigen for TB vaccines. Studies have shown that both Ag85A and Ag85B can induce a robust Th1 immune response with elevated IFN- γ , IL-2 and TNF- α , while Ag85C is not effective [146]. Other targeted antigens were Fbp/Htpx [87], Mtb72F [147], Rv3407 [148], Hsp65 [149], ESAT-6 [150] and MPT64/MPT83 [151], among others. Most of these vaccine schedules consisted of priming with BCG and boosting with the specific DNA vaccine in order to achieve a superior response compared with BCG alone. Other possible ways of improving efficacy are giving vaccines as pools [152], combining the antigen with the correct adjuvant like IL-12 [149] and delivering the construct in cationic lipid formulation [153]. The correct combination of delivery method and route of administration was described by Rosada *et al.* who designed a TB vaccine that consists of a single intranasal dose of DNA-hsp65 complexed with cationic liposomes [154]. This construct was as effective in eliciting a cellular immune response as strong as that induced by four intramuscular doses of naked DNA, and it even reduced the number of bacilli in the lungs.

Different challenge models are required for testing the efficacy and safety of plasmid-based vaccines. Mice, guinea pigs and rabbits are the most frequently used animal models where TB can be studied after either intravenous or pulmonary infection [155]. Histopathological analysis, survival curves and bacterial-load counts are necessary. The best nonhuman primate model of human TB is the cynomolgus monkey, also known as *Macaca fascicularis* [156]. The first successful TB vaccine in a monkey model was reported by Okada when he combined heat-shock protein 65 with IL-12 and obtained better efficacy, chest x-ray findings and immune responses than for BCG [157]. The control of infectious diseases like TB should be high priority. Further work is required to obtain an ideal TB vaccine, but studies have provided evidence that the beginning is promising.

Clinical view: update

To date, there have been several preclinical and clinical studies on DNA vaccines, but what is remarkable is that in the past 4 years, four DNA vaccines for larger animals have been licensed for use in the veterinary field. To be more precise, two of them target infectious diseases like West Nile virus in horses [4], and aquatic rhabdovirus called infectious hematopoietic necrosis in salmon [5]; one is a cancer vaccine for melanoma in dogs [6]; and the last one has a therapeutic purpose in swine [7]; a plasmid-mediated-releasing growth hormone supplementation delivered before specific vaccination demonstrated enhanced protection against *Mycoplasma hyopneumoniae*. Although these animal disease models are not completely similar to a humans, past success with DNA vaccines reveals a promising future towards using this technology to target human diseases.

All the beneficial renovations and improvements performed to this technique merit testing in clinical trials. A large number of them are currently operating mainly to test safety, efficacy

and efficiency of vaccines targeting viruses such as HIV-1, hepatitis B virus and malaria, or cancer cells like melanoma [3,34,158,201–204]. Bacterial vaccines are tested less in these trials because antibiotics seem to be a reasonable solution, a fact that has been changing in the past years with the emergence of resistant strains. TB, typhoid fever and anthrax DNA vaccines are the most popular candidates for entering clinical trials [157]. The majority of these studies were performed using a recombinant vaccine technique, which can induce antivector immunity. Gene therapy-based vaccines against bacterial pathogens have proven to be effective. However, researchers are accumulating data on this platform for upcoming clinical trials [157]. What experts state based on the clinical results is that a combination of vaccine strategies and prime–boost approach will be the future for immunization against pathogens like *M. tuberculosis* rather than a single vaccine candidate [159].

Conclusion

Current licensed vaccines for human use have limitations in terms of manufacture, storage, potency and safety. DNA vaccination is a novel but broadly studied vaccine strategy that offers a completely different system to trigger long-lasting cellular and humoral responses for a wide variety of pathogens without any of the limitations mentioned. The beauty of DNA vaccines lies in its simple concept and versatility to accomplish immunization against infectious diseases as well as cancer.

Although few DNA vaccines targeting bacterial pathogens have been tested, some encouraging results have been obtained. A wider range of organisms can be targeted by these vaccines, which can be useful to fight against antibiotic-resistant strains or trigger immune responses in children under 2 years of age, facilitating the control of epidemics. New approaches were studied to create vaccines against all types of bacteria virulence factors, such as polysaccharide capsules or toxins. In our opinion, it is a worthy field to pursue because of its potential advantages for public health.

