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DNA and RNA-based vaccines: principles, progress and prospects

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Abstract

DNA vaccines were introduced less than a decade ago but have already been applied to a wide range of infectious and malignant diseases. Here we review the current understanding of the mechanisms underlying the activities of these new vaccines. We focus on recent strategies designed to enhance their function including the use of immunostimulatory (CpG) sequences, dendritic cells (DC), co-stimulatory molecules and cytokine- and chemokine-adjuvants. Although genetic vaccines have been significantly improved, they may not be sufficiently immunogenic for the therapeutic vaccination of patients with infectious diseases or cancer in clinical trials. One promising approach aimed at dramatically increasing the immunogenicity of genetic vaccines involves making them 'self-replicating'. This can be accomplished by using a gene encoding RNA replicase, a polyprotein derived from alphaviruses, such as Sindbis virus. Replicase-containing RNA vectors are significantly more immunogenic than conventional plasmids, immunizing mice at doses as low as 0.1 μg of nucleic acid injected once intramuscularly. Cells transfected with 'self-replicating' vectors briefly produce large amounts of antigen before undergoing apoptotic death. This death is a likely result of requisite double-stranded (ds) RNA intermediates, which also have been shown to super-activate DC. Thus, the enhanced immunogenicity of 'self-replicating' genetic vaccines may be a result of the production of pro-inflammatory dsRNA, which mimics an RNA-virus infection of host cells.

Keywords

Genetic vaccines; Dendritic cells; Replicase

1. Why use genetic vaccines?

DNA vaccination has become the fastest growing field in vaccine technology following reports at the beginning of the 90's that plasmid DNA induces an immune response to the plasmid-encoded antigen [1,2]. This unexpectedly successful new method is considered by some to be one of the most important discoveries in the history of vaccinology [3,4]. However, DNA vaccination in many cases is hampered by poor efficacy. Thus, as discussed later, various strategies are being developed to improve immune responses induced by genetic vaccines.

In contrast to vaccines that employ recombinant bacteria or viruses, genetic vaccines consist only of DNA (as plasmids) or RNA (as mRNA), which is taken up by cells and translated into protein. In case of gene-gun delivery, plasmid DNA is precipitated on to an inert particle (generally gold beads) and forced into the cells with a helium blast. Transfected cells then express the antigen encoded on the plasmid resulting in an immune response. Like live or attenuated viruses, DNA vaccines effectively engage both MHC-I and MHC-II pathways

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allowing for the induction of $CD8^+$ and $CD4^+$ T cells (reviewed in [5]) whereas antigen present in soluble form, such as recombinant protein, generally induces only antibody responses.

Because genetic vaccines are relatively inexpensive and easy to manufacture and use, their immunogenicity and efficacy have been analyzed in a large number of systems and results from preclinical studies have supported human clinical trials [6,7]. Studies have rapidly moved from small laboratory animals to primates and clinical trials are currently being conducted for diseases such as cancer [8], HIV-infection [9], or malaria [10,11]. DNA vaccine studies for disease-models have intensively been reviewed elsewhere [12-15]. The focus of this review will be on experimental strategies aimed at improving genetic vaccines and in particular on those capable of self-replication.

2. A variety of factors determine the success of genetic vaccination

The quick acceptance of genetic vaccines in experimental settings is due to the many advantages this strategy has over traditional vaccines. However, the efficacy of genetic vaccines in many systems has not proven to be satisfactory, leading some to conclude that genetic vaccines are not a viable alternative to conventional vaccines and will never replace them (reviewed in [16]). Some studies, however, purport that DNA vaccines are more efficacious than some established vaccines based on recombinant proteins [17], recombinant viruses [18], or both [19]. Indeed, DNA vaccines can circumvent many of the problems associated with recombinant protein-based vaccines, such as high costs of production, difficulties in purification, incorrect folding of antigen and poor induction of $CD8^+$ T cells. DNA also has clear advantages over recombinant viruses, which are plagued with the problems of pre-existing immunity, risk of insertion-muta-genesis, loss of attenuation or spread of inadvertent infection [8,20]. Perhaps, the primary goal of genetic vaccines should not be to replace well established conventional vaccines with a good track record, instead the focus should be on diseases for which conventional vaccine approaches are ineffective.

Genetic vaccines can be delivered into the host by several routes and methods. Needle-injection into muscle tissue and into the skin is the most commonly used method [1,21,22]. Also the spleen and a variety of mucosal surfaces [23-25] including those of the nose [26] and gut [27] have been targeted. Scale-up from small rodents to larger animals and humans may not be an obstacle: A given DNA-dose may effectively induce an immune response regardless of body size [28,29]. Despite the large number of genetic vaccine-studies conducted so far, many of the results are difficult to compare and inconsistent. A number of factors determine the magnitude and type of immune response induced by plasmid DNA (summarized in Table 1).

