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DNA vaccination: an immunological perspective

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VACUNACIÓN CON DNA: UNA PERSPECTIVA INMUNOLÓGICA

RESUMEN

La vacunación con DNA es una nueva y prometedora estrategia para la prevención y el tratamiento de muchas enfermedades debido a su habilidad para inducir una respuesta inmunitaria tanto humoral como celular, frente al antígeno codificado por el DNA recombinante. Una vez inoculado en el huésped, el DNA se introduce en las células, donde el antígeno se expresa y se procesa para ser después reconocido por el sistema inmunitario como si se tratase de una infección natural. Esta tecnología presenta grandes esperanzas para ser usada en la inmunoterapia del cáncer y se espera que llegue a ser el método preferido para la próxima generación de vacunas, particularmente adecuadas contra infecciones intracelulares para las que no hay vacunas efectivas. Esta revisión se centra en los mecanismos por los cuales la vacunación con DNA es capaz de inducir una respuesta inmunitaria y los abordajes que se están llevando a cabo para su optimización. También se ofrece una visión general sobre las aplicaciones de la vacunación con DNA.

PALABRAS CLAVE: Vacunación con DNA / Vacunas genéticas / Inmunoterapia / Presentación cruzada.

ABSTRACT

DNA vaccination is a promising new approach 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. After inoculation into the host, DNA enters the cells, where the antigen is expressed and processed and subsequently recognised by the immune system as in a natural infection. This technology holds great promise for cancer immunotherapy and it is also expected to become the preferred tool for the next generation of vaccines, particularly those suitable against intracellular infections for which available vaccines are not effective. This review focuses on the mechanisms by which DNA vaccination induces immune responses and the approaches being followed for its optimisation. An overview of the applications of DNA vaccination is also presented.

KEY WORDS: DNA vaccination / Genetic vaccines / Immunotherapy / Cross-presentation.

INTRODUCTION

Vaccination is one of the most important discoveries in the medical sciences. It was described over 200 years ago by Jenner and today it is 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[1].

DNA vaccination was introduced in the early 90s, following the observation that an intramuscular injection with naked DNA triggered the expression of the coded

antigen[2]. Two years later, Tang et al. showed that this approach could elicit an immune response against the expressed antigen[3]. The interest on DNA vaccination increased when it was described that the immune response induced after DNA injection was strong enough to protect mice or chickens against a challenge with an experimental influenza virus[4, 5]. DNA immunisation is, indeed, a promising new approach to prevent infectious diseases for which classical vaccines, consisting on inactivated or attenuated pathogens or, more recently, recombinant proteins, do not have positive effects. Live or live attenuated vaccines represent the best choice for those diseases requiring both humoral and cellular responses, based on the broad range of effector cells generated and the memory response that they induce[6]. However, there are diseases for which the use of live or attenuated pathogens can be problematic. For instance, they can cause disease in people whose immune system is compromised, like in cancer patients treated with chemotherapy, AIDS patients, newborns or the elderly. But even more dangerous is the possibility that attenuated viruses revert to virulence through mutation. For some diseases such as AIDS, the risks of reversion to virulence are intolerable. By comparison, DNA vaccines also elicit strong and long-lasting humoral and cell-mediated immune responses without any of the risks associated with live and attenuated vaccines[7].

DNA vaccines generally consist of plasmids (small rings of double-stranded DNA) originally derived from bacteria but totally unable to produce infection. The intracellular transcription and translation of the recombinant DNA mimic the replication of a virus during infection. A conventional DNA vaccine is made by cloning the antigen of interest into a bacterial plasmid under the control of an eukaryotic promoter. The essential elements required in the plasmid are: 1) a bacterial origin of replication for the propagation of the plasmid in a bacterial system, 2) an antibiotic resistance gene for plasmid selection during bacterial culture and, 3) an expression cassette consisting on a promoter for expression in the host cells, the coding sequence of interest, and sequences for the stabilisation of the mRNA transcripts (Fig. 1). The backbone of the plasmid also contains some immune stimulatory sequences consisting on unmethylated CpG motifs. These sequences are able to induce an unspecific immune activation, acting as adjuvants for DNA immunisation[8, 9].

DNA vaccines can be delivered through many different routes, including the intramuscular[10], intradermal[10], subcutaneous[11], oral[12, 13], intranasal[10, 14-19], intraperitoneal[10], intravenous[10], and vaginal routes[20, 21]. Intramuscular and intradermal inoculations have become the most effective ways to deliver the DNA vaccines by needle injection. An alternative and very efficient method for intradermal delivery is carried out by particles bombardment with the «Gene gun», which consists on covering gold microparticles with recombinant DNA and shooting them by gas pressure, normally helium, on to the skin[22, 23].