This revolutionary technology is facing the challenge of enhancing potency to stand as the next-generation vaccine platform. Although DNA vaccines are already being tested in clinical trials, new methods to augment immunogenicity from codon optimization and adjuvants to delivery methods including electroporation and nanoparticles are under evaluation in preclinical studies. All of these reveal the evident potential of this platform in both *in vitro* and *in vivo* models.

Expert commentary & five-year view

It has been over 18 years since the field of DNA vaccines emerged into the biomedical limelight. During this period, the field has had some very difficult times. Recently, however, several important technical improvements in their design, formulation, synthesis and delivery, as listed in this article, have now contributed to a recent resurgence of interest as they have started to exhibit improved performance in larger animals and in the clinic. The concept of DNA vaccination has been studied against a wide array of pathogens and tumor antigens, both in preclinical and clinical stages, with clear measures of progress. However, only a few bacterial pathogens have been targeted by DNA vaccines. Although antibiotics and conventional vaccines have been useful in the past, the emergence of resistant strains of bacterial pathogens has become a major roadblock in the clinic. Clinical trials in a number of countries with conventional vaccines like BCG, the TB vaccine, for example, have revealed a very heterogeneous degree of protection, ranging from 0 to 80%. Similar disappointments have been seen in cases of several other bacterial pathogens. Recently, the paradigm of protection generated by DNA vaccines, which hitherto relied mainly on the generation of cellular immunity, is now shifting to incorporate the contribution of humoral components of immune

protection against such pathogens. Accordingly, these vaccine approaches have renewed interest against bacterial pathogens. Specifically, DNA vaccines are being developed against mycobacterium species, including *Mycobacterium leprae*, *Mycobacterium avium* and *Mycobacterium ulcerans* among other targets, through the availability of suitable murine models to test vaccine efficacy. With the effort to translate these studies to humans, the next 5 years of testing will be crucial for the generation of clinical success based on immune potency. Improved vaccine design, including better formulation, better delivery techniques and incorporation of adjuvants, supported by the joint efforts of research organizations, industries and regulatory authorities, could finally pave the way towards successful DNA vaccines against bacterial pathogens in the not too distant future.

Key issues

- DNA vaccines involve the intramuscular or subcutaneous injection of a DNA plasmid containing a transgene that encodes the sequence of a target protein from a pathogen under the control of a eukaryotic promoter. This induces both long-lasting humoral and cellular immune responses.
- The construct consists of a plasmid backbone and a transcriptional unit, which contains the promoter and an insert or gene encoding the antigen of interest, followed by a transcript termination/polyadenylation sequence.
- Multiple methods exist to enhance the immunogenicity of DNA vaccines at every step of the way, such as codon optimization, molecular adjuvants or electroporation.
- Four DNA vaccines for larger animals were licensed for veterinary use. Current clinical trials have also shown its safety, efficiency and efficacy, and predict that a combination of vaccine strategies and prime–boost approach will be the future for immunization against pathogens like *Mycobacterium tuberculosis*.
- DNA vaccines have major advantages over existing vaccines platforms, making them a suitable option for controlling bacterial infectious disease in the era of antibiotic resistance.
- DNA vaccines targeting bacterial pathogens such as *Helicobacter pylori*, *M. tuberculosis* and *Bacillus anthracis* have been tested with encouraging results.
- The development of peptides that mimic carbohydrate antigens have opened the possibility to induce T-lymphocyte-dependent polysaccharide immunity by the injection of a DNA vaccine.

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Figure 1.
Plasmid DNA vaccine vector design.

Figure 2. Types of vaccines currently in use

APC: Antigen presenting cell; CTL: Cytotoxic T lymphocyte; Th: T-helper cell.

Table 1

Currently available bacterial vaccines in the USA.

Category	Bacteria	Type of vaccine	Administration	Type of response	Immune effector	Ref.
Intracellular	<i>Mycobacterium bovis</i>	Live-attenuated	Intradermal	Th1	CD4 ⁺ T cells/CD8 ⁺ T cells	[160]
	<i>Salmonella typhi</i>	Live-attenuated	Oral	Th1/Th2	CD8 ⁺ T cells/anti-Ag-O IgG-IgA	[161]
	<i>Salmonella typhi</i>	Polysaccharide	Intramuscular	Th1/Th2	CD8 ⁺ T cells/anti-Vi antibody	[162]
Toxoids	<i>Clostridium tetani</i>	Toxoids adsorbed	Intramuscular	Th2	Anti-TT antibodies	[163]
	<i>Bacillus anthracis</i>	Proteins adsorbed	Intramuscular	Th2	Anti-PA antibodies	[164]
	<i>Bordetella pertussis</i>	Acellular	Intramuscular	Th2	Anti-FHA, PRN, PT and BPT antibodies	[165]
	<i>Corynebacterium diphtheriae</i>	Toxoids adsorbed	Intramuscular	Th2	Anti-DT antibodies	[166]
	<i>Neisseria meningitidis</i> serogroups A, C, Y and W-135	Polysaccharide–protein conjugated	Subcutaneous	Th2	Anti-capsular polysaccharide antibodies	[167]
Extracellular	<i>Haemophilus influenza</i> type b	Polysaccharide–protein conjugated	Intramuscular	Th2	Anti-PRP antibodies	[168]
	<i>Streptococcus pneumoniae</i>	Polysaccharide	Intramuscular/subcutaneous	Th2	Anti-capsular polysaccharide antibodies	[169]