Genetic vaccines may mimic some aspects of the natural infection of host-cells. However, microorganisms contain surface-molecules such as LPS and a variety of soluble factors that function as adjuvants, alerting the immune system to ‘danger’ by inducing inflammation. The potency of genetic vaccines may be significantly enhanced by mimicking these signals with synthetic adjuvants such as QS21 [26] or monophosphoryl lipid A (MPL) [30,31]. However, DNA plasmids without adjuvant are able to induce remarkably strong immune responses to the encoded antigen. In part, this may be due to immunostimulatory sequences within the DNA itself [32,33].

3. Myocytes likely play a secondary role in successful vaccination

Because myocytes are able to take up some of the plasmid injected into the muscle the mechanism of intramuscular immunization seemed very straightforward (Fig. 1). Some reported that this uptake was enhanced when muscle fibrils were recovering from tissue damage induced by the injection of bupivacaine [34] or cardiotoxin [35,36], although such pre-treatment may not be beneficial for all systems [16]. However, muscle is not considered an

immunologically relevant tissue as myocytes lack the characteristics of antigen-presenting cells (APC) such as MHC-II expression, costimulatory molecules or marked cytokine-secretion. Even the co-expression of costimulatory molecules or cytokines like GM-CSF or IL-12 is insufficient to turn non-hematopoetic cells into efficient APC [37]. Successful antigen presentation to naive T cells in vivo by 'non-professional' APC like fibroblasts may be restricted to some viral antigens that are immunogenic enough to overcome costimulatory requirements [38]. The insignificant role of muscle cells in intramuscular DNA-immunization is further supported by a study, in which the surgical removal of the injected muscle within 10 min of injection of DNA-plasmid did not affect the magnitude or longevity of the antibody response to the encoded antigen [39]. Thus, it seems unlikely that muscle tissue is the immune activating component. Indeed, there is little or no local inflammatory infiltrate at the DNA injection-site, especially after the acute effects of the vaccination have disappeared [40].

Two possible scenarios could explain the mechanism of immune-priming by intramuscularly injected genetic vaccines (Fig. 1). First, myocytes are the antigen-factories that supply professional APCs with antigen for the induction of an immune response in the form of full-length protein or peptides [41,42]. Alternatively, resident APCs may be transfected directly and the antigen that is expressed by transfected myocytes is an irrelevant side-product. In either case, the antigen-expressing bone marrow-derived APCs then migrate to lymph nodes where they activate the T and B lymphocytes. Transfected myocytes may also serve as plasmid-depots for continued APC-transfection. Because myocytes expressing the antigen are subject to CTL-lysis [43], plasmid released from these myocytes may be picked up by monocytes migrating through the muscle.

4. Bone marrow derived APC are required for the induction of an immune response after DNA-vaccination

The crucial role of bone marrow-derived APC (BM-APC) has been well established by using BM-reconstituted chimeras [42,44-46]. The migration of plasmid-carrying macrophages from the injected muscle to lymph nodes has been observed. Minute amounts of the immunizing plasmid have been demonstrated in regional lymph nodes and the spleen [47]. In the case of gene gun delivery, only a small number of transfected dendritic cells (DC) can be found in local lymph nodes after the immunization leading some to question whether these few cells are sufficient to induce an immune response [48]. Alternative explanations such as antigen-transfer to DC or antigen-presentation by non-hematopoetic cells are not consistent with data from experiments in which the transfected cells were selectively depleted in vitro, severely impairing the ability of the remaining non-transfected APCs from immunized animals to induce T cells [48]. The crucial role of DC is also supported by the observation that subcutaneous DNA-injection is very inefficient since this tissue lacks Langerhans cells [49].

The role of DC is not unique to DNA-based vaccines. In case of recombinant vaccinia viruses, the strongest immune response is not induced by the construct with a promoter that showed the highest in vitro expression but the construct using a promoter that was active in DC [50]. Likewise, the function of DNA immunogens should be optimized in DC.

5. Intramuscular and intradermal genetic vaccination may employ different mechanisms in inducing immune responses

In contrast to muscle, skin has important immunological functions as it represents the 'first line of defence' of the immune system. Throughout the epidermis, specialized DC form a 3-dimensional network to assure tight immune-surveillance of the skin. Infections agents stimulate DC to pick up antigen and after migrating to the local lymph nodes initiate an immune response [51,52]. The main methods of plasmid-DNA delivery to the skin, by needle injection

or by gene-gun, differ in several respects. While needle injection requires relatively large amounts of plasmid (similar to the 50–100 μg dose used in intramuscular immunization), the amount of plasmid required for gene-gun immunization has been titrated down to a few nanogram [53,54]. As in myocyte-transfection after intramuscular immunization, plasmid can be actively taken up by skin cells [55] but only few cells are transfected after intradermal injection [22].