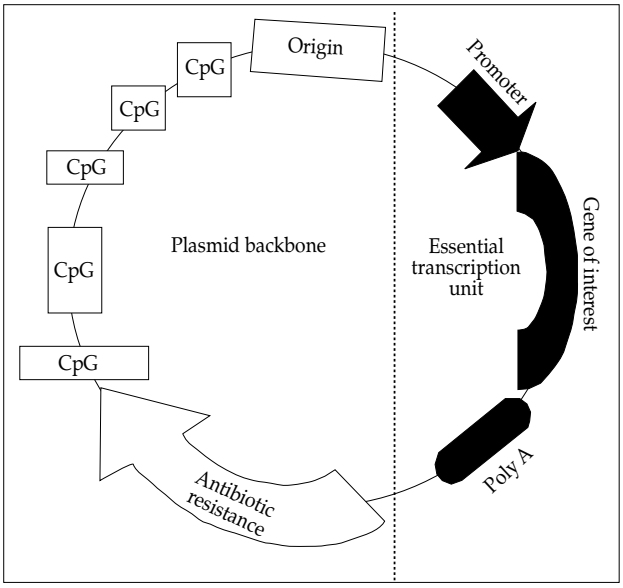

Figure 1. ***Components of a plasmid DNA vaccine.*** *A DNA plasmid consists in several sequences, summarised as the essential transcription unit and the backbone. The essential transcription unit consists on a viral promoter, for example cytomegalovirus, recombinant DNA sequence encoding for the antigen of interest and the required sequences to provide mRNA stabilisation. The plasmid backbone contains other expression cassettes encoding for antibiotics resistance and other sequences necessary for bacterial propagation. As the plasmid backbone contains many CpG motifs, the regulatory influence on the immune response depends on the quantity and sequence of these motifs present in the plasmid.*

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[4, 24, 25]. The capacity of DNA vaccines to induce both humoral and cellular immune responses[24-27] is one of the most important characteristics. 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[28].

IMMUNOLOGY OF DNA VACCINATION

The innate immune response provides a first barrier against many microorganisms and is essential for the control of common bacterial infections[29]. However, there are many pathogens that cannot be recognised or eliminated by the

innate immune system. Pathogens have different lifestyles that require different mechanisms, not only to ensure their elimination, but also for their detection and recognition. The adaptive immune system recognises antigens through two different types of receptors: the surface immunoglobulin of B cells and the antigen receptor of T cells. These surface receptors are adapted to recognise antigens in two different ways: B cells recognise extracellular antigens while T cells recognise intracellular antigens. The intracellular antigens must be displayed as peptides bound to the major histocompatibility complex (MHC) of the presenting cell. The two classes of MHC molecules transport peptides from different intracellular compartments to present them to distinct types of T cells. $CD8^+$ T cells recognise peptides derived from proteins synthesised in the cytosol, bound to MHC class I[30]. Secreted or exogenous proteins undergo endocytosis or phagocytosis to enter the MHC class II pathway to stimulate $CD4^+$ T cells. Activated $CD8^+$ T lymphocytes kill cells showing a determinate peptide-MHC class I complex, early in the microbial life cycle, thereby blocking the replication and spread of pathogens. Activated $CD4^+$ T cells stimulate antibody production by B cells and provide a helper function to $CD8^+$ T cells. The two main subsets of T helper cells, Th1 and Th2, have different functions. Th1 cells activate macrophages and B cells, can act themselves as cytotoxic cells and secrete interferon gamma (IFN-γ) as well as other effector molecules. On the other hand, Th2 cells activate B cells and secrete interleukins, such as IL-4, IL-5, and IL-10, among other molecules[31-35]. The cytokines produced by the T helper cells play an important role in determining the subclass of antibodies generated by the B cells. Thus, the IFN-γ produced by Th1 cells induces the production of the IgG2a isotype in mice while cytokines produced by Th2 cells induce the generation of the IgG1 isotype[36].

All arms of the immune response come into play after DNA immunisation.

ANTIGEN PRESENTATION

The classic ways to present antigens through MHC complexes involve: 1) *de novo* antigen synthesis within the professional antigen presenting cells (APC) to be presented through an MHC class I-dependent pathway to stimulate naïve $CD8^+$ T cells; 2) endocytosis or phagocytosis of the secreted or exogenous proteins by professional APC to enter the MHC class II pathway to process and present antigen to $CD4^+$ T cells; 3) secreted peptides or proteins from somatic cells (or from other origins such as immune complexes or inactivated microbes) can also be processed by APC and presented by MHC class I molecules. This mechanism of processing and presenting antigens is known as cross-priming/cross-presentation.

All these three mechanisms become into action to process and to present the antigen coded by the plasmid to induce an immune response (Fig. 2). This is so because after delivery, the DNA can transfect different cell types and, depending on that, the antigen will be produced and presented differently. The following possibilities have been described: a) direct transfection of somatic cells; b) direct transfection of professional APC; c) cross-priming (somatic cells and/or professional APC are transfected, and the secreted protein, peptide etc, is taken up by other professional APC and presented to T cells).

Direct transfection of somatic cells

The recombinant DNA transfects those somatic cells that are present at the place of inoculation (myocites, keratinocytes, etc.). These cells synthesise the antigen, which undergoes processing in a TAP-dependent pathway to be presented by MHC class I molecules to the $CD8^+$ T cells (Fig. 2.2). Although somatic cells express MHC class I molecules, they do not express the costimulatory molecules (CD80, CD86, etc) required to prime naïve T cells. Therefore, direct transfection of somatic cells alone cannot prime a cellular immune response. This is so because, generally, when a naïve T cell encounters a cell presenting the correct antigen-MHC class I complex in the absence of costimulatory molecules, the T cell is driven to a non-responsive state (anergy), rather than to activation[37]. In fact, it has been shown that surgical ablation of the inoculated tissue within ten minutes of intramuscular injection does not affect the magnitude or longevity of the antibody and the CTL responses[38]. Therefore, antigen production by somatic cells alone seems not to be the main mechanism responsible for initiating an immune response.

