DNA Vaccines

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Abstract

The use of DNA based vaccines is an alternative to conventional vaccines and which is a novel strategy now under development and evaluation for the prevention and treatment of many diseases because of its ability to induce both humoral and cellular immune responses against antigens encoded by recombinant DNA. A large amount of data has been generated in preclinical model systems, and more sustained cellular responses and more consistent antibody responses are being observed in the clinic. It can further be a choice of great interest for its simplicity, safety, stability and potentiality. However it lacks a major disadvantage of delivering genetic material to the immune cells, which must be targeted. This review focuses on the background and origin of DNA vaccines, the mechanisms involved in inducing an immune response, examples of possible useful experimental DNA vaccines, as well as an overview of safety issues, advantages and disadvantages and future promising perspectives.

Keywords: DNA; Vaccines; Plasmids; Clinical trials

Abbreviations: hAAT: human α-1 Antitrypsin; DNA: Deoxyribonucleic Acid; MHC: Major Histocompatibility Complex; APCs: Antigen Presenting Cells, TH: T Helper; IS: Immunological Synapse; CTL: Cytotoxic T-Lymphocyte; TCR: T Cell Receptor; NP: Nucleoprotein; HA: Hemagglutinin Antigen; TLR: Toll-Like Receptor; MHC-I: Major Histocompatibility Complex Class I**;** DLN: Draining Lymph Node; TCR: T Cell Receptor; HPV: Human Papillomavirus; HSV: Herpes Simplex Virus; CTE: Cis-Acting Transcription Element; HBV: Hepatitis B Virus; TAAs: Tumor-Associated Antigens.

Introduction

Vaccines represent the most commonly employed immunologic intervention and discovery in medicine today with documented clinical success. Current estimates by the Centres for Disease Control indicate that greater than 5,000,000 doses of vaccine against some infectious organism are administered yearly in the United States, making vaccines the most commonly administered immunotherapeutic [1]. Current vaccines target only a

tiny fraction of infectious diseases, since prophylaxis against some of the most common and deadly infections in the third world are limited by expense and ease of distribution, in addition to the public health concerns of expense and distribution, other features of current vaccines limit their efficacy [1]. Therefore, the use of DNA based vaccines is however an alternative to conventional vaccines and which is a novel strategy now under development and evaluation.

DNA vaccines are bacterial plasmids constructed to express an encoded protein following in vivo administration and subsequent transfection of cells [2]. According to the immunogenic use of DNA was first reported by Tang and his colleagues, by the demonstrating the production of a human growth hormone (HGH) and human α-1 antitrypsin (hAAT)-specific anti- bodies following injection of HGH DNA into mouse skin [3]. Since then, DNA vaccines have shown promising results in a number of trials for prophylaxis of bacterial, viral, parasitic, autoimmune and neoplastic diseases [2].

The molecular breakthroughs in cell transformation and gene therapy induction has created the new field of DNA vaccinology and its enormous potential to provide safe, inexpensive and effective DNA-based vaccines [4]. Basically, the concept of DNA vaccines is to deliver plasmid DNA encoding for protective proteins into the cells of the host animal where they can direct transcription and translation, effectively transforming the vaccinate into a mammalian bioreactor for the production of its own vaccine [4].

Background

One of the most important applications in the field of immunology in the last century was the invention and development of the vaccines. It was and still is significant in the prevention of infectious diseases that saved lives of millions of people. It all begun with the experiment conducted by Edward Jenner in 1798, when he demonstrated that inoculation with pus from cowpox lesions was conferring protection and assurance against smallpox infection [5-7]. This was a milestone that prompted the termination of smallpox through an innovative contribution to immunization, bringing about the establishment of the premise of vaccinology, which is the principle of isolation, inactivation, and administration of disease causing pathogens and hence treatment of infectious diseases [5].

Shortly, there have been rapid advances in producing safe and highly efficient vaccines against a number of common diseases. These vaccines contained bacterial toxoids (diphtheria and tetanus); killed entire organisms (*e.g.* typhoid, cholera, pertussis and the Salk polio vaccine); or live attenuated organisms (reduce its pathogenicity) (*e.g.* Bacillus Chalmette Guerin, yellow fever, the Sabin polio vaccine, measles, mumps and rubella) [5-7]. Today vaccines are widely used to prevent or reduce the infection by many pathogens. Despite the development and widespread use of vaccines against a broad collection of infectious agents, there is not yet an effective vaccine available against any of the three most dangerous infectious diseases of our days, namely AIDS, tuberculosis and malaria [8].

Presently, with the advance in the biotechnology and the utilization of novel techniques in molecular biology, it is very feasible to make new vaccines. A typical example would be the utilization of yeast cell to express hepatitis B antigens, which was the first and strikingly fruitful recombinant protein vaccine [5]. This vaccine has been highly effective in preventing hepatitis B viral infection and thus became the first vaccine, which has the capability to prevent a human cancer, the hepatocellular carcinoma, associated with early acquired, persistent hepatitis B infection [5].

Additionally, development of conventional vaccination can be time and labour intensive, not permitting a quick action to the need of a new vaccine, as in the occurrence of influenza pandemics [5,6]. Also there are hypothetical safety concerns linked with the approaches of using both non-live and attenuated concepts [5,7]. To overcome all these challenges, new approaches have been applied to vaccine advancement in the last 30 years [5]. These updated approaches in vaccination technology included recombinant DNA, polysaccharide chemistry and more recently reverse vaccinology, structural vaccinology, and synthetic RNA vaccines are all opening up the perspective for the outlining and advancement of third generation vaccines, which were formally characterized as impossible to make [5].

There has been increasing pressure applied by the regulatory authorities, both human and veterinary, to specifically define the protective antigens and produce vaccines free from pathogen-associated toxins and immunosuppressive components. Subunit vaccines based on recombinant protein immunogens, deoxyribonucleic acid (DNA) immunogens, and non-pathogenic vectors are currently the most cost-effective methods of producing

antigens free from exogenous material characteristic of conventional vaccines [4]. .

Conventional Vaccines

Conventional vaccines or traditional vaccines are based on inactivated or live attenuated microorganisms or on purged pathogen subunits, such as toxins, polysaccharides and proteins. These vaccines have been extremely productive in the prevention of infections by pathogens. The mechanism by which these vaccines works are based functional antibodies that can neutralize viral invasion, neutralize bacterial toxins and induce opsonophagocytosis or complement dependent bacteriolysis [5]. Today most of the licensed vaccines are conventional vaccine. However, using conventional vaccines is time consuming and has taken decades of research. Also, some microorganisms are difficult to cultivate or even to attenuate and may bring about undesirable immune responses [5,6].

While most current vaccines typically elicit reasonable antibody responses, cellular responses (in particular, major histocompatibility complex [MHC] class l-restricted cytotoxic T cells) are generally absent or weak [7]. For many infectious diseases, such as tuberculosis and malaria, humoral responses have been shown to be of little protective value against infection [1]. Another limitation of most current vaccines relates to the limited duration of immunologic memory, as an ideal vaccine would provide lifelong prophylaxis, a goal generally not achieved by current formulations [1].

Polysaccharide Chemistry and Glycol Conjugate Vaccines

In the course of the most recent decades, capsular polysaccharides have been successfully used in the preparation of antibacterial vaccines and the commercialisation of several polysaccharide-protein conjugate vaccines was a breakthrough aimed at filling the gaps in many areas, which can prevent most childhood deaths [5].

Immunisation by vaccines made out of plain bacterial polysaccharides has been acquainted subsequent to the 1970s to control diseases caused by clinically important bacteria such as *Haemophilus influenza* type B, *Streptococcus pneumoniae* (*S. pneumoniae*) and *Neisseria meningitidis* (*N. meningitidis*) group [5]. Conversely, one of the major immunological problems faced in the development of polysaccharide vaccines is due to the fact that carbohydrates are usually poorly immunogenic and cannot induce a T cell-dependent immune response that

is necessary for protective immunity and therefore, it is less effective especially in children aged below two years and infants who represent the main target population of vaccination [5].

To solve this predicament of poor immunogenicity, the carbohydrate molecules have to be coupled to a carrier protein, to enhance their immunogenicity and by facilitating access to structures of increasing complexity many carbohydrate-protein coupling techniques have been applied to develop several polysaccharide-protein conjugate vaccines [5]. The current progress in glycol-chemistry has facilitated the design of adequate and highly sophisticated glycol conjugate vaccines using synthetic saccharide components, which are derivatives of epitopes that naturally involved such protection [5].

Protein-polysaccharide conjugate vaccines were introduced in the 1980s against *Haemophilus influenzae* type B, inducing a better and persistent antibody response in all age groups [5]. Today, different approaches to prepare conjugate vaccines can be followed and adequate glycol conjugate vaccines are available for *S. pneumonia* and the different strains of *N. meningitides* [5].

Subunit Vaccines

Subunit vaccines have improved conventional attenuated or killed vaccines in many aspects, including safety and production [9]. The systems mostly used to produce these vaccines are based on bacteria, yeast, insect or mammalian cells [6,9]. However, production of recombinant vaccine proteins in these expression systems is expensive in many cases, requiring large scale fomenters' and stringent purification protocols. Worldwide, only a small number of facilities exist with capabilities to produce kilograms of a specific protein to be used as immunogenic, and the construction, validation, and final approval of new production facilities take many years implying important investments in capital and human resources [6].

Additionally, some antigens require post-translational modifications that cannot be achieved using all expression systems. In the last decade, non-fermentative alternatives based on living organisms have been developed to solve such problems and provide low cost technologies for vaccine production [6]. Insects and plants have been adapted for subunit vaccine production with clear advantages to conventional fermentative systems, especially in terms of time of development, scaling-up production and cost-efficiency [6]. Despite the improvements in the recombinant technology, these

vaccines remain hard to produce due to their inherent toxicity for the bacterial/viral expression system (e.g., Human Papilloma Virus type 16-E2, wild-type p53) [6].

Synthetic Peptide Vaccines

Identification of individual epitopes within protective proteins allows the development of peptide vaccines as alternative approach respect to using a whole protein as a vaccine [6]. Selected peptide epitopes represent the minimal immunogenic region of a protein antigen and allow for precise direction of immune responses aiming at the induction of T-cell immunity [6]. A peptide vaccine should ideally include epitopes recognized both by B and T cells, and take into account the MHC restriction of the T-cell response. In some cases B and T-cell epitopes can overlap substantially within the sequence of an antigen and, in others, they might be present in separate discrete regions of the antigen or present in different antigens from the targeted pathogen [6].