When delivered by gene-gun, the plasmid solubilizes when the plasmid-coated gold bullet penetrates the cells in the skin. Thus, plasmid is directly deposited into cells transfecting up to 20% of the cells in the target-area [56]. Tissue stress resulting from the blast may contribute to the activation of DC. Indeed, the total number of DC in the skin-draining lymphoid tissue increases enormously after gene-gun immunization, although the majority of these cells do not carry the plasmid. Such massive DC-immigration into lymph nodes could be induced simply with plasmidfree gold bullets demonstrating the ‘adjuvant effect’ of gene-gun immunization [48]. In case of gene gun immunization, an antibody-response to the plasmidencoded antigen has been reported not to occur if the target area is excised within 24 h. There is little or no effect when the excision occurs later [39,57]. This time period may coincide with the time it takes an activated DC to migrate from the skin to the lymph nodes. In contrast to these findings, the removal of the gene-gun immunized mouse-ear immediately after immunization does not abrogate the immune response (Johnston SA, personal communication) most likely because some plasmid can rapidly leave the tissue after it is delivered directly into the bloodstream, possibly transfecting cells in blood vessels close to the surface of the skin.

Larger amounts of plasmid delivered by needle-injection together with increased quantities of immunostimulatory DNA sequences may steer the immune system towards a Th1-type response [58-60]. Indeed, the intramuscular injection of relatively small amounts of plasmid elicits Th2-type responses that are characterized by IgG1 [61]. The small amounts of immunostimulatory DNA delivered with the gene gun may not be sufficient to mediate a Th1-type response allowing Th2-type responses to emerge (reviewed in [61]). It is important to note that needle immunization with large amounts of DNA does not always induce Th1-type responses and gene gun immunization does not always yield a Th2-response [62,63]. By modifying the immunization-regimen, we have shown that either IgG1 or mixed IgG1/IgG2-responses can be induced by gene-gun immunization [63]. Furthermore, a gene gun-induced Th2-type response can be switched to a Th1-type response by co-delivering the genes for IL-2, -7 or -12 [64] (Fig. 2).

6. Enhancing the efficacy of genetic vaccines

A large number of approaches have been used in an attempt to improve the often poor efficacy of DNA vaccines. Because the efficacy of DNA vaccines in many systems has not been satisfactory, the most simple and unexpectedly effective strategy is increasing the intervals between immunizations and, thereby, the ‘rest-period’ of the immune system [63,65]. In addition, many elements of the plasmid can be optimized for use of the vector as a DNA vaccine [66]. Based on the idea that more antigen is better, most DNA vaccines use strong viral promoters and are geared towards maximum expression. Other sequences that can be optimized in a plasmid include introns, enhancers and poly-adenylation signals [67-69].

6.1. Antigens can be modified to make them better immunogens

To improve the immunogenicity of an antigen encoded by a genetic vaccine, functional sequences like the intracellular domain or the trans-membrane sequence can be eliminated [70]. Antigens can be targeted to the Class-I or Class-II processing pathways with the addition of sequences designed to direct intracellular trafficking. Finally, immunodominant epitopes from antigens can be expressed as minigenes, or they can be buried within unrelated, but highly

immunogenic core-sequences [71]. This may be especially useful in cases where 'full-length' proteins are not suitable as vaccine candidates, because they are toxic for the host [61] or immunosuppressive [72]. Antigenic proteins can be maximally truncated, leaving only defined epitopes for B or T cells. Antigens consisting of $CD8^+$ T cell-epitopes alone are sufficient to induce CTLs as shown with an ova-epitope in vaccinia [73] and this approach has also successfully been used for $CD4^+$-T cell epitopes [55]. To overcome MHC-restriction of individual epitopes or to induce a broader range of effector-cells, it is possible to deliver multiple contiguous minimal epitopes in form of a 'polytope'. This approach has successfully been used for vaccinia as a carrier [74] as well as for an analogue DNA-construct [75]. To improve MHC-I-loading, endoplasmic reticulum (ER) insertion signal sequences can be attached to minigenes. These sequences can facilitate the targeting of the antigen to the ER, where MHC-I molecules are complexed with antigen. This approach was pioneered in the vaccinia system [76] and also works for peptide-immunization [77]. An adenovirus leader-sequences has successfully been used to target DNA vaccine encoded $CD8^+$ T cell epitopes to the ER [71].

Helper epitopes, such as the hepatitis B core-antigen, can activate B cells and elicit strong T cells responses adding significantly to DNA-based vaccines against hepatitis [78,79]. In another example, a short sequence of a malaria antigen encoding a known helper-epitope (*P. berghei* CSP57-70) with a nested and previously unidentified H2-K^d $CD8^+$ T cell epitope (PbCSP58-67) was engineered into a DNA plasmid together with the hepatitis core antigen-sequence. When this plasmid was delivered as a DNA vaccine by gene gun, we were able to induce the highest level of protection seen with any PbCSP-DNA vaccine tested (Lyon JA, Leitner WW, unpublished observation).