Direct transfection of professional APC

Professional APC such as dendritic cells (DC) can be transfected directly at the inoculation site, and migrate to the draining lymph nodes while expressing and presenting the antigen by the MHC class I and II pathways to naïve $CD8^+$ and $CD4^+$ T cells, respectively (Fig 2.3). Keratinocytes and Langerhans cells constitute the major cell types transfected after DNA inoculation in the skin[22, 39]. Casares et al.[40] showed that isolated DC, but not B cells or keratinocytes from DNA vaccinated mice were able to efficiently present antigen to T cells *in vitro*. Torres et al.[38] showed that, contrary to what occurs after muscle ablation, removal of the skin inoculated with DNA up to 24 h after inoculation, prevented the generation of the immune response. This study indicated

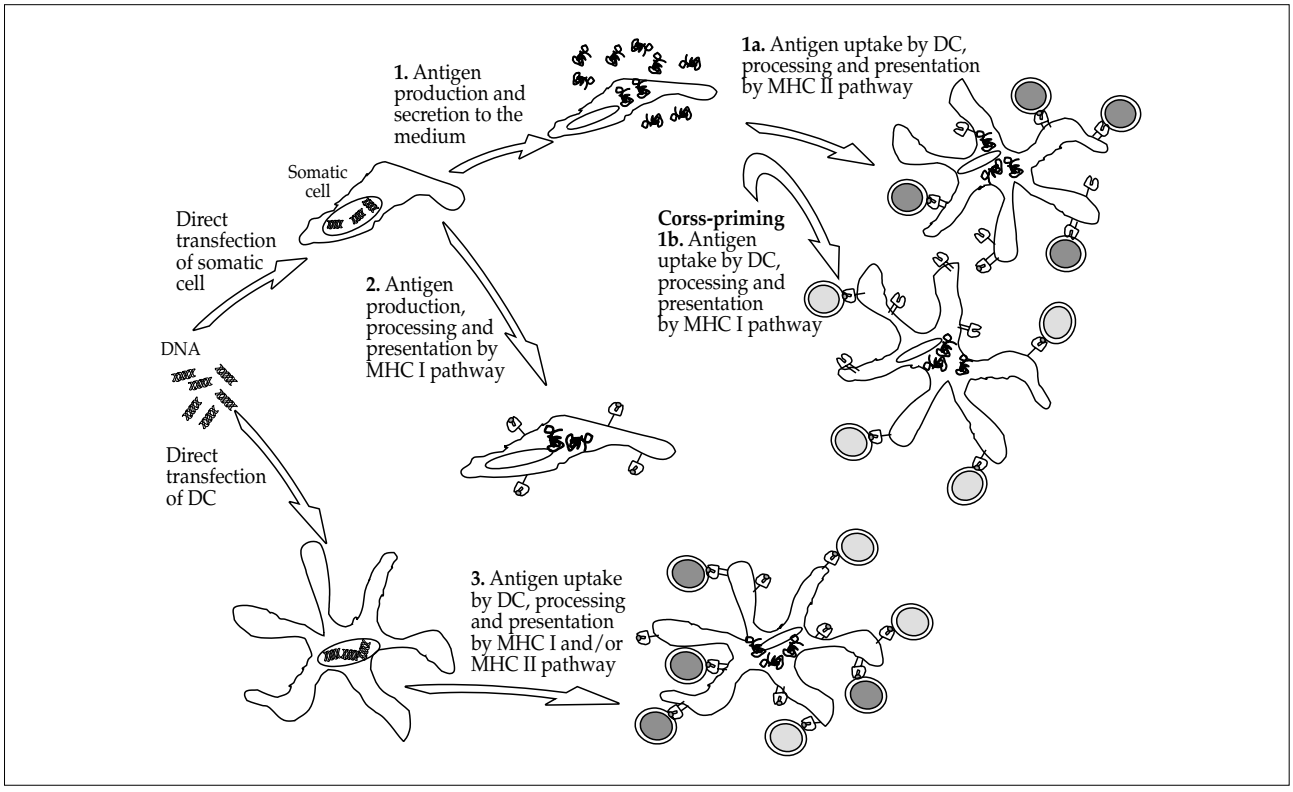

Figure 2. ***Mechanisms of antigen presentation after DNA immunization.*** *Recombinant DNA can transfect somatic cells (such as myocites or keratinocytes), where the antigen will be synthesised and subsequently secreted to the extracellular milieu. DC can uptake the antigen to be processed and presented by MHC class II molecules to naïve CD4+ T lymphocytes (1.a) and/or they can divert it to the MHC class I pathway to be presented to CD8+ T cells by cross-priming (1.b). Transfected somatic cells themselves can also present the antigen via the MHC class I pathway to CD8+ T lymphocytes, but lacking costimulator molecules on their surfaces (2). Another way to synthesise, process and present the antigen is carried out by DC after direct transfection with the injected DNA. The antigen can then be presented by both the MHC class I and II pathways to CD8+ or CD4+ T cells respectively (3). Darked grey circles represent CD4+ T cells and light grey circles represent CD8+ T cells.*