The simplicity of producing clinical grade peptides allows swift changes in the design of peptide vaccines and, therefore, rapid translation of new immunological concepts, which represent a great advantage for the development of vaccines against rapidly changing viruses such as influenza [6]. Despite the potential advantages of this approach, the development of successful peptide vaccines has been limited mainly by difficulties associated with stability, poor immunogenicity of simple peptides and by the MHC polymorphism of the host species [6].

Reverse Vaccinology

Since the gradual advancement in the field of immunology, the defining moment was the publication documented in 1995 of the genome arrangement of the first living organism [5]. By sequencing the genome and by characterizing the entire antigenic repository of the infectious microorganism, several contender protective targets could be distinguished and tested for their suitability as vaccine [5]. This technique, named reverse vaccinology [10], has implemented a change in the viewpoint of vaccine design. The thought of the reverse vaccinology was started to conquer the issues confronted to develop vaccine with high adequacy against MenB [5,10].

Reverse vaccinology is an approach that uses genome analysis to identify the complete repertoire of antigens that are surface-exposed and highly antigenically conserved across multiple strains. The most immunogenic epitopes, once sequenced, are typically patented and evaluated for suitability in various vaccine formulations

[7]. An example is the influenza vaccines under development that are based on recombinant Hemagglutinin protein antigens rather than on live attenuated virus [7].

The precept at the foundation of the reverse vaccinology path was that, felicitous vaccine targets were proteins either expressed on the surface of the microorganism or excreted into the extracellular environs [5]. About 600 surface-exposed proteins were predicted and successfully expressed using bioinformatics analysis. Of these, about 350 were cloned in *Escherichia coli* (*E. coli*), expressed and used to immunize animal model [5]. Through this process three protective antigens that are common to multiple MenB strains have been filtered and characterised and named as factor H-binding protein, *Neisseria* adhesin A, and neisserial heparin-binding antigen and combined with a MenB outer membrane vesicle, resulting in the first universal vaccine against MenB [5].

Since then, the reverse vaccinology technology has been utilized in a wide range of other clinically important pathogens, such as *S. pneumoniae*, *Streptococcus pyogenes*, *Chlamydia pneumonia*, *Chlamydiatrachomatis*, *Streptococcus agalactiae*, *E. coli*, and *Leishmania major*. Consequently, the genome-based reverse vaccinology approach can manipulate adequate and innovative strategies to design vaccines that were found to be difficult or even unattainable to develop using conventional approaches [5].

Conclusion

Current licensed vaccines are predominantly composed of either killed pathogens, pathogen subunits, or live attenuated viruses. Nonlive vaccines, which confer protection primarily through the induction of CD41 T- cell and humoral mechanisms, generally do not provide life-long immunity [11]. In contrast, live-attenuated vaccines can mobilize both the cellular and humoral arms of the immune response and generally induce more-prolonged immunity. However, their degree of attenuation can significantly lower the immunogenicity of live vaccines, and the development of live vaccine strategies can be especially challenging when the goal is to target multiple viral subtypes or pathogens [11].

DNA Vaccines

In the last three years, DNA vaccines have burst onto the scene as a radically new approach to infectious disease prophylaxis [1]. One of the most surprising and

important features of DNA immunization is that purified "naked" DNA appears to be taken up and expressed by cells in vivo with much greater efficiency than would have been predicted by the experience with DNA transfection in tissue culture [1]. In addition, naked DNA can be produced in large scale with tremendous purity, allowing for freedom from contamination with potentially dangerous agents [1] and finally, its tremendous stability relative to proteins and other biologic polymers, a feature likely to be more relevant for the production of vaccines than the recreation of dinosaurs [1].

Demands for effective vaccines to control parasitic diseases of humans and livestock have been recently exacerbated by the development of resistance of most pathogenic parasites to anti-parasitic drugs [12]. Novel genomic and proteomic technologies have provided opportunities for the discovery and improvement of DNA vaccines which are relatively easy as well as cheap to fabricate and stable at room temperatures [12]. However, their main limitation is rather poor immunogenicity, which makes it necessary to couple the antigens with adjuvant molecules [12].

DNA vaccination is also known as genetic immunization which is a rapidly developing technology that has been described as a third generation of vaccines and seems to offers new approaches for the prevention and therapy of several diseases of both bacterial and viral origin [5].

Successful in vivo transfection of mammalian cells following injection of purified DNA was first reported over 40 years ago. However, its potential went largely unrealised until 1990 when Wolff and colleagues demonstrated that a reporter gene encoding an enzyme protein could be expressed in murine skeletal muscle *in vivo* and the tissue retained its transgenic biological activity for up to 60 days after inoculation [5]. These observations were extended by several studies such as those of Tang, et al. (1992) who demonstrated that mice injected with plasmid DNA encoding human growth hormone elicited antigen-specific antibody responses [5]. Based on these findings, it is concluded that this technology is promising as it can enhance both cellular and humoral immunity against parasites, bacteria and disease-producing viruses [5], although it is still experimental.

DNA Plasmid and its Components

DNA-based vaccines are composed of purified closed-circular plasmid DNA, originally from bacteria [8] or non-

replicating viral vectors containing genes that encode viral antigens [13], but incapable of eliciting a disease. Therefore, depending on the viral gene carrier system, the DNA vaccines fall into two categories. Viral (bacterial) recombinant vaccines are genetically engineered viruses or bacteria cells, modified by inserting a foreign virus gene (encoding the desired vaccine protein) into their genome [14]. The foreign gene carriers are usually attenuated (non-pathogenic) virus species, such as vaccine or canary poxviruses, non-pathogenic adeno- or retroviruses etc [14]. In contrast, the naked DNA (plasmid) vaccines are vectors encoding viral antigens, which become expressed in the immunised host where they generate both cytotoxic (cellular) and humoral (antibody) responses [14].

A typical DNA plasmid or vector consists of several genetic elements required to drive intracellular expression of the foreign gene insert. (Figure 1) These include
a) an origin of replication allowing plasmid propagation in *E. coli*
b) a transcriptional promoter, which is the incorporation of a strong viral promoter to achieve optimal expression in mammalian cells, such as cytomegalovirus or simian virus 40 which provide the greatest gene expression
c) An optional enhancer element to augment gene expression
d) The foreign gene encoding an antigenic gene product (e.g., a viral protein)
e) RNA-processing elements, primarily a polyadenylation signal and an optional intron element. [5,6,13-15].
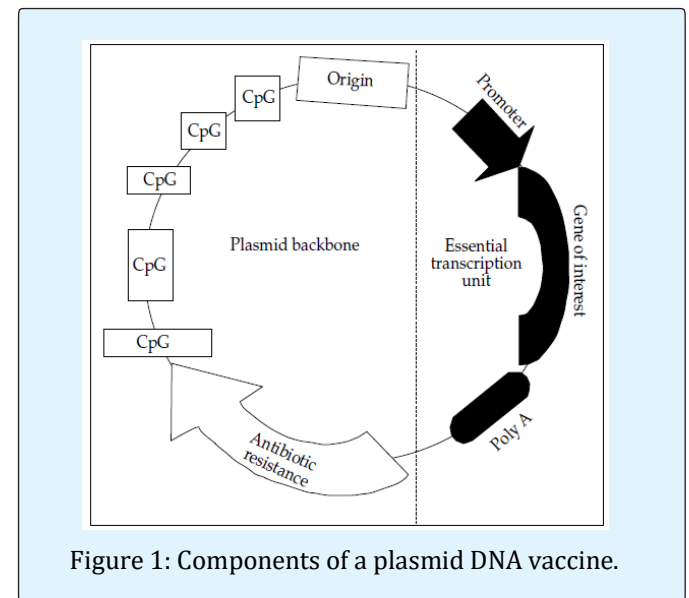

Figure 1: Components of a plasmid DNA vaccine.

A DNA plasmid consists in several sequences, summarised as the essential transcription unit and the backbone. The essential transcription unit consists on a viral promoter, for example cytomegalovirus, recombinant DNA sequence encoding for the antigen of interest and the required sequences to provide mRNA stabilisation. The plasmid backbone contains other Commonly, a marker gene conferring resistance to an antibiotic is included. This is also known as a bacterial antibiotic resistance gene which allows plasmid detection and selection during bacterial culture. (e.g., neomycin neomycin phosphoryltransferase, which confers resistance to genetic in [Neor] [5,13]. In addition, the plasmid contains two bacterium-specific genetic sequences to allow large-scale production of the DNA: an antibiotic selectable marker to permit identification and isolation of bacterial cells successfully transduced with the gene of interest, and a bacterial origin of replication to facilitate large scale amplification of the plasmid within this host cell [13]. Once the DNA enters the mammalian cell, the encoded antigens are expressed through normal cellular transcription and translation mechanisms [13-15].

Incorporation of the alpha virus replicate gene into the plasmid DNA vectors has been an exciting approach towards improving DNA vaccination [16]. Alpha virus replicate- based vectors have been shown to elicit better immune responses than the conventional plasmid vectors encoding the same antigens and are effective when used as vaccines against cancer or viral infections [16]. The enhanced immunogenicity of the replicate based plasmid vectors has been attributed not only to the levels of antigen production but also to various other mechanisms [16].

Different strategies can be used for enhancing the plasmid DNA vaccine potency. The first strategy allows improving the vector construct i.e. by working on the plasmid backbone design and construction; a second strategy allows improving the codon usage in order to maximize the antigen synthesis [6,17].

Mechanism of DNA Vaccines

DNA vaccination involves the introduction of nucleic acid into host cells where it directs the synthesis of its encoded polypeptide(s) and stimulates an immune response without genetic integration such that construction of a DNA vaccine is designed to permit localized, short-term expression of the target antigen [5].

Although several attempts have been made to study the cellular pathways for the processing of antigens and their presentation to T lymphocytes, the precise mechanism based on cellular and molecular events involved in the induction of immune responses following DNA immunisation are not fully understood [5]. However, it is well documented that the magnitude and type of immune response induced after DNA immunisation are influenced by a number of different parameters, some of which are represented by the type and components of the expression plasmid [5].