6.2. Exogenous cytokines can enhance or direct the immune response

Cytokines can significantly improve vaccine-induced immune responses, accelerating and augmenting it as well as directing it, for example, towards a Th1- or Th2-type response [80, 81]. In the case of cancer, adjuvant cytokines are especially important, because tumor-associated-antigens are generally of poor immunogenicity. Irvine et al. demonstrated that gene gun delivery of a model antigen was protective by itself, but was only therapeutic when co-delivered with IL-2, IL-6, IL-7 or especially IL-12 [82]. Other useful cytokine adjuvants include GM-CSF, a cytokine thought to recruit and mature dendritic cells [83]. Besides using exogenous factors, cytokines [84] as well as chemokines [85,86] encoded on plasmid DNA or as cDNA have been used to study, modulate or enhance a DNA vaccine induced immune response. One study shows the conversion of a non-immunogenic antigen into a DNA vaccine by fusing it to the genes for chemokines [87].

6.3. DNA-encoded costimulatory molecules can enhance APC-functions

To develop a T cell response, APCs have to deliver two signals to the T cell: one signal is from the MHC/peptide complex to the T cell receptor, the second is from a costimulatory molecule, of which B7 is perhaps the most important and best characterized. In the absence of costimulation, T cells may become anergic preventing self-reactive cells from producing auto-reactivity. B7.1 and B7.2 are expressed on professional APC and on a variety of other tissues after exposure to inflammatory cytokines [88]. Transfection of tumor cells with either isoform has successfully been used to overcome the poor immunogenicity of tumor cells [89-91]. Both molecules bind CD28, the constitutively expressed T cell ligand, as well as CTLA-4, an inducible ligand expressed upon T cell activation. There is an ongoing controversy over how these two B7-molecules differ functionally. Blockade of B7.1 has been shown to abrogate primary and secondary antibody responses, while B7.2 blockade suppressed only primary responses [92]. The differential expression of B7.1 and B7.2 on APC could result in differences in the T helper cell phenotype [93]. Alternatively, some investigators challenge the role of B7

as a costimulatory molecule altogether and describe B7.1 and B7.2 as 'late' triggers of IL-2 production and T cell survival that are not involved in the initial T cells stimulation [94,95].

The use of B7 for DNA-immunization has shed some light on the differential roles of the two isoforms. Interestingly, co-immunization with the B7.2-gene yielded better results in some studies while B7.1 codelivery had little beneficial effect [37,96]. We, however, observed that co-delivery of B7.1 with a malaria antigen (*Pb*-CSP) by gene gun significantly increased the protective effect of a low-expressing plasmid, but not of a high expresser plasmid (Lyon JA, Leitner WW, unpublished observations). The enhanced efficacy was strongly dependent on the immunization interval and, surprisingly, repeated B7.1 co-immunization was suppressive when used with longer intervals. Importantly, enhanced immune responses are observed in some studies only when the two genes are encoded on the same plasmid, suggesting that the antigen-specific and costimulatory signals must emanate from the same cell [84].

6.4. Bacterial DNA-sequences called immunostimulatory sequences can be potent adjuvants

Non-methylated, palindromic DNA-sequences containing CpG-oligodinucleotides (CpG-ODN) can activate an 'innate' immune response by activating monocytes, NK cells, dendritic cells and B-cells in an antigen-independent manner (immunostimulatory DNA sequences, ISS). Indeed, methylation of the CpG-ODN reportedly abrogates the immunogenicity of the DNA vaccine [97]. Thus, the use of large amounts of plasmid for immunization may not only overcome the low transfection eficiency in vivo, but may also serve as an adjuvant, driving a Th1-type response (reviewed elsewhere [98]). Immunostimulatory DNA sequences may activate skin-derived DC in vitro and in vivo [99]. Because resting DC are weak APCs, this activation may be critical for successful plasmid-vaccination [100,101]. CpG-ODN have been reported by one group to be as effective an adjuvant as Complete Freund's Adjuvant and to be without significant toxicity [33]. In the case of DNA vaccines they can either be co-administered with plasmid-DNA in the form of oligonucleotides or the number of ISS on the plasmid-backbone can be increased. Limited information is available so far about the species-specificity and restrictions of the DNA-sequences outside the central CpG-ODN [97]. Nevertheless, the study of the immunostimulatory properties of DNA may be an important area of research because of the limited number of adjuvants for use in humans (reviewed in [102]).

