DNA Vaccines

Molecular Medicine
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In order to stay ahead of ever changing pathogens, vaccinology must forever be moving forward. Eliciting potent, targeted, safe immune responses against pathogens is a challenge that can be addressed in many ways. DNA vaccines have been touted as the next step forward, the "third generation" of vaccines. However challenges of poor immunogenicity still need to be addressed. Several methods of doing this are reviewed here.

Introduction

One of the major hopes for future vaccine development is the progress of DNA vaccines, touted the "third generation" of vaccine technology. DNA vaccination involves the introduction of genes encoding the vaccine antigen, to a host, whose cells then expresses the antigen, inciting an immune response. The concept was first shown in 1992, when Tang *et al.* (1992) injected the genes coding for a protein into mice and elicited the same immunological response as injecting the pure protein. There are many advantages to this form of vaccination over current methods: pDNA (plasmid DNA) is cheap, easy to manufacture and work with. Unlike conventional vaccines, they do not require refrigerated transport and storage, a huge bonus for the distribution of vaccines to certain regions, such as rural African areas. This is due to DNA being a relatively stable biomolecule, compared to other molecules used in vaccines such as various proteins (McMichael *et al.* 1983). Another attractive feature is its safety. As they only encode certain antigens, there is no danger of the vaccine reverting to its viral form, as can happen in attenuated vaccines. Attenuated vaccines can also be harmful to immunocompromised recipients (Jean-Philippe *et al.* 2007), and some pathogens

are considered too risky to deliver even if attenuated, DNA vaccines can safely deliver immunity to a broader range of pathogens. DNA vaccines can be constructed chemically, instead of from potentially dangerous virulent sources, making safety another plus for DNA vaccination.

Perhaps the biggest advantage of DNA vaccines is their ability to generate a cellular as well as humoral immune response, spanning all areas of adaptive immunity (antibodies, helper T cells, cytolytic T-lymphocytes (CTLs)) through the activation of both MHC class-I and class-II molecules. A vaccine such as this, which can generate CTLs, is very desirable as highly conserved epitopes across various strains tend to be internal or functional proteins, making access difficult for antibodies. Generating a CTL immune response against these epitopes is important in delivering heterologous immunity, as the antigens that can be reached by antibodies, are likely to mutate causing pathogen resistance to the vaccine. CTLs can reach the highly conserved epitopes, resulting in a broader range of immunity. It has been shown that CTLs can provide immunity against viral challenges, even if pre-existing antibodies to the specific strain of virus do not exist (McMichael *et al*. 1983). This CTL response cannot be stimulated by normal vaccines, as upon injection of the exogenous protein or inactive vaccine, the particles are guided down the endolysosomal pathway, activating major histocompatibility complex II (MHC II) on antigen presenting cells (APCs), which in turn stimulate CD4+ (helper T) cells. These produce cytokines and trigger B cells to produce antibodies. In order to produce a substantial CTL response, intracellular production of the foreign antigens needs to take place (such as during viral infection of a cell). These antigens are processed by proteasomes and picked up by nascent MHC class I molecules. The complex is then transported through the Golgi apparatus onto the cell surface (Fig. 1). Immature CD8+ (CTLs) bind and are activated, begin dividing, and start destroying cells presenting the antigen. The ability to deliver a gene encoding antigens straight into cells, which will then be processed through the endogenous pathway described above, is the main motivation behind the development of vector based vaccines. In particular the ability the generate CTLs is particularly exciting in the area of therapeutic cancer application, "breaking" the bodies

tolerance towards the cancer cells. The area of cancer vaccines, in particular DC vaccines will be explored later in this article.

Initial trials demonstrating the safety and immunogenicity of DNA vaccines, lead to great excitement at the potential for the technology. Early fears that the DNA vector could elicit an autoimmune response (Klinman *et al.* 2000) proved to be unfounded, and DNA vaccines are now being developed to treat autoimmune conditions. There are currently four DNA vaccine products licensed for use in animals (Liu 2011), however the majority of clinical trials in humans to date, have resulted in lower than anticipated immune responses. The mechanism by which immune responses are generated from DNA vaccines proved more complex than initially thought. The next section will deal with methods used to enhance immune response, to a clinically relevant level.