The crucial event responsible for the initiation of an immune response against a foreign antigen is recognition by specialized cells namely the antigen presenting cells (APCs), uptake and presentation of the antigen to naïve lymphocytes and induction of effector T helper (Th), cytotoxic (CTL) and B lymphocytes [6]. In this context the mechanism of action of DNA vaccines looks very simple [6]. Once the DNA vaccine is delivered into the skeletal muscle, the plasmid DNA is taken up by the resident DCs and by the muscle fibres [6]. While transfect muscle cells behave as permanent antigen reservoir as well as target of immune effector cells, resident DCs have the property to leave the muscle tissue and move to the closest draining lymph nodes in order to process and present the antigen to T lymphocytes [6]. DCs are specialized in capturing extracellular antigens by receptor-mediated endocytosis and pinocytosis mechanisms and following antigen uptake they undergo a complex multi-step maturation process [6]. DC maturation depends also on the microbial and pathogens-derived signals which increase their capacity to migrate towards the draining lymph node [6]. While DCs move to the lymphoid organs, they interact with various chemokines which contribute further to their maturation process [6].

Once in the lymph nodes, DCs shift from an antigen-capturing cell to a T sensitizing cell, being capable to present antigen in association with the class I and class II MHC molecules to CTLs and Th lymphocytes [6]. Interaction between the DC and the T lymphocyte induces formation of the immunological synapse (IS) via complex MHC antigen- T cell receptor (TCR) resulting in the clonal expansion of the T lymphocyte and differentiation in T memory cell. Professional DCs can also capture antigens released in the interstitial space by skeletal muscle fibres or in form of apoptotic bodies activating the cross-presentation pathway [6]. This route allows presentation of extracellular/exogenous antigens through the MHC-I restriction pathways [6].

Therefore, extracellular antigens which normally induce a humoral immune response can also access to the MHC-I compartment through endoplasmic reticulum, leading to simultaneous stimulation of the CTL immune response [6]. Antigen synthesized by DC or skeletal muscle cell can also be released in the extracellular environment and activate directly the B lymphocytes through antigen-antibody interaction [6]. Considering the mechanism described above, plasmid DNA vaccines (Figure 2) are able to stimulate all the principal effector cells of the adaptive immune system but due to the presence of CpG islands intrinsic to the DNA structure they can also can mimic some aspects of live infection, activating important signals of the innate immune system [6,15,18].

There are a number of strategies available that have the potential to improve the potency of DNA vaccines [19]. These strategies include

a) Vector modification to enhance antigen expression, which may involve targeting of the expressed protein to a particular cellular location, the inclusion of immuno stimulatory sequences, or the elimination of inhibitory sequences in the plasmid;
b) Improvements in DNA delivery; or
c) The inclusion of adjuvants, either as a gene or as a co administered agent [19,20].

According to Zonouzi AA, et al. [3], at least three diverse mechanisms have been suggested to have key role in the immunogenicity of DNA vaccines

a) The host-synthesized antigens are presented by somatic cells (keratinocytes or muscle cells) by their MHC class I molecules to CD8 T cells;
b) DNA vaccination lead to direct transfection of professional APC such as dendritic cells and
c) Transfect somatic cells are phagocytised by professional APCs and the recombinant antigen is present to T cells [3].

Figure 2: Mechanism of action of plasmid DNA vaccination [6].
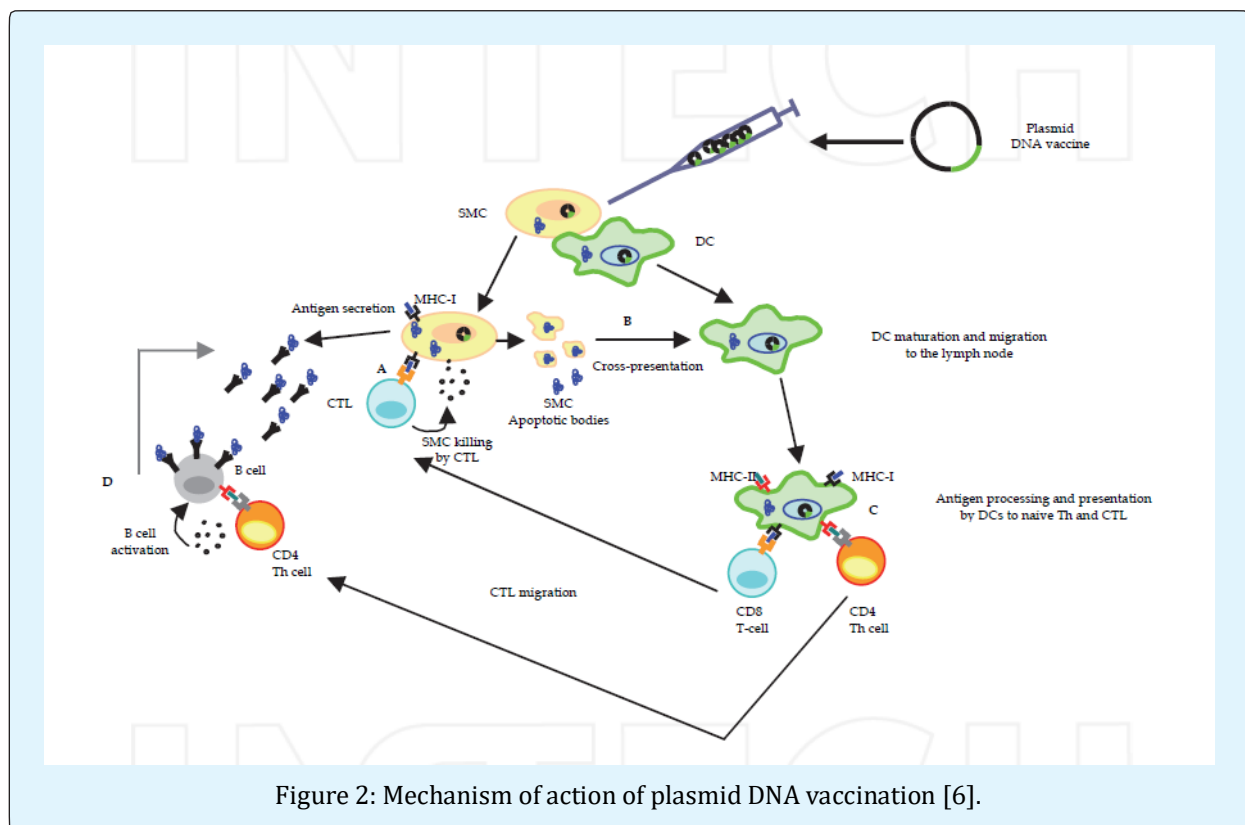
Generally, the optimized gene sequence of interest is delivered to the skin (intradermal), subcutaneous or muscle by one of several delivery methods. Using the host cellular machinery, the plasmid enters the nucleus of transfect local cells (such as myocytes or keratinocytes),

including resident antigen presenting cells (APCs) [21]. Here, expression of plasmid-encoded genes is followed by generation of foreign antigens as proteins that have been converted to peptide strings [21]. These host-synthesized antigens can become the subject of immune surveillance

in the context of both major histocompatibility complex (MHC) class I and class II molecules of APCs in the vaccinated host [21]. Antigen-loaded APCs travel to the draining lymph nodes where they 'present' antigenic peptide– MHC complexes in combination with signalling by co-stimulatory molecules to naive T cells [21]. This interaction provides the necessary secondary signals to initiate an immune response and to activate and expand T cells or, alternatively, to activate B cell and antibody production cascades [21]. Together, both humoral and cellular immune responses are stimulated.

Generation of an Immune Response

An important step in the design of DNA immunisation constructs is to understand the immune correlates of protection. Antigen peptides expressed after DNA immunisation are usually presented by antigen presenting cells (APCs) in the context of either MHC class II or class I molecules to CD4+ and CD8+ T cells, respectively [5,13,14,22]. There are at least three means by which MHC class I restricted cytotoxic T-lymphocyte (CTL) might be elicited following administration of plasmid DNA:

a) Transfection of professional APCs,

b) Antigen presentation mediated directly by transfect myocytes

c) Cross priming [5].

From an immunologic perspective, the unique ability of DNA to either integrate stably into the genome or be maintained long-term in an episomal form provides the potential for long-lived antigen expression [1]. Despite the flurry of reports documenting the ability of naked DNA vaccines to induce both immunologic and protective responses in animal models, the mechanism by which DNA injections activate the immune system against the encoded antigens remains somewhat mysterious [1]. Nonetheless, given what is now understood about pathways of antigen processing and the requirements for T cell activation, exposing the mechanisms of immune activation by naked DNA may reveal some provocative clues to how the immune system deals with different forms of antigen [1].

It is now well established that injection of naked DNA through any of a number of routes reproducibly induces both humoral and cellular immune responses against the encoded antigens [1,23]. The initial report that genetic immunization could elicit immune responses measured the induction of antibodies against human growth hormone (HGH) subsequent to ballistic injection of DNA-coated gold particles with a gene gun [1,13].

Subsequently, Liu and colleagues demonstrated that antigen-specific CTL responses could be induced by intramuscular injection of naked DNA [1]. They utilized an influenza A model to emphasize the advantage of a vaccine strategy that could induce CTL responses, as humoral responses to influenza A tend to be strain specific and poorly cross protective [1]. This is because the major antibody responses are directed against the Hemagglutinin (HA) antigen, which varies significantly among different influenza strains. In contrast, epitopes of the influenza nucleoprotein (NP) antigen, a major target for CTL responses, demonstrate sig demonstrate significantly less inter strain variability [22]. Using a plasmid containing the NP gene driven by either an RSV or CMV promoter, they demonstrated specific CTL responses against the NP 147-155 epitope presented by the H-2Kd MHC class I molecule [1,22]. Importantly, animals immunized intramuscularly with NP DNA were protected from intranasal challenge with 102.5 TCID 50 of an influenza isolate, AIHlV66, which arose 34 years after the strain from which the vaccinating NP gene was isolated (A/PR/A/34) [1].