6.5. Heterologous prime-boost-regimens can enhance the efficacy of genetic vaccines

The delivery of the same antigen multiple times using carriers with little or no immunogenic crossreactivity (heterologous prime-boost-regimen) provides several advantages over the repeated delivery of an antigen with the same carrier (homologous boosting). The repeated use of any given recombinant virus-based vaccine may be impaired by anamnestic responses to the carrier itself [8]. Including DNA in these regimens may also shift the response towards Th1, even when a Th2-type response was initiated with recombinant protein [103]. Heterologous boosting yielded full protection in the *P.berghei* malaria model when plasmid immunization was followed by administration of recombinant vaccinia virus. Homologous boosting was weak or ineffective in this model [104,105]. Similarly, Irvine et al, reported greatly improved immune responses and tumor treatment when delivering a model tumor antigen by gene gun, cowpox or fowlpox in a heterologous prime-boost-regimen [106].

The order of carriers used in a heterologous primeboost-regimen may be important. Going from the antigenically simpler vector in the priming, to the antigenically more complex (and potentially more powerful) vector in the boosting may help the immunotherapist to focus the immune response. For example, priming with DNA-priming and boosting with recombinant vaccinia virus was the only effective regimen in some studies [104,105]. This might be due to viral products that are immunosuppressive such as soluble, secreted cytokine receptor homologues, the expression of which could interfere with immune priming, especially to weak

antigens [107-110]. Furthermore, epitopes derived from viral vectors can be immunodominant over weak antigens. Such interference might have less impact on a secondary response that had been primed with a DNA vaccine. Finally, in humans, pre-existing immunity to some viral vectors might add another level of complexity to the outcome of heterologous prime-boost strategies.

7. Self-replicating genetic vaccines have some clear advantages over conventional vectors

'Self-replicating' genetic vaccines are designed to overcome the poor efficacy of some current DNA-based and RNA-based genetic vaccines. The idea and the elements for this new generation of vaccines come from members of the Alphavirus genus, which includes Sindbis virus, Semliki Forest virus (SFV) and Venezuelan equine encephalitis (VEE) virus. These RNA viruses contain a single copy of positive-stranded RNA encapsidated by a protein/lipid envelope. The viral RNA encodes its own RNA replicase, an autoproteolytic polyprotein that cleaves itself into four non-structural protein components (nsP1-4) [111,112]. Upon infecting a cell, the viral RNA first translates the replicase complex, which in turn drives its own RNA replication. The replicase complex then synthesizes a genomic negative-strand (anti-sense RNA), which is used as a template for the synthesis of the genomic positive-stand RNA as well as a subgenomic RNA encoding the structural viral proteins (Fig. 3). The genes for structural proteins can be replaced with the gene for the antigen of interest to construct powerful replicase-based vaccines [113].

Theoretically up to 200,000 copies of RNA can be produced in a single cell within 4 h and expression of the encoded antigen can be as much as 25% of total cell protein [114]. The alphavirus replicase functions in a broad range of host cells (mammalian, avian, reptilian, amphibian and insect cells) [115]. Replication takes place in the cytoplasm of the host cell and, therefore, is independent of the host's replication system. All the above features, i.e. high level expression, broad host range and cytoplasmic replication, are useful features in genetic vaccine development.

To facilitate vaccine production, genomic alphavirus RNA alone can be used as a vaccine vehicle. The in vitro transcribed self-replicating RNA contains sequences coding for the SFV replicase and a model antigen. A single intramuscular injection of a self-replicating RNA elicited antigen-specific antibody and $CD8^+$ T cell responses and was shown to be significantly more effective than non-replicating RNA [115,116]. DNA-based vaccines can also be constructed by inserting a strong promoter like the human CMV immediate promoter/enhancer element to initiate the transcription of the full length 'genomic' RNA in the nucleus [112, 117-119]. Replicase-based DNA vaccines may be significantly more immunogenic and efficacious than conventional DNA-plasmid vaccines when low doses of the vaccine are given. Indeed, nanogram amounts of replicase-based vaccine can induce antigen-specific antibody and $CD8^+$ T cell responses [118-120] (and Leitner WW et al., in preparation).

8. Mechanisms that could account for the high efficacy of self-replicating genetic vaccines

A major rationale for putting antigen-coding genes under the control of the alphaviral RNA replicase was to enhance antigen expression and presentation. Unexpectedly, the level of antigen expression of replicase-based constructs in vitro was not necessarily higher than that obtained with conventional DNA or RNA-vectors [118,120] (and Leitner WW et al., manuscript in preparation). The discordance between antigen expression level and the increase in immunogenicity suggests that other mechanisms are involved such as those illustrated in Fig. 4. A fundamental difference between replicase-based DNA vaccines and conventional

DNA vaccines is the virus-like RNA-replication inside transfected host. Transfection of host cells with replicase-based genetic vaccines could trigger a series of 'danger signals'[121].