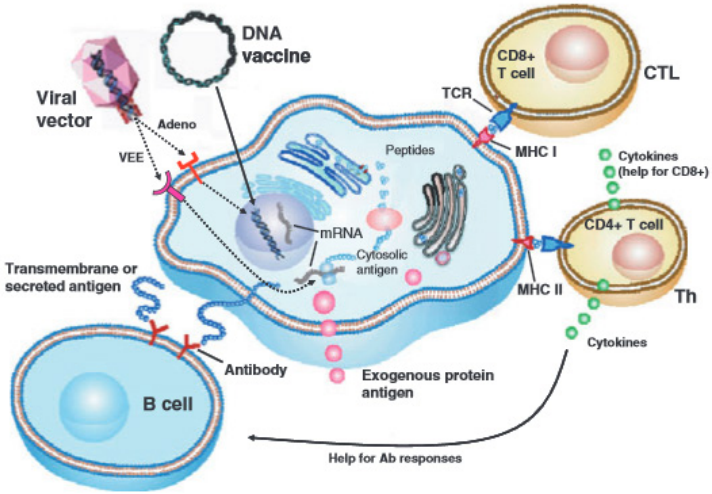

Figure 1: Plasmid DNA entry via viral vector/electroporation. *Encoded antigens are then expressed and presented for immune response from T+B cells (Liu 2011).*

Methods of Enhancing Immune Response

Various methods of enhancing the, until now, disappointing immunogenicity of vector vaccines, have been researched. The use of adjuvants, both native and foreign, the method of administration, the construction of the DNA vector with various promoters and enhancers, different vector types (viral, bacteria etc.) have all been shown to be effective.

Cytokine Adjuvants

The method that seems to be showing the most promise is the addition of genes encoding adjuvants to the vaccine. Adjuvants are compounds that potentiate/modulate the immune response of a vaccine towards an antigen in a desired direction. Molecules such as cytokines, growth factors, TLR ligands and enzymes have all proved to be effective adjuvants. Cytokines will be dealt with in detail here as their clinical use as adjuvants has been well established. Purified recombinant cytokines are very expensive and there are dangers associated with their use in vaccines, so Raz *et al.* (1993) developed a new approach, injecting cytokine encoding genes straight into the muscle, resulting in *in vivo* production of cytokines. This effect was shown to last several weeks, showing that administration of vectors encoding cytokines can regulate humoral and cellular responses. Cytokines also have disadvantages as adjuvants, they have a relatively short half-life in the body, and one type of cytokine can moderate the activities of another (Trinchieri 2003), leading to an unpredictable immune response. Despite this, the immunodulatory effects of cytokines as a way of amplifying DNA vaccine effects have been well established. When the cytokine, IL-2, was administered as an adjuvant alongside a DNA vaccine against HIV-1, the HIV-1-specific delayed-type hypersensitivity response and CTL activity were significantly amplified (Xin *et al.* 1998*)*. Further studies found that the effect of the cytokine IL-2 as an adjuvant was improved by the creation of an IL-2/immunoglobulin fusion protein, due to the increased half-life of the new protein. This protein augmented cellular immune responses in HIV-1 (Barouch *et al.* 1998). These results have been carried forward to human trails with promising results.

There are two main subtypes of cytokines, type 1 which enhance the proliferation, stimulation and/or function of T cells (INFγ, TNFα, etc.), and type 2 which favour the enhancement antibody responses through the recruitment of APCs, increasing their ability to prime adaptive immune response. The timing of the administration of the plasmid cytokine in relation to the DNA vector, as well as dosage, an individual's genome and combination of adjuvants used, all have to be considered.

Delivery Methods

The traditional method of administering vaccines (intramuscular injection) may not be the most effective form of delivery for DNA vaccines. The bulk of the vectors injected do not actually succeed in transfecting any cells (Dupuis *et al.* 2000). The majority of those that do, end up transfecting skeletal muscles cells (monocytes), instead of the desired professional APCs. Monocytes are at best are only partially effective at presenting antigens, and do not have any MHC molecules while under physiological conditions (Wiendl *et al.* 2005). This makes them unable to directly activate CTLs. Instead these cells produce the antigen encoded by the vector, and then by the process of cross presenting, the antigen is transferred to an APC, and the appropriate MHC class-I CTL response elicited. Needle type, size and speed of injection all have effects on optimisation of vaccine effects (Bins *et al.* 2013). The high speed implantation of vaccine loaded polymer films, to carry pDNA and bio degradable polycations into the immune cell rich epidermis has been shown to induce up to 140 times more gene expression than a straight forward intradermal DNA injection, in primate skin. This "multilayer tattoo" type of needle injection is used widely in animal studies but not yet in a human clinical context (DeMuth *et al.* 2013).