Innate response to DNA vaccines: DNA sensing by pattern recognition receptors has been shown to be essential for vaccine responses. Many of the pattern recognition receptors used by cells to detect infection are based on the recognition of chemicals that are outside their usual location, so the presence of naked DNA outside of the nucleus is inherently inflammatory [24]. Transfect immune and somatic cells are able to sense the presence of "foreign DNA" in the cytosol, using a range of intracellular pattern recognition receptors including RIG-I, AIM2, ZBP1/DAI, and HB2 histones [24]. However, their role in the initiation of an innate signalling cascade to DNA vaccination is unclear, as knockout studies showed minimal contributions, suggesting redundancy [24].

The detection of un methylated CpG motifs inherently present in DNA vaccines by toll-like receptor (TLR) 9 is assumed to have a role in the detection of DNA vaccines, but TLR9-/- mice had immune responses to DNA vaccines similar to control mice, again suggesting some redundancy [15,24]. It has been deduced that the downstream signalling molecules STING and TBK1 are essential for instigation of an innate immune response to DNA vaccines, as their deletion abrogated type I interferon production [24]. The induction of an innate immune response to the DNA vaccine is critical in activating the APCs that present the expressed antigen to the T cell [14,24].

Adaptive responses to DNA vaccines: The adaptive immune response to a DNA vaccine is heavily influenced by the cell that is transfect by the DNA. As described above, antigen is either expressed by non-antigen-presenting cells such as myocytes or keratinocytes or by APCs that have taken up the DNA. Which cell expresses the DNA is influenced by a number of factors including the route of delivery, the device used to deliver the vaccine, the formulation of the vaccine, and the use of adjuvants [24]. Speculatively, if APCs are directly transfect, they are most likely to present antigen on major histocompatibility complex class I (MHC-I) molecules, thereby initiating aCD8+ T cell response [14,24]. If somatic cells are transfect, the antigen will be either displayed on MHC-I, secreted as processed antigen, or released upon cell death [24].

Antigen that is displayed upon MHC-I acts as a trigger for activated CD8+ T cells to kill the transfect cell, reducing the expression of the vaccine and potentially dampening the immunogenicity. Secreted antigen is either taken up by APCs or presented on MHC-II molecules to CD4+ T cells or interacts with B cells, inducing an antibody response [24]. Finally, antigen released after cell death most likely enters APCs and is loaded onto MHC-I by the cross-presentation pathway [24]. Cross-presentation of DNA vaccine derived antigen has been demonstrated in vitro and in vivo studies suggest that antigen expressed from non-antigen-presenting cells is more important in the induction of the immune response to DNA vaccines than antigen from APCs [24].

The clinical trial experience suggests that DNA vaccines (Figure 3) are better at inducing T cell responses than B cell responses [24]. There are a number of reasons why this might be the case, including incorrectly folded antigen, low expression levels, poor activation of the innate response via pattern recognition receptors, and differences in MHC-I and MHC-II loading [24]. Our understanding of the immune response to expressed antigen can be used to target either the DNA vaccine or the expressed antigen to different cells to alter the immune response [24]. Fusing the antigen to an anti-MHC-II single chain fragment variable molecule that targets it to APCs has been shown to increase the response. Optimizing the expression, presentation, and secretion of the antigen to engage different arms of the adaptive response is critical for improving immunogenicity [24].
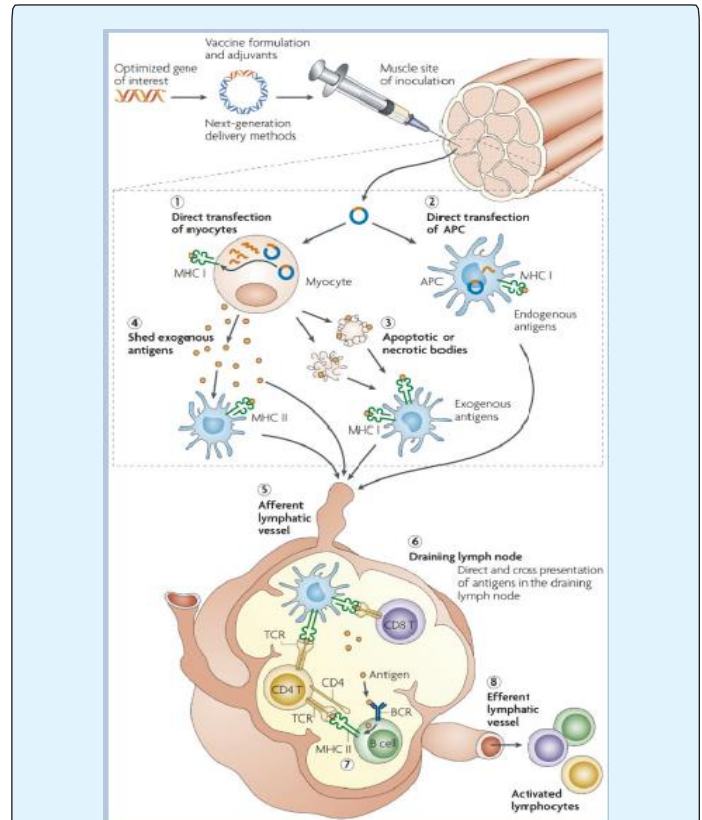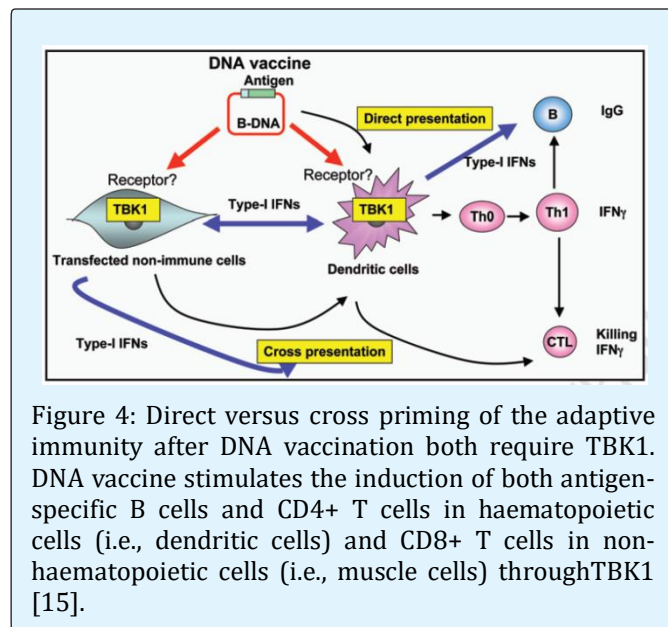

Figure 3: Induction of cellular and humoral immunity by DNA vaccines. Using the host cellular machinery, the plasmid enters the nucleus of transfect myocytes and antigen presenting cells [1]; this can follow either direct transfection by the plasmid vaccine [2] or cross-presentation of cell-associated exogenous antigens; for example, owing to APC engulfment of apoptotic transfect cells [3]. APCs mediate the display of peptides on MHC II molecules after secreted protein antigens that have been shed from transfect cells are captured and processed within the endocytic pathway [4]. Antigen-loaded APCs travel to the draining lymph node (DLN) via the afferent lymphatic vessel [5] where they present peptide antigens to naive T cells via MHC and the T cell receptor (TCR) in combination with co-stimulatory molecules, providing the necessary secondary signals to initiate an immune response and expansion of T cells [6]. In response to peptide-bound MHC molecules and co-stimulatory secondary signals, activated CD4 T helper cells secrete cytokines during cell-to-cell interaction with B cells and bind to co-stimulatory molecules that are required for B cell activation [7]. These processes co-ordinately elicit specific immunity against plasmid-encoded antigen by activating both T and B cells, which, now they are 'armed', can travel through the efferent lymphatic system [8] and provide a surveillance system. Together, the two arms of the immune system, which are induced specifically following DNA vaccination, can create a powerful defence against most infectious diseases [21].

Cross priming: Despite the earlier cross-priming (Figure 4) experiments of Bevan in the 1970s the notion that host-derived APCs could efficiently ingest released exogenous antigens for processing and presentation on MHC class I molecules in vivo had been considered improbable, because the defined cellular pathways of MHC class I antigen presentation require that the antigen be expressed endogenously [1].

Figure 4: Direct versus cross priming of the adaptive immunity after DNA vaccination both require TBK1. DNA vaccine stimulates the induction of both antigen-specific B cells and CD4+ T cells in haematopoietic cells (i.e., dendritic cells) and CD8+ T cells in non-haematopoietic cells (i.e., muscle cells) throughTBK1 [15].

Adjuvants

The protein antigen is processed within the cell and presented by the MHC-I and –II pathways thereby eliciting specific immune responses essential for controlling pathogenic infections [25]. Although DNA vaccines have been successful in generating strong immune responses in smaller animal model such as mouse, they have not been as efficient in larger species such as primates and humans [25]. Stimulating both the arms of the immune system is often desirable for efficient control of infectious diseases especially in the larger animals. In the case of recombinant protein vaccines, immune-enhancers technically known as adjuvants, such as Freund's adjuvants and Alum (Table 1), are in use to enhance antigen specific immune responses [25]. However, no such adjuvants are available for use in the context of DNA vaccines. The lack of suitable adjuvants for DNA vaccines is one important reason for the poor performance of the DNA vaccines in larger animals [25].

Various vector modifications can affect both the vector backbone and the gene sequence incorporated into the plasmid, which can include adjuvant-like sequences with stimulating activity on the immune system [6]. By using this approach, enhanced antigen specific immune responses were observed, suggesting that this could be a general method for targeting antigen to selected cell types [6].

Class of Adjuvants	Adjuvant name	Nature of adjuvant
Genetic adjuvants	Interleukin-2 Interleukin-12 Granulocyte Monocyte-colony Stimulating Factor T-helper epitopes of toxins	Cytokine Cytokine Cytokine peptides
Adjuvants targeting pattern recognition receptors	Monophosphoryl Lipid A ASO2 ASO1 QS-21 CpG-DNA	Lipid derivative oil in water emulsion Liposomal formation Saponin Oligodeoxynucleotides
Aluminuim-based compounds	Aluminuim Phosphate Aluminuim Hydroxide	Mineral salt Mineral salt

Table 1: Adjuvants used in vaccination with naked DNA [6].