Replicase-based DNA or RNA induce apoptotic death of the host cell in vitro just as alphaviral infection induces apoptosis in host cells [116,122]. These apoptotic cells may be picked up by dendritic cells for presentation to the immune system [123]. Transfection with self-replicating genetic vaccines may also cause the production of heat shock proteins in transfected or bystander cells [124]. The activity of the viral replicase may provide a powerful adjuvant-effect because of the requisite production of double stranded RNA (dsRNA) intermediates (Fig. 5). dsRNA itself is a potent inducer of the interferons and virus-derived dsRNA can function as a strong adjuvant for cellular and humoral immune responses [125]. Several molecules are known to bind to and can be activated by dsRNA. The best characterized are 2′–5′ oligoadenylate (2-5A) synthetase and protein kinase-RNA activated (PKR). The 2-5A system contributes to the antiviral effect of the interferons through the synthesis of 2-5A and its activation of RNAse, which degrades both viral and cellular RNA. PKR-expression both induces and is induced by the interferons. PKR is then activated by dsRNA to phosphorylate its substrates, including eIF2. This results in the inhibition of translation, further diminishing viral replication. The cellular death observed in response to dsRNA is likely to be mediated by both the 2-5A system-induced RNAse as well as some substrates of PKR [126,127]. INF-γ potentiates the apoptotic effects of dsRNA [128].

The mediators involved in the apoptosis of virally infected cells are subject to viral and cellular inhibitors, like vaccinia E3L [127], HIV-1 Tat protein [129] or the cellular P85IPK [130]. Inhibitors encoded by viruses allow efficient viral replication despite the host cells defense mechanisms. Although apoptosis of cells transfected with self-replicating genetic vaccines might contribute to their enhanced immunogenicity, their efficacy might be limited by the rapid death of host cells due to the absence of vaccine encoded apoptosis-inhibitors that delay the apoptotic death. The manipulation of apoptosis is likely to be a rich area of exploration for vaccinologists.

9. Conclusion

Ideally, a vaccine should be: safe, highly immunogenic, non-integrating, easy to manipulate, genetically stable and inexpensive to produce. In addition to these features, a therapeutic vaccine must not be compromised by pre-existing immunity of the patient against the vaccine vehicle. While 'conventional' DNA vaccines are frequently hampered by low efficacy, replicase-based vaccines may significantly improve efficacy. 'self-replicating' genetic vaccines may be effective in the fight against diseases that have so far successfully resisted conventional vaccination strategies using recombinant proteins, viruses or bacteria.

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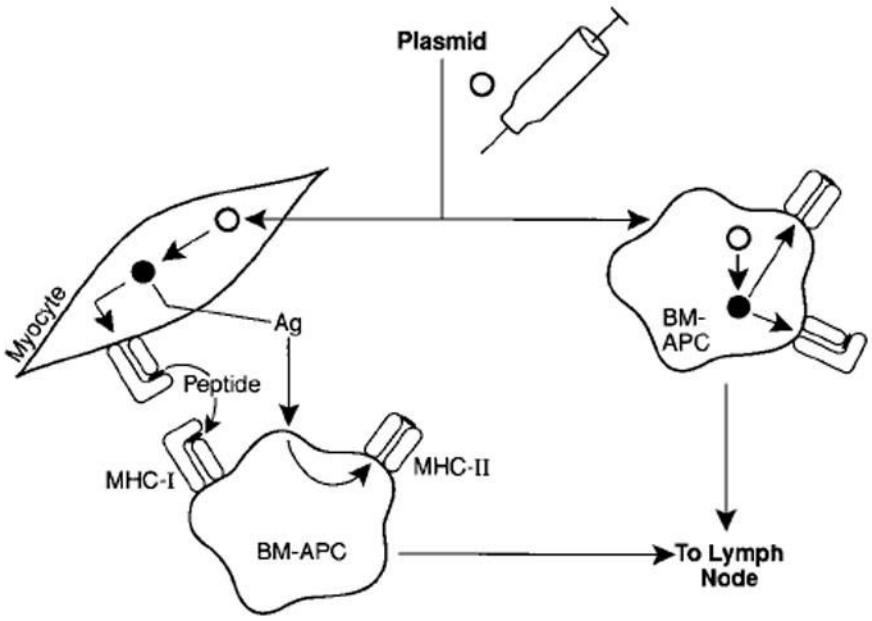

Fig 1. Transfection of host cells with plasmid DNA

Plasmid (○) is actively or passively taken up by host cells. Antigen (v) produced by transfected myocytes can be taken up by bone marrow (BM)-derived APCs. Alternatively, BM-APC can be transfected directly. Antigen-bearing APC then can process and present peptides complexed with MHC-molecules to the immune system after migrating to lymphoid tissue.