that, after DNA inoculation by gene gun, the migratory transfected cells in the skin play a central role in initiating antibody and CTL responses. Another study showed that transplantation of inoculated skin 12 h post-inoculation could elicit an immune response in naïve animals[41]. By contrast, when the period of transplantation was longer than 24 h, little or no immune response could be initiated. Finally, it was shown that the magnitude of the primary immune response increased when the inoculation site was left intact[41]. These data suggest that cells migrate from the epidermis within 24 h and induce the primary immune response after DNA inoculation, while antigen-expressing nonmigratory cells such as keratinocytes may continue to produce antigen to augment the immune response[41]. Moreover, in some studies it has been shown that DNA inoculation leads to the direct transfection of a small number of DC although large numbers of non-transfected DC are also activated and migrate to the lymph nodes[40, 42-46]. By contrast, Garg et al., have shown that the number of antigen-bearing DC that migrate to the draining lymph node after gene gun inoculation is 100-fold higher than previously estimated and that they persist for approximately 2 weeks[47]. According to these data, it seems clear that transfection of DC plays a crucial role in the induction of the cellular immune response after DNA vaccination.

Cross-presentation

More than 20 years ago, Bevan et al, observed that under certain conditions, exogenous antigens could entry the MHC class I-restricted pathway[48]. It is known now that, in the presence of «danger» signals[49, 50], as it occurs after a viral infection inducing massive apoptosis, DC can present apoptotic cells antigens to $CD8^+$ T cells[51] (Fig 2.1b). This mechanism is called cross-presentation. Even in the absence of «danger» signals, apoptotic cells can stimulate DC maturation and trigger a CTL response[52, 53].

Cross-presentation has since been recognised as one of the most important mechanisms to trigger a CTL response[54-56]. After DNA immunisation, particularly when the antigen is secreted, cross-presentation also plays an important role in triggering CTL responses, as has been reported by many authors[51, 57-63]. In general, somatic cells are the predominant cells transfected after DNA immunisation and therefore the main antigen suppliers. Nevertheless, they are not able to prime an immune response. DC provide the MHC class I-restricting elements as well as costimulation for the CTL response to DNA vaccination. DNA delivery to the skin by either gene gun or needle injection produces physical microinjuries resulting in local irritation, which has been shown to stimulate the recruitment of transfected and nontransfected DC from the skin to the draining lymph nodes[43]. This physical stress associated with invasive DNA delivery acts as a type of immunological adjuvant. The same effect of physical stress would be occurring after inoculation of the recombinant DNA by intramuscular injection, recruiting DC to the muscle. DC can up-take peptides or proteins secreted by transfected myocites and migrate to the draining lymph nodes. So, the presenting antigen mechanism used to induce a CTL response by DC is preferentially cross-presentation. Many studies support this hypothesis. Huang et al.[64] showed MHC class I-mediated presentation by DC of tumour antigens produced by plasmid-transfected tumour cells. Several studies showed that chimeric mice were able to induce a protective humoral and CTL response restricted to the MHC haplotype of the bone marrow-derived APC when stably transfected mioblasts were transplanted[57,58,60,65].

In summary, it is well accepted that professional APC are the main responsible for the priming of an immune response after DNA immunisation even when the transfection rate of APC is very low.

GENERATION OF THE IMMUNE RESPONSE AFTER DNA VACCINATION

CD8+ T cell (cytotoxic T-lymphocyte) priming

An important characteristic of DNA immunisation is its capacity to generate antigens endogenously, making them accessible to CD8+ T cells via MHC class I, either as a result of direct transfection or through cross-presentation by DC (see above)[4]. Because of that, DNA immunisation can compete with other vaccination protocols for diseases where a CTL response is required. Up to date, only live vaccines, but not protein-based vaccines, generate CD8+ CTL responses. When also considering the many safety issues associated to the use of live vaccines, DNA immunisation becomes the most appropriate tool against, for example, intracellular pathogens[28]. Depending on the antigen and the model system used, DNA vaccination can induce CTL responses that are similar in both number and breath to those induced by a live viral infection[66-68]. Like live vaccines, DNA vaccines mimic viral infection by inducing a high frequency of precursor and memory CTL. In different studies, DNA vaccination was shown to induce CTL responses against both dominant and subdominant epitopes, suggesting that they can elicit broad primary and memory responses to multiple epitopes[13, 59].

CD4+ T cell priming

The functions that CD4+ T cells mediate in the immune response are very important: they are responsible for promoting B cell survival and antibody production[69], they provide helper function to CD8+ T cells through costimulatory molecules such as CD40 and/or through the production of IL-2[70-72] and finally, they produce different cytokines required to drive the immune response. For example, Th1 cells produce IFN-g whereas Th2 cells produce, among others, IL-4, IL-5 or IL-10[34, 73]. Whereas the presence of IL-12 induces the differentiation towards the Th1 phenotype, the presence of IL-4 leads to a Th2 differentiation of the CD4+ T cell[73]. DNA vaccines are able to induce both types of Th subsets, depending on the inoculation route used and the nature of the antigen (secreted, transmembrane or intracellular)[74, 75]. While the intramuscular injection predominantly triggers a Th1 response induced by IL-12 production, the intradermal delivery by either injection or particle bombardment with a «gene gun» induces a Th2 response driven by IL-4[76]. One explanation given for why particle bombardment induces a Th2 response is based on the way that DNA is delivered to the cell. Gene gun delivers DNA directly to the nucleus bypassing the interaction of the CpG motifs of the plasmid backbone with their receptors[26]. CpG motifs can lead the immune system to induce a variety of proinflamatory cytokines including IL-12[8, 9]. Therefore, CpG motifs should provide DNA vaccines the capacity of inducing a Th1 response. However, as mentioned above, intradermal injection of DNA also leads to a Th2 differentiation of naïve CD4+ T cells and elicit a humoral response characterised mainly by the production of the IgA and IgG1 antibody isotypes (in mice). The differences in the type of immune response generated by the inoculation route must be attributable to the professional APC network residing in the target tissue and the quantity of DNA administered[77]. In the muscle, there are fewer resident DC than in the skin; therefore more DNA is required to induce the same magnitude of response. This provides more CpG, which would deviate the response towards the

Th1 type. But other explanations are possible. For instance, that the DC involved are functionally different and therefore prime distinct immune responses.