The biolistic particle delivery system, or "gene gun" method, originally designed for plant transformation procedures, has been shown to be by far the most efficient way of delivering DNA vaccines (Fynan *et al.* 1993). It uses compressed helium to accelerate heavy metal particles coated with pDNA directly into cells (direct transfection), as opposed to the extracellular space, where a normal

injection will deliver its load. The heavy metal tends to be gold, as it is stable and its high molecular weight lends itself to the task. As with the similar "multilayer tattoo" method, the delivery is directed to skin, where many APCs are present, through particle-mediated epidermal delivery (PMED). This direct delivery system means that far less pDNA needs to be used (1-3μg in mice, has to be scaled up depending on organism) compared to a saline injection (2-20μg) (Feltquate *et al.* 1997). The potency of the technology was also demonstrated when a gene gun delivered DNA vaccine, induced antibody production in 12/16 subjects that had failed to produce a response to a licensed conventional attenuated vaccine (HBsAg vaccine) (Rottinghaus *et al.* 2003). However the procedure needs to be performed several times in different places to overcome the weak immunogenicity of pDNA, and also to counteract the small amount of pDNA that can be delivered per bead. Instability of the DNA on the beads themselves is also a hindrance to the development of this technology.

Perhaps the single strongest method of increasing immunogenicity of DNA vaccines is the Electroporation (EP) method. One of the reasons for the poor immunogenicity of DNA vaccines in cells is the lack of uptake into cells of the foreign DNA molecules. Transfection of DNA into cells has long been aided through the use of EP, where an electrical field induces a voltage across the membrane, opening temporary pores, allowing pDNA (or any desired macromolecule) to pass through inside. The cell reseals slowly (seconds-minutes) after the destabilisation. It is still debated whether the DNA passes through by electrophoretic facilitation or passive diffusion. This EP method is used both in the lab and also on the macro scale, where it is used alongside the administration of the pig gene therapy product described in a previous section. Indeed this method has shown to increase antigen delivery by up to 1000 fold compared to delivery of naked DNA alone (Sardesai & Weiner 2011). EP induces a large Th1 response, with an immune response more than 10 times that of other pDNA methods. The use of EP is an exciting area of investigation, and can be used in conjunction with other forms of transport to incite an even larger immune response. Currently there has been at least one phase 1 clinical trial using the EP method (Heller & Heller 2010).

Vectors

Artificially constructed plasmids are used as vectors for the purposes of DNA vaccination. A vector is a DNA molecule that can be used to transport foreign DNA into cells. The very makeup of the vectors used in a DNA vaccine can be altered to elicit better immunogenicity. A good vector design is important in maximising gene expression. Various factors need to be considered when developing a vector for use as a DNA vaccine, and gene capacity, safety, simplicity to work with and expression of heterologous protein all need to be taken into account. The consideration of the immune response to the vector itself was, and still is, not fully understood. Using DNA encoding for HIV-1 envelope proteins (gp120 and gp41) as model antigens, various vector manipulations and their effects have been studied. Viral gene transcription and translation in host mammalian cells can be hindered by biased codon usage (the favouring of one particular codon across the genome over others that code for the same amino acid) (Wang *et al*. 2006)2006 . Codon optimisation, from wild type codons to codons with a higher frequency of mammalian tRNA, has been carried out successfully on gp120 encoding sequences. *In vitro* expression of the HIV envelope protein was greatly increased, *in vivo*, it resulted in increased CTL reactivity and antibody titres (André *et al.* 1998)1998. Thus optimising codon usage on synthetic vectors represents a workable way to increase efficiency of expression in DNA vaccines.

The significance of Toll-like receptor (TLR) driven adaptive immune response has resulted in TLR ligands emerging as attractive adjuvants to DNA vaccines, expressed through the vaccine vector. TLRs recognise broadly persevered molecules across pathogens, known as pathogen-associated molecular patterns (PAMPs). TLRs are expressed on various APCs, with 13 different subtypes having been discovered so far. Activation of TLRs leads to proinflammatory immune responses. Of specific interest in relation to DNA vaccines is TLR9, the only TLR to recognise DNA. TLR9 is activated by unmethylated CpG (cytosine –phosphate- guanine) motifs. This is used to alert the immune system to the presence of viral or bacterial infection, both of which have DNA with high levels of unmethylated CpG, in contrast to low levels present in mammalian genomes.

Oligonucleotides containing CpG motifs have shown to induce significant B-cell immune response (Krieg *et al.* 1995)1995. Cytokine secretion is also enhanced. Generally DNA plasmids are produced on bacterial vectors, and hence have large amounts of these CpG motifs. It would thus be naïve to think that the plasmid backbone is immunologically inert and has no effect on the immunogenicity of the vaccine. The activation of the TLR leads to triggering both innate and adaptive immune response. Initial phase 1 and 2 trials, of the effects/effectiveness of oligonucleotides containing CpG motifs as vaccine adjuvants in humans have been promising. Subjects given the motif compound showed higher IgG (class of antibody) levels then subjects who had been given just the vaccine and saline (used as a control). The test subjects given higher doses of the motifs also tended to have higher CTL levels compared to control groups (Cooper *et al.* 2004), only relatively mild side effects were observed during the study. This demonstrates the potential for exploiting these CpG motifs, as anti-tumour agents (through the stimulation of CTLs) and as standard vaccine adjuvants.