DNA based Vaccine Development

The development of DNA vaccines began in experiments investigating whether the direct injection of DNA or RNA expression vectors for gene therapy could abrogate the need for live-virus vectors [13]. Studies have found that intramuscular (i. m.) injection of non-replicative DNA expression vectors in cationic lipid vesicles resulted in the expression of gene products in muscle cells but surprisingly, they found that for plasmid DNA vectors this occur red even without the lipid delivery system [13], where by reporter genes such as the bacterial Chloramphenicol acetyl transferase, firefly luciferase (luc), and bacterial b-galactosidasegenes were used to assess gene expression. Expression of the luc and b-galactosidase reporter genes in mice was shown to be more efficient in regenerating muscle than in mature muscle [13].

Mode, Site and Routes of Administration of DNA Vaccines

DNA immunization is a novel technique used to efficiently stimulate humoral and cellular immune responses to protein antigens. The direct injection of

genetic material into a living host causes a small amount of its cells to produce the introduced gene products. This inappropriate gene expression within the host has important immunological consequences, resulting in the specific immune activation of the host against the gene delivered antigen [20]. Several studies have shown that the type of immune responses induced by plasmid immunisation is significantly affected by

a) The mode and site of gene delivery,

b) The dose of plasmid and

c) The administration of booster injections and the interval between immunisations [5].

Immunization with DNA-based plasmids has been successfully attempted in several tissues by various routes of administration. DNA vaccines can be delivered through many different routes, including the intramuscular, intraderma, subcutaneous, oral, intranasal, intraperitoneal, intravenous, and vaginal route [3,6,15,18,20,26]. Intramuscular and intradermal inoculations have become the most effective ways to deliver the DNA vaccines by needle injection [8]. An alternative and very efficient method for intradermal delivery is carried out by particles bombardment with the Gene gun, which consists on covering gold micro particles with recombinant DNA and shooting them by gas pressure, normally helium, on to the skin [20].

The skin and mucous membranes have been considered as the best site for immunization due to the high concentrations of dendritic cells (DC), macrophages and lymphocytes, whereby the plasmid DNA can be diluted in distilled water, saline or sucrose [20]. Delivery of DNA vaccines via intramuscular injection tends to promote an immune response that is characterised by the production of INF-γ and IgG2a isotype immuno globulins of the Th1 phenotype [5,6,13]. In contrast, delivery via gene gun to the skin tends to promote a Th2 lymphocyte response predominantly characterised by IL-4 and IgG1. In order to balance the immune response, genes encoding various cytokines and co-stimulatory molecules can be included or co-administered with the gene of interest [4]. A large number of studies have been performed to date in order to evaluate the efficacy of co-administered plasmids encoding biological adjuvants, including INF-γ, IL-2 and IL-12 [4].

Intramuscular, intravenous, intranasal, intradermal, subcutaneous, and intraperitoneal routes of DNA administration have tested for their ability to raise protective immunity in mice by Fynan EF, et al. [27]. With the exception of the intraperitoneal injections, each of these routes of inoculation raised at least some

protection. Fynan EF, et al. [27] found that the level of protection varied, with from 67% to 95% of test groups surviving, where by all of the survivors developed transient signs of influenza. Therefore, excellent survival occurred in groups receiving intramuscular inoculations, intravenous inoculations, or inoculations by each of three routes (intramuscular, intravenous, and intraperitoneal) [27]. Thus, the intramuscular, intravenous, and intranasal routes of administration each provided good protection.

The *in vivo* transfection with recombinant DNA, whatever the technique used, normally leads to the expression of the coding proteins provided the adequate promoter is used. The capacity of DNA vaccines to induce both humoral and cellular immune responses is one of the most important characteristics [8]. This capacity also makes them the best candidate tool for the prevention of diseases caused by intracellular pathogens such as tuberculosis, malaria, leishmania and AIDS, for which a cellular response is, required [8]. In general, immunisation with DNA can be accomplished in two fundamentally different ways. One approach is the use of needle injection into different tissues, the most effective route being intramuscular injection into the hind leg quadriceps or tibia is anterior, followed by intradermal injection. An improvement in efficacy of plasmid transfection was achieved by injection of DNA into regenerating skeletal muscle, achieved by prior injection of either cardio toxin or local anaesthetic such as bupicaine [5]. Several methods have been investigated to improve delivery of DNA vaccines including

a) Mechanical delivery consisting of microinjection by various types of needles including pressure injection

b) Electrical delivery such as electroporation, ionophoresis

c) Chemical liposomes and various polymers, in addition to mucosal delivery [5]. Each one of these methods of delivery introduces plasmid DNA into distinct areas of immune surveillance and therefore primes the immune system in distinct ways.

Gene gun delivery of DNA which propels the DNA-coated gold particles into the epidermis resulted in a more Th2 biased antibody isotype response and efficient humoral and cellular responses [5]. The distinct Th1- or Th2-biased immune responses elicited by intramuscular injection or gene gun delivery, respectively, are however not fully understood. Bacterial DNA contains CpG motifs that induce non-specific Th1-dominant responses [15]. Gene gun delivery requires 100-1000 fold less DNA to stimulate immune responses to that achieved by intramuscular injection. The reduced number of Th1-promoting CpG motifs involved in gene gun immunisation

may therefore explain the Th2-bias response to gene gun DNA vaccination [5].

According to Fynan EF, et al. [27] the efficacy of different routes of DNA immunization would reflect both the efficiency of in vivo transfection (DNA uptake and expression) and the efficiency with which transfect cells presented proteins to the immune system. Studies in rodents on the transfection efficiency of injected DNA have demonstrated that muscle is 100-1000 times more permissive than other tissues for the uptake and expression of DNA [27].

Tissues also differ in the efficiency with which they present antigens to the immune system [27]. Tissues, such as the skin and the mucosal linings of the respiratory tract and the gut, that serve as barriers against the entry of pathogens have associated lymphoid tissues that provide high levels of local immune surveillance [27]. Such tissues also contain cells that are specialized for major histocompatibility class II restricted presentation of antigens to T-helper cell [27]. T-helper cells produce the lymphokines that induce growth and differentiation of lymphoid cells. In view of the above, DNA inoculations were undertaken
a) By a route that supports unusually efficient transfection (muscle)
b) By routes that support less efficient transfection but represent routes frequently used for the administration of an antigen to a test animal (subcutaneous, intraperitoneal)
c) By routes that support less efficient transfection but deliver DNA to tissues with high levels of local immune surveillance (skin and respiratory passages) [27].

Demonstrate that many routes of DNA inoculation can be used for raising protective immune responses [27]. Two of these we consider particularly promising
a) Vaccination by gun delivery of DNA into the epidermis
b) Vaccination by administration of DNA to mucosal surfaces. Both of these routes of administration should raise responses that will provide systemic immunity as well as specialized surveillance for major portals of pathogen entry [27].

Following intramuscular (needle) or intra epidermal (gene gun) administrations, a small proportion of the plasmid enters the surrounding myocytes and/or epidermal cells [14]. The injected DNA becomes internalised within 5 min by muscle cells near to the injection site. Over several hours it appears within phagocytes located along the muscle fibres [14]. Then it is transported to the draining lymph nodes and the uptake of plasmid is thought to occur via endocytosis. The engulfed plasmid possibly travels through the endosome-lysosome compartment of the recipient cell, where it becomes partially degraded [14].

Examples of Possible Useful Experimental DNA Vaccines

DNA vaccines can lead to a strong and long-lasting immune response through the inoculation of a plasmid containing a gene for a particular protein antigen, which is subsequently expressed by the cellular machinery of the person receiving the vaccine [7]. DNA vaccines first sparked the interested of the scientific community in the early 1990s, when it was reported that plasmid DNA, delivered into the skin or muscle, induced antibody responses to viral and non viral antigens [11].

Theoretically, DNA vaccines could generate broad immune responses, similar to the live-attenuated virus platform, without the need for a replicating pathogen. Owing to the promise of DNA vaccines in small animal studies, clinical trials soon ensued [11]. Although only veterinary DNA vaccines have been approved to-date (e.g., equine vaccine for protection against west Nile virus), there are numerous DNA vaccines in various stages of development for targets such as HIV, cancer, and multiple sclerosis [7]. The first of several of phase I trials, conducted almost 2 decades ago, evaluated the efficacy of a DNA vaccine targeting human immunodeficiency virus type 1 (HIV-1) for therapeutic and prophylactic applications [11]. Other studies shortly followed that targeted cancer or other HIV-1 antigens, influenza, human papillomavirus (HPV), hepatitis, and malaria [11]. However, the results of these early clinical trials were disappointing. The DNA vaccines were safe and well tolerated, but they proved to be poorly immunogenic but these studies provided proof of concept that DNA vaccines could safely induce immune responses (albeit low-level responses) in humans [11].

According to Ferraro, et al. [11] there are currently 43 clinical trials evaluating DNA vaccines for viral and non viral diseases listed in the clinical trials (Figure 5). The majority (62%) of these trials are investigating vaccines for HIV (33%) or cancers (29%). Almost half (38%) of cancer vaccines currently being investigated are targeting melanoma. The remaining 38% of enrolling or active clinical trials are investigating vaccines for influenza, hepatitis B and C, HPV, and malaria. This review highlights DNA vaccines for influenza, HPV, and HIV-1 as examples of antibody, cellular, and complex immunological targets, respectively.
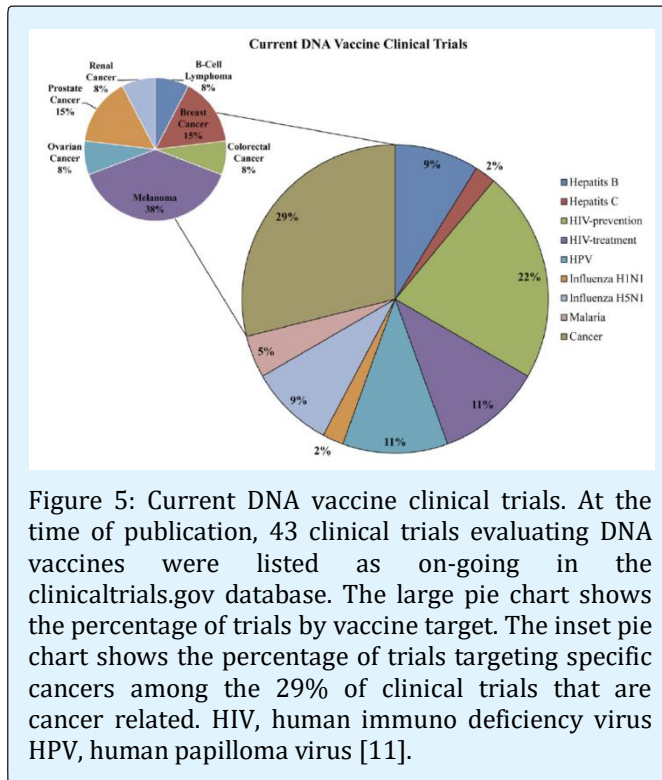
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Figure 5: Current DNA vaccine clinical trials. At the time of publication, 43 clinical trials evaluating DNA vaccines were listed as on-going in the clinicaltrials.gov database. The large pie chart shows the percentage of trials by vaccine target. The inset pie chart shows the percentage of trials targeting specific cancers among the 29% of clinical trials that are cancer related. HIV, human immuno deficiency virus HPV, human papilloma virus [11].