B cell priming

DNA immunisation induces antibody responses to a wide diversity of antigens in different animal models[78-81]. Moreover, the humoral response produced has been shown to be protective in several challenge models[82, 83]. This humoral response peaks 4-12 weeks post-immunisation, as with protein or live virus immunisation[78, 84]. Antibody production is increased in a dose dependent manner, with a single dose or with several doses of DNA and by different inoculation routes[78, 84]. Once the dosage reaches the plateau to induce an immune response the amount of antibody produced is not affected[78, 84]. The antibody isotypes induced by DNA immunisation include IgG, IgM and IgA. In mice, an IgG2a isotype production correlates with a Th1 response, while a Th2 response is correlated with an IgG1 isotype production. Boyle et al.[74] have shown that DNA encoding secreted antigens induces higher levels of IgG1 than that encoding membrane-bound antigens, indicating a Th2 response. So, not only the inoculation route, but also the nature of the antigen can influence the isotype produced. According to other studies, DNA immunisation can induce neutralising antibodies[82]. This demonstrates that the antigen expressed *in vivo* presents a native conformation, because these antibodies recognise conformational epitopes[82, 83].

Long-term memory

A successful vaccine must be able to induce long-term memory. Vaccines based on proteins or live attenuated pathogens induce durable humoral responses. DNA vaccines are also able to induce long lasting humoral responses after either intradermal or intramuscular inoculation[39,66,68,78], although this capacity can be dependent on the type of antigen encoded by the DNA vaccine.

On the other hand, CTL responses were reported by Raz et al.[39] to be present more than 60 weeks after intradermal DNA injection. Later, in a different study, Davis et al.[85] showed that CTL responses could be detected 4 months post-DNA inoculation. Akbary et al.[86] have showed that antigen-specific $CD4^+$ T cells are activated in the draining lymph nodes, by a small number of transfected DC after DNA immunisation, and migrate to the spleen, where they can persist for more than 40 weeks in the absence of antigen. Another study showed that DNA vaccination induced long-term Th1 responses and suggested that DNA immunisation could be more effective at this than a protocol consisting on protein plus adjuvant[87].

The characteristics of cellular memory are reviewed by Seder and Hill[28]. Memory T cells can be divided into two different subsets: «resting» or central and «effector» cells. Effector memory $CD4^+$ T cells readily secrete cytokines, whereas effector memory $CD8^+$ T cells have direct cytolitic activity. Central memory $CD4^+$ or $CD8^+$ T cells need to be restimulated with the antigen before producing cytokines or acquiring cytolitic activity, respectively. There is a big controversy about the constant need of antigen to sustain cell memory. Some reports show that antigen is required to maintain memory cells[88-90] whereas others observed that memory $CD8^+$ T cells can be maintained in an antigen-independent way[91-93]. However, there is strong evidence suggesting that antigen is required to maintain a protective memory response in diseases where $CD8^+$ T cells are important, such as malaria or AIDS[94-101]. Regarding $CD4^+$ T cells, it has been shown that antigen is not required for sustaining memory Th1 cells[102, 103], but for a protective Th1 memory response, as in $CD8^+$ memory T cells, the presence of antigen might be required[104]. In summary, to maintain central memory T cells, antigen is not necessary; however, if long term protection is required, then antigen is indispensable for the maintenance of the effector memory T cells. DNA immunisation allows a continuous and persistent antigen supply and so the persistence of protective immunological memory.

OPTIMISING DNA VACCINES

DNA vaccines have important advantages over current vaccines, but some potential risks have also been proposed (Table I), which could be exacerbated by the large amounts of DNA needed to immunise clinically relevant animals and humans[105, 106].

Most of the potential risks associated with DNA vaccination are being currently assessed in many animal models and Phase I clinical trials. Mutation rates occurring from integration of plasmid DNA into the host chromosomes have been calculated in animal studies and found to be much lower than the spontaneous mutation rate for mammalian genomes[107, 108]. One study conducted in fish[109], demonstrated that the inoculation of DNA could elicit an effective immune response without initiation of autoimmunity or host-chromosome integration. A clinical trial carried out in humans of a therapeutic DNA vaccine for HIV-1 infection, has demonstrated that DNA inoculation induces no local or systemic reaction, no anti-DNA antibodies, and no muscle-enzymes elevation[110, 111]. Another clinical study with a malaria DNA vaccine demonstrated that the intramuscular inoculation of up to three doses of 2.500 µg of plasmid DNA was well tolerated[112].