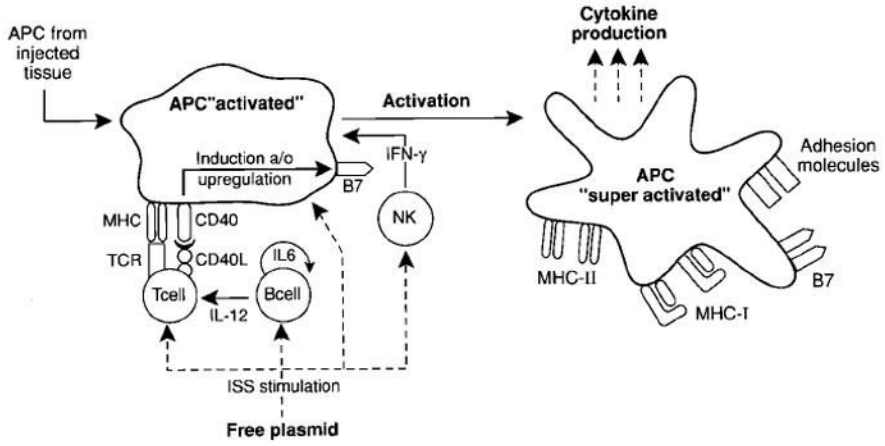

Fig 2. 'Super-activation' of APC after vaccination with genetic vaccines

APC are initially stimulated in the injected tissue by either needle or gene-gun delivery of plasmid. Immunostimulatory sequences (ISS) stimulate host APCs, T cells and NK cells. APC interact with T cells through both TCR/MHC and CD40/CD40L, triggering the upregulation of B7 expression on the APC. ISS also polyclonally stimulate B-cells and induce the release of IL-6 and IL-12, further promoting a Th1 response.

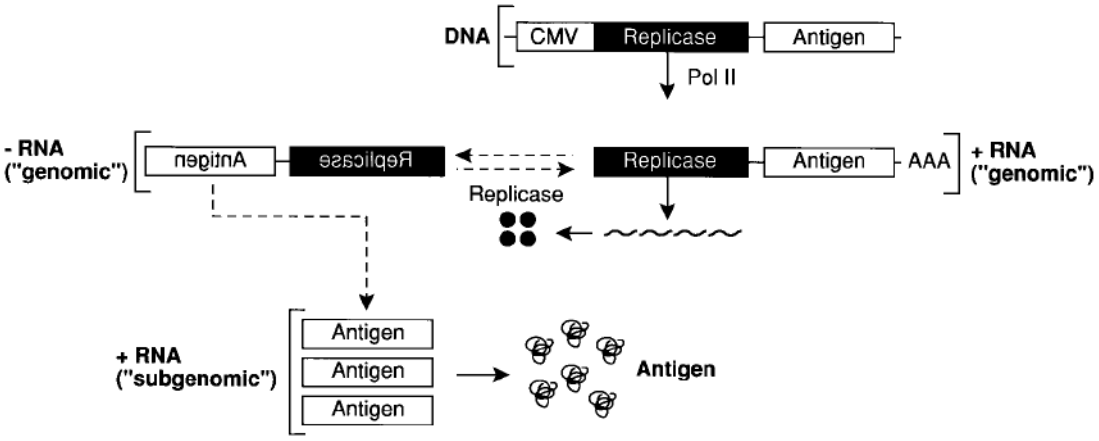

Fig 3. Self-replicating genetic vaccines

The first product of the self-replicating RNA is a four-subunit-replicase which uses the (+) strand RNA as a template to make (−) strand RNA and more copies of full length (+) strand 'genomic' RNA and (+) strand 'subgenomic' mRNA for the encoded antigen. Due to the high number of RNA-copies, the main product of the transfected cells becomes the encoded antigen. The host cell eventually undergoes apoptosis.

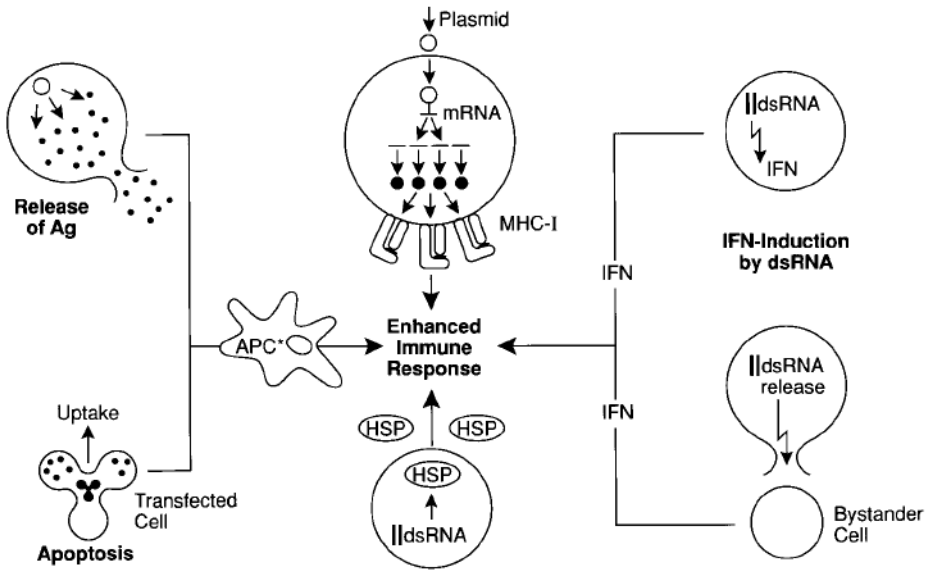

Fig 4. Potential factors contributing to the high immunogenicity of self-replicating genetic vaccines