Herpes simplex virus (HSV) 1 and 2 vaccines: Animal studies following the development of humoral and cell mediated immune responses upon injection of plasmid DNA constructs encoding gB, gC, gD and gE were performed in mice and guinea-pigs. Immunisation with plasmids expressing gB and/or gD, or both, exerted a clear cut prophylactic effect, which could be demonstrated in mice as well as in guinea-pigs, when immunised animals were challenged with virulent HSV-1 and/or 2. DNA immunisation with the HSV-2 gD vaccine inoculated by the i. m. route, protected mice from lethal disease and prevented the development of genital herpes [14]. A similar effect was demonstrated in guinea-pigs as well. The strength of protection against lethal challenge was comparable to that elicited by sub lethal natural infection [14]. In knockout mice, the establishment of immunity following natural infection required predominantly T/CD8 cells and B lymphocytes, while the efficiency of gD plasmid immunisation depended predominantly on T/CD8 cells [14]. The expression of full-length gD polypeptide remained cell associated due to the trans membrane anchor sequence and induced a Th1 type response (activation of T/CD4 and T/CD8 cells) [14]. In contrast, the expression of secreted gD (lacking the trans membrane sequence) resulted in a Th2 type helper cell response mediating higher antibody levels [14]. The

Th1 type protective CTL response following gD DNA vaccination could be enhanced by co-injection with cDNA encoding IL-8 and/or the chemo attractant RANTES [14].

Human immunodeficiency virus (HIV) 1 vaccines: As soon as the direct injection of a plasmid was shown to express proteins in recipient host cells and to promote immune responses, several workers demonstrated the ability of HIV-1 env gene-based DNA vaccine to induce expression of corresponding glycoproteins (either the un cleaved precursor gp160 and/or the envelope gp120) in immunised hosts, such as mice, guinea-pigs and non-human primates [14]. The HIV-1 env DNA vaccines provided only partial protection, being effective at challenge with low pathogenic virus strains only [14]. However, in comparison with recombinant protein vaccines, their ability to reduce the virus load (i.e. the levels of viral RNA within the bloodstream) was firmly established [14]. An interesting feature of the HIV-1 DNA vaccine is that it primes for subsequent boost with a corresponding recombinant protein vaccine and the combined immunisation procedure generates a satisfactory antibody response.

CTLA-4, the Trans membrane protein expressed on activated T lymphocytes, has a damping role at co-simulation and launching intracellular signalling pathways; activation of this receptor prevents derepression of silenced genes [14]. Over expression of CTLA-4 following combined DNA vaccination results in an increased Th2 type response rather than in eliciting the Th1 type CTL response [14]. Indeed, application of a plasmid encoding HIV-1 g120/CTLA-4 fusion protein, when given in three i. m. injections, elicited a clear-cut and specific antibody response [11,14]. Such an approach might hamper the undesired bystander stimulation of T cells carrying the latent HIV-1 provirus, which increases the infectious virus load in blood [14].

Another approach for enforcing stronger protection is a combined DNA vaccine encoding the envelope gp160 precursor and regulatory proteins such as rev Rajcani, et al. [14]. The expression of structural HIV genes may be further enhanced in the presence of a cis-acting transcription element (CTE) [14]. Combination of CTE þrev sequence with the env sequence was used for the experimental type A HIV-1 vaccine; this plasmid provided a stronger immune response of Th1 type [14].

A serious problem of any HIV-1 vaccination comes from the variability of antigenic domains of the envelope gp120 [14]. Several observations confirmed the differences in progression to AIDS, depending on association with A or

non-A HIV- 1 subtypes (clades) [14]. Thus, neither the design of future DNA vaccine can avoid 'tailoring' env protein antigenic domain sequence in order to achieve cross-protection against several virus subtypes circulating in a larger geographical region [14].

The concept of combining a vaccine platform that induces T-cell responses (DNA or viral vector vaccines) with one that induces antibody responses (recombinant protein vaccines) to induce broad HIV-1–specific immunity has shown promise in a recently completed efficacy trial (RV144) [11]. This trial incorporated a multiple-antigen viral vector prime (ALVAC) to induce HIV-1–specific T cells, followed by a recombinant gp120 protein boost (AIDSVAX) to generate HIV-1–specific antibodies [11]. In a modified intent to treat analysis, this heterologous prime-boost approach demonstrated 31% efficacy for prevention of HIV-1 acquisition, but it did not affect viral load in subjects who were not protected [11].

The most potent recombinant vector platform in humans for generation of cellular immunity seems to be the adenovirus platform [21]. The viral vector generated using adenovirus serotype 5 (Ad5) is the most potent [10], although vectors based on new and promising primate serotypes, as well as rare human serotypes, are also being developed. Much of the work on Ad5-based vaccines is being applied to the HIV-1 vaccine arena [28]. Important studies from Merck and the Vaccine Research Centre at the National Institutes of Health have directly compared CTL induction generated in humans by plasmid vectors versus the Ad5 recombinant vector [21].

Human papillomavirus (HPV) vaccines: Over 90 HPV types have been identified so far, which fall into two categories, namely low risk and high risk papilloma virus types (HPV 16, 18, 31, 33, 45, 51, 56, 58 and 66), of which the HPV types 16 and 18 are known to be firmly associated with cervical cancer in women [14].

Two different strategies were introduced in this respect [14]. One is based on recombinant vaccines containing DNA vaccines expressing the structural L1 protein, which assembles into non-infectious virus like particles (VLP) [14]. The second strategy implies the expression of non-structural virus coded regulatory proteins (such as E6 or E7) [11], which act as cofactors of cell transformation and immortalisation [14].

According to Rajcani J, et al. [14], injection of DNA-based HPV16L1 vaccine with the co-stimulatory molecule B7-2 in mice elevated the specific IgG levels to HPV16. In addition, increased proliferation of T lymphocytes and

production of IFN-_ was noticed when leukocytes from immunised mice were examined in vitro [14]. The modified E6/E7 on co protein based HPV vaccine efficiently delays cancer development and/or prevents or causes regression of benign papillomavirus induced skin tumous in rabbits [14].

Hepatitis B virus vaccines: The currently used hepatitis B virus (HBV) vaccine is a recombinant S glycoprotein (gp27) prepared in yeast [14]. HBsAg is secreted by hepatocytes into blood either during acute and/or chronic hepatitis B infection [14]. An alternative recombinant HBsAg glycoprotein, produced in CHO cells, became widely used for vaccination in China [14]. In developing countries, where baby or childhood vaccinations have not been introduced, the congenital and chronic HBV infection represents a great health care problem [14].
In fact, the shortest surface (S) polypeptide covers the C-terminal portion of the L polypeptide (S2þS1þS) such that the S1 and S2 domains of the full length surface (L) glycoprotein have an important immunogenic role effective in HBsAg clearance, in eliciting anti-HBs antibodies and corresponding CTL response [14]. The balance maintained between the Th1 and Th2 type anti-HBV responses, plays an essential role in the clearance from chronic hepatitis B [14]. Th2 type cytokines (IL-4, IL6, IL-10 and IL-13) help B cells to produce antibodies [14]. The Th2 subset of CD4 T lymphocytes seems essential for eliminating the production of infectious virus in hepatocytes as witnessed by monitoring the HBsAg and HBV DNA levels [14].

Plasmids carrying genes encoding various HBV envelope glycoproteins were tested in mice; the results confirmed that CTL as well as anti-HBs antibodies develop post-vaccination [14]. An HBsAg and pan-DR helper T cell epitope encoding plasmid was constructed for eukaryotic cell expression and found convenient for studying the optimal cellular and humoral immunity against HBV [14]. It is known that some chronic HBV-infected individuals spontaneously clear HBV DNA from the serum, which is often accompanied by increased T/CD4 cell responses [14].

Cancer vaccines: Cancer is a leading cause of mortality worldwide, and conventional therapies, including surgery, radiation and chemotherapy are highly invasive without offering lifelong protection. It is now clear that tumor co-opt certain immune-checkpoint pathways as a major mechanism of immune resistance, particularly against T cells specific for tumor antigens [12]. Thus, immunotherapy has received increasing attention over the last few decades as a strategy for cancer treatment,

and new immunotherapeutic approaches have been developed that are positively influencing the clinical outcome for cancer patients [12].

A major goal of immunotherapy is to induce the immune system to elicit specific immune responses against tumor-associated antigens (TAAs). In this context, DNA vaccination represents a simple, safe and promising strategy for harnessing the immune system. DNA vaccines for cancer immunotherapy are designed to deliver one or several genes encoding tumor antigens or, to modulate immune responses, thereby eliciting or augmenting immune responses against tumor antigens that play central role in tumor initiation, progression and metastasis [12]. Vaccine efficacy can be significantly improved not only by selecting appropriate tumor antigens but also by implementing strategies for improving antigen presentation and immunogenicity, such as new delivery systems, addition of molecular adjuvants and immuno stimulatory signals, optimized prime-boost strategies or blockade of immune checkpoints [12].

DNA cancer vaccines can induce both innate immunity activation and adaptive immune responses in order to suppress tumor growth and achieve total tumor rejection. New developments in vaccine delivery methods improve the efficacy of tumor antigens to evoke protective immune responses with the aim to overcome the primary hurdle of poor transfection efficiency and subsequent lower immunogenicity.