TABLE I. Advantages and potential risks associated to DNA vaccines

Advantages	Potential risks
Easy to manufacture and deliver, eliminating the need for a «cold chain»	Capacity of the plasmids to persist *in vivo* for months or years could lead to tolerance or even auto-reactivity
Stable and prolonged antigen expression that continuously stimulates the immune system	Stable integration of the recombinant DNA into the genome of the host could lead to the activation or deactivation of some genes such as oncogenes or tumour suppressor genes
Antigen processing and loading into both MHC class I and class II molecules, resembling more closely a viral infection	Encoded bacterial genes, antibiotic resistant genes and other sequences needed only for propagation or production (these genes are expressed even at low rates in the transfected cells)
Elicit qualitatively different immune responses	CpG sequences contained in the backbone of the plasmid can induce a non-specific immune response
Opportunity to manipulate the antigenicity of the protein at the cDNA level	
Possibility for enhancement or modulation of the response to the encoded antigen, when co-delivered with plasmid-DNA-encoded cytokines or co-stimulatory molecules	

However, many efforts have been devoted to improve the efficacy of DNA vaccines. One of the main advantages of DNA vaccination is the ease by which plasmid can be manipulated in order to alter the quantitative and/or the qualitative aspects of the immune response. But there is still room for improvement. Some of the strategies being followed are reviewed below.

Vector optimisation

Although plasmids have been the vectors of choice in most DNA vaccination protocols, some of their features make them unsuitable for a wide use as vaccines: 1) the presence of a long backbone with sequences needed for their expansion in bacteria but not for the vaccine itself (Fig. 1) renders them very inefficient as a vaccine tool; 2) the plasmid backbone also contains one or more antibiotic resistance genes, whose inoculation into either humans or animals of the human food chain may constitute a major safety concern[113, 114]; and finally 3) the unmethylated CpG motifs present randomly in the backbone can be effective adjuvants for some applications, but counterproductive in others[115]. Moreover, the recognition of the CpG motifs by receptors of the innate immune system[116] seems to be species-specific and depends on the surrounding nucleotides[117]. Thus, the delivery of CpG motifs in an uncontrolled fashion, as happens when using plasmids, is undesirable.

Some attempts have been made to develop safer and more efficient DNA vectors for vaccination[118, 119]. However, due to difficulties in large-scale production, it is highly unlikely that they ever progress from the research setting to the clinic. One alternative that is showing very promising results is the use of «Minimalistic Immunogenically Defined Gene Expression» (MIDGE) vectors. MIDGE are linear, covalently closed double-stranded DNA vectors containing all the basic information for gene expression, and none of the non-essential and potentially dangerous plasmid backbone sequences[27, 120]. The covalently closed ends of MIDGE avoid exonuclease degradation of the DNA and provide unique specific sites for the binding of targeting signals, immunomodulatory proteins, etc. MIDGE vectors are linear, not supercoiled, and significantly smaller than plasmids. These features make it easier for MIDGE to go through the different membranes of the cell, and to get integrated into the genome of the host cell (integration into mammalian genomes probably requires DNA lengths of more than 30,000 bp and MIDGE are 1,000-1,200 bp long on average)[121]. The construction of MIDGE vectors is very straightforward, easy to scale-up and allows for chemical modifications, which can be designed to improve uptake, increase expression, modulate the immune response, etc. Thus, the binding of a Nuclear Localisation Signal (NLS) peptide allowed a reduction in the dose of DNA required to induce an effective immune response (both humoral and cellular) and a shift towards the Th1 type after intradermal inoculation[122]. This makes MIDGE-NLS vectors excellent candidates to be used in diseases in which a Th1 response is required[123]. MIDGE vectors are, in general, a safe and efficient alternative to plasmids and even to recombinant viruses for genetic vaccination[123].

Antigen optimisation

It is generally believed that the level of gene expression obtained *in vivo* after DNA vaccination correlates with the strength of the immune response generated. This has led to many efforts to improve antigen expression, such as the use of strong viral promoters, codon optimisation, etc.[26]. However, once the dosage reaches a plateau, the amount of antibodies produced is not affected any more[78, 84]. We[122] and others[124] have also demonstrated an improved immune response without increased antigen expression. Therefore, efforts to improve the efficacy of DNA vaccines should be directed not only to obtain a higher expression of the antigen but also to optimise the form of the antigen itself. Several studies have shown that the type and magnitude of the immune response depend on whether an antigen is secreted, membrane-bound, or intracellular. Secreted antigens induced higher IgG titres than the same antigen localised either intracellularly or bound to the membrane[74, 125-127]. These studies demonstrate that B cells require free or membrane-bound linear determinants or conformational epitopes to initiate the process of clonal expansion for efficient antibody production. The evidence that the nature of the antigen (secreted vs intracellular) can preferentially bias the T-helper response is less clear[74,125-127].

The use of minigenes and multiple epitopes may be advantageous, because a combination of antigenic epitopes can generate a broader immune response than a DNA vaccine coding for a single antigen. One single vaccine could be utilised to induce immunity against a range of pathogens by including epitopes from several different microbes. Several groups have successfully used minimal-epitope vaccines to induce CTL responses[128-135]. Hanke et al.[131] have shown the generation of CTL after immunisation of mice with a novel vector containing a polyepitope construct from the human immunodeficiency virus (HIV) and *Plasmodium falciparum*. Likewise, Thomson et al.[132] have shown the induction of CTL responses against each of the epitopes derived from five separate viruses and parasite epitopes derived from a malarial protein, all encoded in a single plasmid. Furthermore, these CTL were protective after infectious viral challenge. Huebener et al.[136] have shown the induction of protective immunity in a syngeneic murine neuroblastoma model following vaccination with minigenes comprising three novel natural MHC class I ligands.