Another Toll-like receptor, TLR5, has also been the subject of research for enhancing DNA vaccine immunogenicity. TLR5s are unique as they are the only TLR with a protein as their antagonist. They are stimulated by flagellin, the primary protein building block of bacterial (both gram positive and negative) flagella (Hayashi *et al.* 2001). Stimulation results in maturation of DCs and production of cytokines. DNA vaccines encoding for this protein on the vector have been shown to function well as adjuvants (Applequist *et al.* 2005)2005. A transmembrane bound version of *Salmonella* flagellin, FLiC was used in this study. The *in vivo* expression of FLiC by mammalian cells activated monocytes, inducing local inflammation. Significant antibody and cellular responses were also noted.

Prime Boost Immunisation

An interesting, if slightly confusing observation is the increased potency of DNA vaccines when used as part of a mixed modality or prime-boost immunization. A mixed modality vaccine is where two different types of vaccine are given sequentially, the priming shot,

such as a DNA vaccine, followed up with a boost shot of a different vaccine modality such as a viral vector or protein boost. This results in greater immunogenicity compared to one type being given on its own, regardless of the dosage (Liu 2011).

Interestingly, the sequence of the shots has a large effect on the resulting potency of the treatment. J Schneider *et al.* (1998) showed this, while at the same time bringing about the very concept of heterologous prime-boosting , when they found that injecting mice with a plasmid DNA followed by a boost consisting of a recombinant viral vector vaccine (encoding the same antigen), resulted in very high levels of CD8+ T cell response. When they reversed the order of administration, this effect was not observed. The initial prime-boost sequence generated immune response greater than either of the vaccines given alone, or in reverse order, leading to better protection to malarial challenge (Schneider *et al.* 1998). This was exciting news in the search for greater DNA vaccine immunogenicity even if the exact immunological reason for the observation remains unknown. There has been a few explanations proposed, with the true reason probably being a combination of these and other, as of yet undiscovered, clues. The use of DNA vaccine as the prime, as it only presents the antigen of interest, may focus the immune response, and eliminate vector specific immunity, which would reduce immune response (de Mare *et al.* 2008). Another possibility is that, while DNA vaccines are excellent at eliciting cellular immune responses, other pre-existing technologies have had better results in relation to humoral immunity, producing larger antibody responses. This has been seen in numerous studies, using a recombinant protein to greatly increase antibody immune response (Otten *et al*. 2005). Bacterial/viral vectors may also be used as boosts, they may be advantageous to use due to the innate and adaptive immune responses of the organism to the vectors (Liu 2010). There have been numerous trials of this prime boost technology, the largest being a 16,000 human phase 2 trial for a HIV vaccines in Thailand, with results showing "modest benefit" with vaccine efficacy (reduction in incidents of disease among vaccinated subjects) around 31.2%, but show exciting possibilities for the future (Rerks-Ngarm *et al*. 2009). A follow up study a few years later showed that the vaccination did not affect the clinical course of AIDs once the subject was infected (Rerks-Ngarm *et al.* 2013). Another tri-

al, a prime-boost vaccine for malaria, carried out on rhesus monkeys showed that while total dosage of the DNA prime was unimportant, the amount of DNA injections and interval between prime and boost were critical in optimising vaccine efficacy. Intervals ranged from 7-21 weeks (Weiss *et al.* 2007). The most important thing to take away from this study is the finding that shorter intervals between boost and prime lead to less protection, something that must be applied to a human clinical context.

Conclusions

The use of prime boost technology is probably the most likely way for DNA vaccines to initially make their way into mainstream pharmaceutical use in humans, given that trails have already been carried out on thousands of humanoid subjects. Given the efficacy the prime boost system lends to DNA vaccines, along with the fact that they are being used in conjunction with established, licensed, proven vaccines, this approach seems the most likely way forward. However none of these methods should be considered in isolation. The benefits of DNA vaccines are too much to ignore, the possibility of a cancer vaccine, not to mention possible treatments of diabetes and allergies (Isakovic *et al.* 2014, Tasyurek *et al.* 2014), the ability to elicit meaningful CTL response and the lack of transport issues are just some of them. Combinations of vector design, delivery methods, use of suitable adjuvants along with the use of prime boosts, are the best bet for delivering maximum DNA vaccine immunogenicity in the future.

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