In mouse models, DNA vaccines have been successfully directed against a wide variety of tumors, almost exclusively by driving strong cellular immune responses in an antigen-specific fashion. Furthermore, tumor burden has been decreased or even obliterated by novel DNA vaccine strategies that deliver cytokines as plasmids directly into tumor [21].

Influenza vaccine: Every year, the scientific and medical communities are charged with the task of determining the appropriate influenza strains to include in the seasonal influenza vaccine. Current vaccine platforms require months to generate sufficient quantities of antigens because of the requirement for the growth of the virus in chicken eggs. This can delay the availability of viral stocks or result in a mismatch between the vaccine strains selected and the actual circulating strains [11]. In contrast, development of a DNA vaccine for a particular influenza strain could shorten this timeline 2-4 fold and could potentially provide a product in a few months with little chance of mismatch [11].

One preclinical study of an H5N1 influenza DNA vaccine showed that protective antibody titres were induced to multiple cades of H5N1 using a single consensus H5 antigen [11]. In further support of this cross-protection approach, it has recently been shown that cross-protective titres can be achieved to viruses that circulated over 90 years apart; namely, the 1918 "Spanish Flu" and the 2009 "Swine Flu" [11]. The concept of cross-neutralization of different influenza strains may be of great significance in future influenza vaccines and this concept applies not only to influenza strains with the potential to cause pandemics but also to strains included in seasonal vaccines.

There are currently several DNA-based influenza vaccines in various stages of phase I clinical trials, including vaccines against potentially lethal pandemic strains such as H5N1 (Invivo Pharmaceuticals) and H1N1 (National Institutes of Allergy and Infectious Diseases) [11]. The ultimate success of these vaccines could reshape the way physicians and researchers view influenza vaccine development [11].

Parameters Affecting Gene Expression and Immunogenicity of DNA Vaccines

The efficiency of expression of antigen genes and the immunogenicity of DNA vaccines can be influenced by several parameters, including
a) The construction of the plasmid vector, in particular the choice of the promoter used to drive expression of the antigen gene
b) The route of administration
c) The tissue or organ in which the antigen is expressed
d) The physical nature and properties of the expressed antigen that govern whether it is secreted by the cell, remains bound in the cell membrane, or remains sequestered within the cell [13].

Gene expression was observed in all species tested including rats, rabbits, mice, and rhesus macaques [13]. The organs and tissues evaluated for *luc* gene expression were epidermis, dermis, muscle, liver, and pancreas [13]. In the rat, the cytomegalovirus immediate-early promoter showed the highest activity in each tissue tested [13]. The relative strengths of various other promoters showed tissue specificity. The pPEP luc gene promoter and the pmMTluc gene promoter were inducible in liver post transfection at 1 and 5 days, respectively [13].

A second parameter affecting the nature of immune responses obtained with DNA immunization is the localization of the expressed antigen.DNA immunization

evoked antibody responses in mice when the DNA vaccines, such as influenza virus NP or HIV gp120,expressed secreted forms of the antigen and for HIV gp120, antibody titres greater than 10,000 were observed [13]. In contrast, immunization of mice with a plasmid vector containing the *luc*reporter gene (which codes for a no secreted gene product) resulted in undetectable antibody to luciferase, although it was estimated that luciferase was produced at a rate of 0.5 to 5ng/day at the site of injection [13]. It was concluded that secreted antigens proceed to peripheral lymph nodes, where a humoral response is evoked [13]. Thus the muscle would act as a reservoir of antigen that would be released over an extended period [13].

Strategy of DNA Immunisation in the Development of Clinical Trial

The remarkable advance and diverse applications of DNA immunisation attracted the attention of many researchers as an alternative procedure for analysing the structure and expression of genes in general, studies for improving the treatment of several diseases, and clinical trials soon ensued [5].

Owing to the promise of DNA vaccines in studies using small animal model, clinical trials were soon developed. The primary of a few of phase-I trials, performed almost 2 decades back, assessed the adequacy of a DNA vaccine targeting HIV-1 for therapeutic and prophylactic usage [5]. Subsequent studies conducted after that focused on other diseases such as cancer, human papillomavirus, hepatitis, malaria, influenza, and other HIV-1 antigens. Nonetheless, the results of these early clinical trials were upsetting [5] and their effectiveness has not been determined [29].

The DNA vaccines were intact and well abide, yet they turned out to be inadequately immunogenic. The antibody titres induced has been found to be very low or absent; CD81 T-cell responses were desultory, and CD41 T-cell responses were of low frequency [5]. However, these studies provide substantiation of connotation that DNA vaccines could safely induce immune responses. Numerous improvements have been integrated into the present DNA vaccines, and these improvements have assist to gleam a revival of interest in the platform [5]. Although the subsequent or the second generation DNA vaccines seem to influence towards both humoral and cellular immune responses regardless of animal models used, researchers suggested that new modified DNA vaccines can be more efficient by broadly activate CD8+ CTLs in larger animal models, compared with previous approved DNA methods [5]. The reduced level of immunogenicity of precocious DNA vaccines is speculated to stem, due to the inefficiency of cellular uptake of the inoculated plasmids [5].

Current research is focusing on developing neoteric approaches to promote transfection competence and improve other facets of the DNA platform [5]. Such neoteric approaches involve optimization of the antigens encoded by the plasmids to increase antigen expression on a per-cell basis, enhance formulation, and inclusion of molecular adjuvants to promote and direct immune responses [5]. Up to date there are about 43 clinical trials evaluating DNA vaccines for viral and non-viral ailments recorded in the gene database clinical trials [5]. The majority of the recorded trials are investigating vaccines for HIV and cancers. The remaining are investigating vaccines for human papillomavirus, malaria, influenza, and hepatitis B and C viruses [5].

Furthermore, in the available trials there is currently a lack of long term follow up. Ideally, the availability of data from randomized clinical trials featuring robust end points such as biochemical response, progression free and overall survival will provide categorical evidence for DNA vaccination potential [5].

Safety Considerations for DNA Based Vaccines

Safety issues specific for DNA vaccines that are presently being addressed include uptake into cells other than the intended target cells, the potential for oncogenic mutagenesis through integration of the plasmid DNA, anti-DNA immune responses, and uncertainty of the fate of the administered DNA that fails to enter the target cells [13].

In studies to date, plasmid DNA has been shown to exist only extra-chromosomally without integration into the host cell chromosome and myocytes are terminally differentiated and do not undergo further cell division [13]. Thus, these muscle cells, which efficiently take up and express DNA delivered as plasmid vectors, would have a decreased probability of integrating the plasmid DNA into the host chromosome compared with actively dividing cells [13]. Furthermore, PCR amplification of DNA recovered from vaccine-injected muscles as long as 19months after administration has demonstrated retention of a bacterial methylation pattern, indicating that DNA replication did not occur in the mammalian host [13].

Despite the low probability of integration of plasmid DNA [21], the low likelihood that such an event would result in activation or disruption of a gene, and despite existing clinical experience with immunization with live DNA viruses (smallpox and varicella-zoster viruses),the possibility of integration will have to be carefully evaluated [13]. More definitive and sensitive evaluations of the fate of the injected DNA will have to be done to determine that no integration occurs and that other cells do not take up or integrate low levels of the injected DNA [13].

Another potential safety issue is whether the injected DNA will induce anti-DNA antibodies similar to those associated with autoimmune diseases [13]. Double-stranded chromosomal DNA has been shown to be non-immunogenic, despite the immunogenicity of denatured single-stranded DNA complexed to protein [13]. The latter, however, generated antibodies that are specific to the protein in the complex and which do not recognize the mammalian chromosomal DNA [13]. Essentially, studies in nonhuman primates have failed to demonstrate anti-DNA antibodies following immunization with plasmid DNA [13]. These findings are in agreement with studies showing lack of immunogenicity of double-stranded DNA.

However, vectors that are modified or adjuvant with the goal of increasing immunogenicity could increase the chances of integration [21]. A further concern is that an integrated vaccine might cause in sertional mutagenesis through the activation of oncogenes or the inactivation of tumor suppressor genes [21]. In addition, an integrated plasmid DNA vaccine could, in theory, result in chromosomal instability through the induction of chromosomal breaks or rearrangements [21]. However, none of these concerns have been witnessed in the preclinical or clinical evaluation of DNA products [21].

Another issue regarding DNA vaccines involves antibiotic resistance. Large-scale manufacture of a DNA vaccine involves enriching cultured cells for the plasmid by virtue of its antibiotic-resistant marker [21]. Concern has been raised that resistance to the same antibiotic might be introduced in participants and transferred into carried bacteria when the plasmid is used in clinical trials [21]. However, the antibiotic resistance genes contained by vaccine plasmids are restricted to those antibiotics - in particular the kanamycin restriction element that are not commonly used to treat human infections [21].

In response to these various safety concerns, the European Union and the US Food and Drug Association (FDA) have developed specific advice on safety and testing of DNA vaccines [21]. Efforts to examine integration, antibiotic resistance and the induction of autoimmunity are being followed even as an impressive unblemished record of clinical safety is continuing to be expanded upon and based on this outstanding safety record the focus of the field has shifted to optimizing immune induction [21].

Advantages of DNA Vaccines

The ability of plasmid DNA to induce both cellular and humoral immune responses after inoculation has been demonstrated in several animal models, and hopes have been raised that its applications will lead to new therapies for a range of human diseases [5]. It is potentially cheaper to produce than recombinant protein vaccines and it is much easier to transport and use, especially in developing countries [5,13,14,18]. DNA-based vaccines exhibit several important advantages over conventional immunisation strategies such as live-attenuated or killed pathogens, proteins, or synthetic peptides.

It incorporates many of the most attractive features of each approach. One of the important advantages of the DNA immunisation is that the immune response to immunisation can be directed to elicit either humoral or cellular immune responses or both without the need for live vectors or complex biochemical production techniques [5]. Additionally, DNA vaccines are highly specific and the expressed immunizing antigen is subjected to the same glycosylation and post-translational modifications as natural viral infection [18]. Moreover, it is relatively easier to insert multiple variants of an antigen into a single array of plasmid vaccine [5,6,14]. Candidate bacterial antigens can now be chosen from genomic sequences. This is a considerable advantage for curative vaccination against tumor antigens which may be identified only as DNA sequences produced from both human and cancer genomes [5].

Logistic advantages of DNA vaccines include the relative ease and low cost of production and transportation making them more suited for production in the developing world as opposed to other systems [6,13,18].