Finally, the inclusion of target sequences for ubiquitination has also shown to be effective in enhancing the CTL response against the epitopes from the protein to which the ubiquitination signal was fused[137]. However, this approach also resulted in the abrogation of humoral responses and so it is not indicated for infections requiring both arms of immunity.

Cytokines and costimulatory molecules

As mentioned above, cytokines and/or costimulatory cell surface molecules have a crucial role on the activation of T cell subsets as well as in determining the magnitude of the immune response. Because of that, several groups have carried out studies using plasmids encoding different cytokines or costimulatory molecules to enhance or bias the immune response induced by DNA immunisation[26,138,139]. The effects of cytokine-encoded DNA on the humoral and cellular responses are nicely summarised by Gurunathan et al.[26]. They include an increase in antibody production, changes in the isotype, a rise in T cell proliferation and increased CTL responses among others, depending on the cytokine selected for the study. However, as the stimulation induced by these so-called genetic adjuvants cannot be delimited to the desired site, it is difficult to predict if the promising stimulatory results obtained in experimental models will be enough to translate them into clinical applications, particularly in preventive vaccination.

Mucosal immunisattion

Induction of mucosal immunity is one the most important issues in preventing infection, as most pathogens enter the body via the mucosa. A vaccine aimed at protecting from mucosal infections must generate a good mucosal immune response because the systemic response is not enough[140]. Many sexually transmitted diseases lack vaccines. Strong mucosal T cell immunity and IgA antibodies may be critical to provide protection from HIV-1, Epstein-Barr, herpes simplex, and human papilloma viruses. Several studies have been carried out to compare the immune responses elicited by mucosal immunisation with those achieved after systemic immunisation with DNA[15-21]. IgG responses after mucosal immunisation were shown to be comparable to those elicited after systemic immunisation with the same plasmids[17,18,21]. Other groups demonstrated that mucosal immunisation did not lead to an efficient induction of serum IgG responses[15, 16]. Induction of a sustained mucosal IgA response was superior after mucosal immunisation compared with systemic immunisation[17,18 21], but not efficient enough to control viral replication at the mucosal site[141]. The ability of DNA vaccines given mucosally or systemically to induce local T cell responses has not been directly demonstrated. Delayed type hypersensibility (DTH) responses and specific cytolitic activity from splenocytes were comparable between the two routes[17, 18]. Attempts to increase the mucosal immunity induced by DNA vaccination include the use of adjuvants such as cholera toxin[142], DNA encapsulation[143] or complexation with specific proteins[144] as well as the delivery of the DNA inside attenuated enterobacteria[145] or bacterial ghosts[146]. None of these

approaches have yet proven to be effective and/or safe enough to move into the clinic.

Aadministratiion devices and adjuvants

There are several groups pursuing different forms of needleless injection to make DNA immunisation cheaper, easier to administer and painless, both for human and animal use. In animals, as intramuscular immunisation is not very effective and the amount of DNA needed is a limitation, new device systems are required. Although gene gun increases the efficacy, the quantity of DNA administered in a single shot is still a limitation and multiple shots are required to ensure an immune response[106]. But for using the gene gun, hair must be shaved before vaccine administration, making this system labour-intensive and expensive. As few areas of an animal skin are hair less, one of the most suitable sites for DNA vaccines administration are mucosal surfaces of the oral and nasal cavities, leading to mucosal immunity.

A variety of approaches are under evaluation to avoid the traditional needle and syringe commonly used for vaccine administration by several routes, namely intramuscular, intradermal, subcutaneous, intravenous or intraperitoneal routes, in order to increase the efficacy by improving DNA uptake or cell targeting. These include propulsion devices targeting either the mucosa or benefiting from the transfection of Langerhans cells in the skin. A mucosal jet injector device has been utilised in a clinical trial of a therapeutic HIV DNA vaccine[147, 148]. Electroporation devices consisting on the delivery of small electric pulses *in vivo* produce the formation of transient holes in the cells, which permit DNA to enter. These techniques are being evaluated and greatly increase the uptake of DNA into cells and the expression of the encoded proteins[149, 150]. DNA encapsulation in nano or microparticles is another way to enhance the uptake and avoid degradation[143, 151]. Bacteria are also being used as vehicle to introduce DNA into the APC[152, 153].

Conventional aluminium salts are used as adjuvant to increase the potency of DNA vaccines[154]. As successful DNA vaccination involves DC maturation[155], any approach that increases it would result in enhanced immunogenicity. This includes CpG motifs. As described before, unmethylated CpG motifs, present in bacterial DNA, elicit an innate immune response characterised by the production of IL-6, IL-12, TNF-α, and IFN-γ. Evidence suggests that CpG motifs contained in the backbone of plasmid vectors contribute to the immunogenicity of DNA vaccines[8, 9, 156-161]. Recently, Yamada et al.[162] have shown that these sequences can also have an immunosuppressive effect on the immune response. In addition, the immune-enhancing activity of CpG depends on the nucleotides surrounding the motif and this seems to be specific for every species[117]. However, the controlled use of oligonucleotides containing CpG has been shown to enhance the immunogenicity of several types of antigens, including DNA[117].