(Starting in the upper centre and moving clockwise): Accumulation of antigen in the transfected cell can result in highly efficient MHC-I-loading. A number of 'danger signals' may be generated such as interferon production and interferon release from infected cells resulting from the presence of dsRNA. Interferon may also be produced by bystander cells in response to dsRNA released from dead and lysed transfected cells. Heat shock proteins (HSP) have also been shown to be produced in response to the presence of dsRNA in the cells. Ingestion of antigen-loaded apoptotic cells by APCs can also result in the elicitation of powerful immune responses. Finally, the local release of large amounts of antigen at the site of injection by transfected cells may be fed into resident APC.

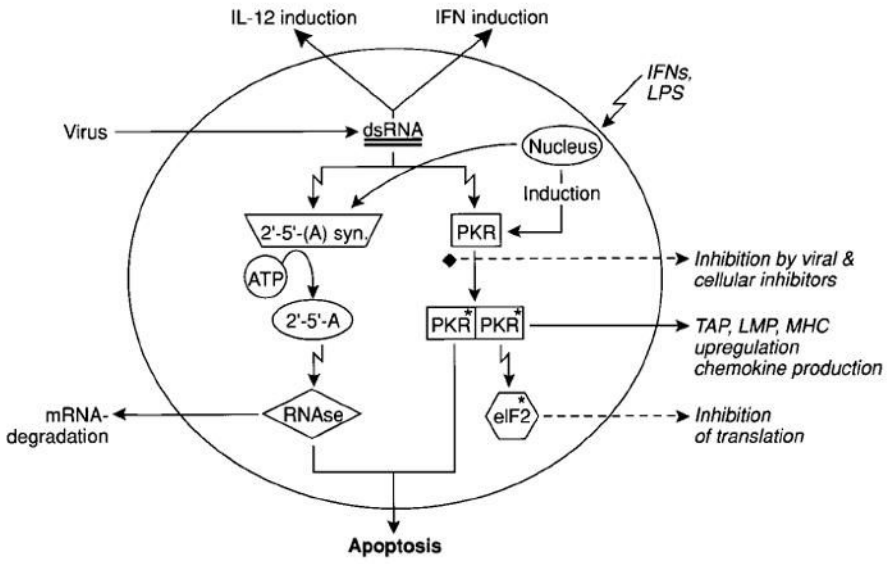

Fig 5. dsRNA might be a central element in the immunogenicity of replicase-based genetic vaccines

dsRNA is produced in cells infected with RNA-viruses, but is also expected to be an intermediate in the replicase-mediated duplication of mRNA after delivery of 'self-replicating' genetic vaccines. dsRNA is a potent inducer of interferon, which in turn induces the expression of PKR and 2-5A synthetase. These enzymes are activated by dsRNA and mediate at least some of the cellular effects associated with viral infection, including apoptotic cell death, aimed at preventing viral spread. The 'danger signals' resulting from the activation of these two enzymes could account for the increased immunogenicity of replicase-based genetic vaccines, providing an adjuvant-effect.

Table 1

Factors determining the immunogenicity of genetic vaccines

Factors affecting the immunogenicity of genetic vaccines	**[[Comments and conclusions|]]**	**[[References|]]**
Structure of the plasmid backbone	Presence of immunostimulatory sequences, introns, poly-A-sequence	[60,67,69,98,99,131-133]
Amount of plasmid delivered	[[More is better|]]	[28,61,134-136]
Expression levels of the antigen	More antigen correlates with stronger response, but not necessarily linearly	[61,63]
[[Immunization schedule|]]	Increasing interval between immunizations can strongly enhance the response	[63,137,138]
[[Route of immunization|]]	Intramuscular, intradermal (needle), epidermal (gene gun), mucosal	[61,63,136,139,140]
[[Target-tissue|]]	Including what muscle is injected or what section of the skin	[39,136]
[[Number of immunizations|]]	DNA-induced immune response can effectively be boosted with DNA	[63,141,142]
Presence or absence of introns in front of the gene	[[Introns increase efficacy|]]	[61,103]
Strain of the particular species	Different mouse strains show qualitative and quantitative differences in DNA-induced immune responses	[61] (and Leitner WW, Lyon JA, unpublished)
[[Age of animals|]]	Stronger response the younger the mice	[61,68]
Toxicity of the antigen for transfected host cell	High expression undesirable for toxic antigens	[61]