DT: Diphtheria toxoid; FHA: Filamentous hemagglutinin; PA: Protective antigen; PRN: Pertactin; PRP: Polyribosylribitol phosphate; PT: Pertussis toxin.

Table 2

More relevant bacterial DNA vaccine targets.

Bacteria	Incidence/mortality per year [†]	Immune response required	Targeted antigens	Ref.
<i>Mycobacterium tuberculosis</i>	9 million/1.7 million	CTL	Ag85A, Ag85B, FbpA, Htpx, Rv3407, Hsp65, Mtb75F, ESAT-6, MPT64	[79,157–159]
<i>Streptococcus pneumoniae</i>	10.6 million/700,000–1 million children	Anti-capsular polysaccharide antibodies	PspA	[170]
<i>Streptococcus</i> group A	~500,000	Anti-capsular polysaccharide, protein and exotoxin antibodies	HtpA	[171]
<i>Haemophilus influenzae</i> type B	3 million/386,000	Anti-capsular polysaccharide antibodies		
ETEC	10 million/380,000	Anti-surface protein antibodies	Ec-FA	[172]
<i>Clostridium tetani</i>	~290,000	Anti-toxin antibodies	TetC	[173]
<i>Clostridium botulinum</i>	~213–293,000	Anti-exotoxin antibodies	AHc	[174]
<i>Salmonella typhi</i>	~216,000	CTL	OmpC	[175]
<i>Neisseria meningitidis</i>	~170,000	Anti-surface protein and capsular polysaccharide antibodies	PorA, NMB0088, MenB CP	[176–178]
<i>Vibrio cholerae</i>	~120,000	Anti-toxin antibodies		
<i>Listeria monocytogenes</i>	2500/500 (USA only)	CTL	Listeriolysin O, p60	[179,180]
<i>Helicobacter pylori</i>	50% of world population/~	Anti-surface protein antibodies	HP-NAP, Ure-B, HP-kat, HspA, HspB	[128,129,131,181]
<i>Streptococcus</i> group B	~/-	Anti-capsular polysaccharide antibodies	LtrG	[182]
<i>Staphylococcus</i>	~/-	Anti-capsular polysaccharide antibodies, proteins and CTL	PBP2a, pCl, pClfa, pSrt	[183,184]
EAEC	~/-	Anti-surface protein antibodies	AAF/1, AAF/2	[185]
<i>Chlamydia trachomatis</i>	17 million/~	CTL	pORF5	[186]
<i>Chlamydia pneumoniae</i>	2–5 million (USA only)/~	CTL	MOMP, Osp2	[187]
<i>Mycoplasma pulmonis</i>	~/-	CTL	A7-1, A8-1	[188,189]
<i>Mycoplasma agalactiae</i>	~/-	CTL	P48	[190]
<i>Borrelia burgdorferi</i>	20,000 (USA only)/~	Anti-surface protein antibodies	OspA, OspC	[191,192]
<i>Brucella abortus</i>	100–200 (USA only)/~	CTL	SOD	[193]
<i>Yersinia enterocolitica</i>	~/-	CTL	Hsp	[194]
<i>Bacillus anthracis</i>	~/-	Anti-exotoxin antibodies	PA	[135]
<i>Pseudomonas aeruginosa</i>	~/-	Anti-surface protein antibodies	OprF, FliC R90A	[195,196]
EHEC	~/-	Anti-surface protein and toxin antibodies	Stx2	[197]

[†] According to the WHO [205] and the CDC [206].

CTL: Cytotoxic T lymphocyte; EAEC: Enteraggregative *Escherichia coli*; EHEC: Enterohemorrhagic *Escherichia coli*; ETEC: Enterotoxigenic *Escherichia coli*.