Mixed-modality protocols

The most successful attempt to increase the potency of plasmid DNA immunisation has been the mixed-modality vaccines. They consist on using a plasmid coding for a particular antigen to prime the immune response, followed by a boosting with another gene-based vector system (such as a recombinant poxvirus or adenovirus) encoding the same antigen. The immune responses and protection are significantly greater than if either vector is utilised alone or the order of administration is reversed[163-165]. This approach has already been used in clinical trials in humans achieving several levels of success[163, 166-169].

APPLICATIONS OF DNA IMMUNISATION

In spite of its use as a preventive tool for infectious diseases, DNA immunisation can also be employed in alternative human immunotherapies for the treatment of diseases such as autoimmunity, cancer, allergy or asthma. The potential capacity to induce Th1 or Th2 responses, depending on the route or the vector used as seen above, make DNA vaccination an efficient approach to deal with the pathogenesis of some autoimmune diseases and allergic reactions. Thus, protective immunity has been induced in an experimental autoimmune encephalomyelitis (EAE) model by using a DNA immunisation method, favouring the induction of a Th2 response[170, 171]. Successful results were also obtained in another autoimmune disease, type-1 diabetes[172]. In contrast, suppression of a Th2 response by the induction of Th1 cells against allergens in an IgE antibody-mediated allergic response has been shown to neutralise the dysregulated production of Th2 cytokines and reduce allergic reactions[173-176].

Regarding cancer, DNA immunisation has proven to be an effective candidate for antitumour therapy. The main difficulty found in cancer immunotherapy relates to the breakage of tolerance against self-antigens present in the tumour cells and activation of a self-directed immune response[177, 178]. Geissler et al.[179] have been able to reduce or inhibit the growth of human tumour cells that produce and secrete a target protein by breaking the tolerance to self-antigens by genetic immunisation with a construct expressing free beta subunit of the human chorionic gonadotropin. Another way to break tolerance has been the use of xenoantigens. DNA immunisation against human melanosomal membrane glycoproteins in mice, generated antibody-

dependent tumour immunity and autoimmune depigmentation or immunity mediated by $CD8^+$ T cells, depending on the glycoprotein used[180].

DNA vaccines are also good candidates to immunise neonates. Newborns are considered not to be appropriate for vaccination because of passive transfer of maternal antibodies and the immaturity of the immune system, which can lead to tolerance instead of immunity. Recent studies in animal models have demonstrated the ability of DNA immunisation to induce an effective immune response in neonates against several viruses[181-184]. In one case, even foetal immunisation into the oral cavity with DNA protected the foetuses from challenge after birth[185].

DNA vaccination is also a good tool for animal immunisation. It is possible to achieve animal protection with DNA alone, but in some cases DNA can be used to prime the animal's immune system and even polarise the induced response by boosting with other kind of vaccines[186]. Since DNA vaccines consist on recombinant genes, they offer the possibility of developing differential diagnostic tests to distinguish vaccinated animals from infected ones, as the immune responses are generated only against the antigen encoded in the DNA vaccine but not against the other antigens of the pathogen[187]. The necessity to discriminate between vaccinated animals from those that have been exposed to the pathogen and could be carriers of the disease is essential in economic and transport business terms. Because the current approach used to eradicate specific diseases by many countries is the slaughter of positive animals for a determinate antigen, such economic cost should be reduced with available differential tests for those animal diseases. Non-tariff trade barriers to control exportation and importation of animals from infected regions could also be carried out[106].

The injection site reactions following immunisation with killed vaccines, where adjuvants are used, are a very important problem in livestock and pets. Such injection site reactions can range from simple granulomas to osteosarcomas in cats[188]. Since DNA vaccines do not need adjuvants, such reactions should be eliminated or reduced, ensuring better meat quality in food-producing animals and the trauma associated with ostesarcoma in pets.

The capacity to immunise neonatal animals with DNA vaccines[189-191] offers the possibility to reduce the «window of susceptibility» where animals do not respond to conventional vaccines because of the presence of maternal antibodies, but they are susceptible to infection. In addition, being able to immunise animals at birth would reduce the cost of immunisation, as large animals can be handled at an early age.

Finally, DNA immunisation can be also utilised as a research tool. As plasmids can be easily manipulated both *in vitro* and *in vivo*, they offer great possibilities in molecular research, providing an easy tool to understand gene function, in terms of proteins. In addition, they are a good and cheap way to produce polyclonal and monoclonal antibodies, because the antigens do not need to be previously purified, they are just produced *in situ* after DNA inoculation[192].

CONCLUSION

DNA vaccination is a simple, safe and cheap alternative for the induction of protective humoral and cell-mediated immunity. This has allowed a very rapid transition for the bench to the clinic. However, the initial enthusiasm was somehow frozen by the realisation that naked DNA alone was highly inefficient at inducing immunity in large animals and humans. The better understanding of the mechanisms responsible for the generation of the immune response after DNA inoculation is leading to the design of more efficacious protocols. It is expected that DNA immunisation will become the treatment of choice for both prophylactic and therapeutic protocols in the very near future. This new generation of vaccines should also be economically accessible to people from all around the world, fulfilling the old ambition of worldwide vaccination exemplified by the Philantropic Vaccine Expedition, which 200 years ago took the smallpox vaccine to America and Asia.

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