Furthermore, the plasmid DNA is relatively stable resisting temperatures over 50°C [9]. Therefore, the DNA would better survive conditions of tropical climate, even outside a refrigerator. Since many recombinant plasmid copies can be prepared in great numbers of bacterial cells, the DNA vaccines provide a cheap method of vaccine preparation [14], which could be used to produce

combination vaccines with antigens that have incompatible formulations when used as traditional vaccines [13].

There is no risk of trace pathogenicity as happens due to incompletely inactivated virus or due to the selection of a partially reverted attenuated virus [14]. The plasmid DNA sequence itself does not elicit a heterologous immune response, at least not in healthy subject. On the other hand, the adjuvant effect of the CpG motifs within the plasmid DNA sequence stimulates an immune response, a process beneficial for vaccination [14]. Additional gene(s) carried by the plasmid, which encode the enzyme(s) providing bacterial resistance (such as Ampicillin resistance etc), cannot be expressed in human cells, since their promoters are active in prokaryotic cells only [14]. Taken together, following DNA vaccination, an undesired heterologous immune response may be far less frequent than in the case of purified subunit vaccines, which are never rid of traces of contaminating proteins [14].

As DNA vaccine plasmids are non-live, non-replicating and non-spreading, there is little risk of either reversion to a disease-causing form or secondary infection [18,21]. In addition to their safety, DNA vaccines are highly flexible, encoding several types of genes including viral or bacterial antigens, and immunological and biological proteins [21]. DNA vaccines are stable, are easily stored and can be manufactured on a large scale [15,21]. They also, for example, bypass concerns that adventitial agents might be transferred from tissue-culture lines to the vaccinated individual.

Disadvantages of DNA Vaccines

The disadvantages of DNA vaccines are based mainly on health, safety and ethical issues (Table 2). Most of the safety issues concerning the system are based on the activation of oncogenes as a result of genomic incorporation of immunising DNA, as well as eliciting anti-DNA antibodies [3,5]. Although this has rarely been detected in experimental studies [5], the outcome could be different in clinical trials and field cases. While these issues are of concern and require careful monitoring, it would not be applied to DNA immunisation of captive animals to produce antibodies, particularly if gene gun is used [5]. This is due to the likelihood of eliciting anti-DNA antibodies when use of the gene gun is minimised because it requires 100-fold less DNA than intramuscular injection to achieve equivalent sero-conversion efficiencies [5].

Other drawback of DNA based vaccines is the reduced level of immunogenicity [5,6,15,17]. Therefore, adequate adjuvants will be necessary to overcome this impediment. Alternatively, plasmid genes can be integrated for those cytokines such as interleukin 4 or granulocyte-macrophage colony-stimulating factor that enhances immune responses or for C3d oligomers as an adjuvant for B-lymphocyte cells [5]. Otherwise, ensuing booster immunisation with the relating antigen as a protein can be an option [5]. However, this may bring about antibiotic resistance which would be a huge drawback [3].

It has been estimated that any foreign line raised DNA fragment would become integrated into positions influencing the expression of a 'housekeeper' proto-oncogene or a cellular regulator gene at frequency far below 10^{-3} [14]. The probability of an oncogenic insertion event may be close to 10^{-9} or 10^{-10} [14]. Thus, even under conditions favouring foreign DNA integration (i.e. in the case of linear ds DNA transposon-like sequences flanked with symmetric repeats), the probability of transformation should not exceed the frequency of spontaneous mutations [14].

Taken together, though the possibility of oncogenic integration of the DNA vaccine sequence into host cell DNA seems rather hypothetical, careful studies are needed to determine which kind of plasmid would fulfil the upmost strict criteria for safety [14]. The probable insertion rates should be defined for plasmids, recommended as DNA vaccine vectors. Limitations assessing the maximal acceptable integration rates should be created as standards in order to prepare the criteria for authorisation [14]. The advantages of the introduction of the recombinant plasmid vaccines are worth these efforts.

One should mention in this context, how some technical problems, accompanying the design and preparation of currently used vaccines, generated safety guidelines, which evolved into recently accepted standards and recommendations [14]. With the exception of recombinant hepatitis B vaccine, current vaccines are mostly live attenuated or inactivated viruses and/or highly purified and concentrated extracts prepared from infected cells [14].

At present, it seems difficult to set clear regulatory issues for the safety of nucleic acid vaccines until questions, which are still a matter of discussion are solved. Examples of unclear problems at this time are:
a) The persistence of the plasmid at the administration site.

b) The local uptake of inoculated plasmid.
c) Access of the plasmid into nuclei of surrounding cells.
d) The rate at which the plasmid gets integrated into host cell DNA.
e) The rate at which the integrated plasmid would exert adverse effects, i.e. transform the recipient cells [14].

A number of issues have limited the translation of DNA vaccines from successful preclinical studies into the clinic, and a better understanding of these issues will greatly help the development of a clinically effective DNA vaccine. One of the simpler issues is scale: the original volumes and doses used in murine studies (often 50 µg in a 50-µl volume) were large relative to the size of the muscle [24]. The delivery of such a large bolus of DNA would have a biophysical effect on the delivery of the DNA, creating shear forces on the cells, inducing inflammation, and generating hydrodynamic pressure increasing DNA uptake [24].

Second, the immune response required to protect a small rodent against infection may not be the same as that required to protect a human [24]. Furthermore, the induction of immune responses, particularly the innate immune recognition of cytosolic and extracellular DNA, may differ among species. Inadequate animal models are an ongoing issue with all preclinical vaccine development and not limited to DNA vaccines [24]. Third, the way in which DNA is taken up, processed, and expressed appears to be different in different species; for example, the DNA scavenger serum amyloid P is more active in humans than mice. Interestingly, inhibition of serum amyloid P is being targeted in an ongoing clinical trial to improve DNA vaccine responses [24]. Steps have been made in recent years to overcome these issues for clinical use. Before exploring the approaches used to optimize DNA vaccines for clinical use, it is necessary to understand how they work, both as gene delivery systems and as vaccine immunogens [24].

Characteristic	Advantage/Disadvantage of Plasmid DNA Vaccines
Antigen	In vivo antigen synthesis with native confirmation
Antigen Presentation	MHC-I, MHC-II, cross priming
Immune Response	Humoral and cytotoxic
Manufacture	Easy and fast
Stability	Stable at various temperature (RT)
Risk	Does not induce the disease related to the encored antigen
Applicability	Prophylaxis and therapy of disease
Indication of Use	Infectious disease, allergy7, cancer. Autoimmune disease
Safety	Low risk of recombination and inflammation
Immunogenicity	Weak

Table 2: Advantages and disadvantages of plasmid DNA vaccines [6].

Future Directions

DNA vaccine immunization is a novel and promising strategy for the development of vaccines against human diseases. Equally promising is the value of this technology as a research tool to study the basic immune mechanisms of vaccination, such as antigen processing and presentation, and the contribution of "professional" antigen-presenting cells (e.g., macrophages) in processing and in presenting antigen secreted from target cells (e.g., muscle cells). Development of DNA-based immunogens as vaccines for human use requires further research and development to ensure the safety of these products.

New assays specifically designed to carefully evaluate this class of vaccine for potency, general safety, purity, and identity should be developed in addition to tests addressing genetic toxicity (i.e., integration), tumorigenicity, and teratogenic toxicity [13]. Further

product development may include the development of methods to enhance the performance of DNA vaccines through improved facilitators or other delivery vehicles designed to optimize uptake and gene expression in target tissues. The use of DNA-based vaccines in multivalent vaccines and combination vaccines designed to immunize against multiple diseases is also a promising area of development.

At a level of vaccine construction, better plasmid constructs can be designed or tissue-specific promoters that target antigen expression to a specific site or cell type may be incorporated. In order to reduce costs of immunization, oral or nasal administration should be explored for delivery of nucleic acid vaccines, as opposed to parenteral route. Subsequently, Oral or nasal delivery may improve the performance of these vaccines through the induction of potent mucosal immune responses, particularly against diseases in which the pathogen enters

via oral, respiratory, or intestinal routes of infection or via genitourinary surfaces [13].

The principal issue regarding the future of DNA vaccines concerns improving their immunogenicity in larger animals and in humans. There are several ways in which antigen expression and immunogenicity can be improved for the DNA vaccine platform. These include optimization of the transcriptional elements in the plasmid backbone with the aim to improve antigen expression levels, strategies to improve protein expression of the gene of interest, inclusion of adjuvants in the formulation or as immune modulators, and the use of next-generation delivery methods [21].

Conclusion

Development of vaccines is one of the most important applications in the field of immunology in the last century and thus a major achievement in the prevention of infectious diseases that saved the lives of millions of people. Currently most of the licensed vaccines are conventional. However, DNA vaccine is potentially cheaper to produce than recombinant protein vaccines. It is much easier to transport and use, especially in developing countries. Importantly, DNA-based immunisation exhibits several advantages over conventional immunisation strategies that involved live attenuated or killed pathogens, proteins, or synthetic peptides. It incorporates many of the most attractive features of each approach.

One of the important advantages of the DNA immunisation is that the immune response to immunisation can be directed to elicit either humoral or cellular immune responses or both without the need for live vectors or complex biochemical production techniques. The disadvantages of DNA vaccines are based mainly on the activation of oncogenes as well as elicitation of anti-DNA antibodies and low immunogenicity in vaccines. These issues need to be resolved based on both scientific and clinical research studies.

It is believed that certain DNA vaccines might become tools for specific immunotherapy of chronic diseases such as herpes simplex, HIV/AIDS and hepatitis B [14]. The immunotherapeutic use of these vaccines might improve the course of a chronic disease, or at least reduce the reactivation rate of the dormant virus or delay the lethal outcome of HIV-1 provirus carrier state [14].

DNA vaccines in humans have yet to live up to the excitement generated by the preclinical studies. This is due to issues with scaling up the dose and differences in both the expression of foreign nucleic acids and the initiation of an immune response to DNA between mice and humans. It has been more than 16 years since DNA vaccines stepped into the scientific limelight. During this time DNA vaccine technologies have generated great deal of excitement as well as disappointment. Its success will however be built on a high level of cooperation between industry, the regulatory authorities, funding by non-governmental organizations, the public and academicians.

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