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REVIEW

DNA vaccines for HIV: challenges and opportunities

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Abstract In December 2005, the UNAIDS and WHO reported that the global epidemic known as acquired immunodeficiency syndrome (AIDS) has claimed the lives of more than 25 million adults and children over the past 26 years. These figures included an estimated 3.1 million AIDS-related deaths in 2005. Despite enormous efforts to control the spread of human immunodeficiency virus (HIV) new infection rates are on the rise. An estimated 40.3 million people are now living with HIV, including 4.9 million new infections this past year. Nearly half of new HIV infections are in young people between the ages of 15 and 24. While drug therapies have helped sustain the lives of infected individuals in wealthy regions, they are relatively unavailable to the poorest global regions. This includes sub-Saharan Africa which has ~25.8 million infected individuals, more than triple the number of infections of any other region in the world. It is widely believed that the greatest hope for controlling this devastating pandemic is a vaccine. In this review, we will discuss the current state of DNA-based vaccines and how they compare to other vaccination methods currently under investigation. We will also discuss innovative ideas for enhancing DNA vaccine efficacy and the progress being made toward developing an effective vaccine.

Keywords Genetic vaccines · CTL responses · AIDS vaccines · Vaccination · Immune therapy

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Difficulties in HIV vaccine development

The development of effective human immunodeficiency virus (HIV) vaccines and immunotherapies has been an elusive goal ever since the virus was first identified. There are several contributing factors that have resulted in the failure to generate a protective or therapeutic HIV vaccine. HIV is highly variable due in part to the two errors made per replication cycle by reverse transcriptase during the replication process. This high mutation rate facilitates the virus in evading the adaptive cellular and humoral immune response. Furthermore, the cumulative interaction of the Vif accessory gene with the Apobec-3G host cell protein dramatically increases viral variability generating twenty five additional point mutations per replication cycle. Exposed parts of the envelope can mutate up to 35% of their amino acids without losing their function, allowing for a large variety of mutations that can act as decoys for immune responses. Traditional vaccines rely on the generation of neutralizing antibodies to confer protection. However, the mutability of HIV is such that neutralizing antibodies effective against one virus may be ineffective at neutralizing viruses from another individual or other viruses within the same person. It is interesting to note that the emergence of viral mutants within an infected individual that escape recognition by neutralizing antibodies does not correlate with a decline in health or disease progression. In contrast, viral mutations that allow the virus to escape detection by cytotoxic T lymphocytes (CTLs) correlates with a decline in health characterized by a decrease in immune function and an increase in viral replication. These data suggest that antibodies play only a limited role in viral control post infection. The results of this study are similar to findings in primate models of lentiviral infection. Depletion of $CD8^+$ cells in infected monkeys with

undetectable viral loads results in rapid SIV (simian immunodeficiency virus) expansion that is once again suppressed when the $CD8^+$ T cells rebound [3]. Together, these data support that CTLs play a critical role in the control of HIV replication. CTL responses are able to target internal epitopes that are not accessible to antibodies, resulting in the destruction of infected cells. This is an advantage for targeting a highly variable virus as it allows for more possible immune targets through the CTL system. However, HIV is able to eventually evade these CTL responses through incorporation of new mutations, resulting in the selection of escape variants that are not recognized and survive. The selective pressures of both antibody and CTL responses keep the virus in a continual state of change while the immune system is undermined through the destruction of $CD4^+$ T cells both by direct lysis due to infection and through the destruction of infected cells by CTL responses. In addition, the roles of several of the accessory genes of HIV in immune modulation and corruption likely play in favor of the virus. High mutation rates have also resulted in the development of many distinct genetic subtypes reinforced by regional boundaries known as clades. Therefore, a vaccine that may prove effective at protecting against one clade may be ineffective against another due to divergence.

The infection pattern of HIV is likely to play a role in hindering the development of effective immunotherapies. The first cells believed to be infected by HIV are dendritic cells (DCs). DCs are professional antigen presenting cells (APCs) that stimulate both $CD4^+$ and $CD8^+$ T cell-mediated immunity [10, 92]. These specialized APCs are able to present exogenous cell-associated antigens through both the MHC class I and MHC class II pathways, a process termed cross-presentation [24, 43, 53, 54, 63]. DCs orchestrate innate and adaptive immune responses through the secretion of cytokines such as type-1 interferons, IL-4, and IL-12p70 in response to "danger signals" encountered in the periphery, known as "signal 3" [30, 49, 59]. After infection, DCs migrate to draining lymph nodes in the gut where HIV is then free to infect and deplete $CD4^+$ T-cell populations that are critical for the development of optimal humoral and CTL-mediated immune responses [17]. Recent evidence demonstrates the ability of chronic infection to suppress cross-presentation in dendritic cells through the continual stimulation of Toll-like receptors (TLRs), inhibiting the induction of immune responses [103]. While this mechanism of immune suppression has not been tested in HIV infection, it may play a role in hindering immune responses especially in the context of immunotherapy for HIV-infected individuals.

In order for a vaccine to be effective on a global scale, it must elicit CTL responses directed against conserved regions of HIV. Furthermore, immune responses directed against single epitopes increase the likelihood that the virus will be able to mutate and escape immune recognition. Therefore, the breadth of the immune response will likely be as critical to developing an efficacious vaccine as the type of immune response that is elicited.

Traditional vaccines rely on the production of antibodies through the injection of live attenuated virus, killed viral particles, or recombinant viral proteins. Killed viruses and recombinant proteins tend to elicit humoral immune responses directed toward variable regions of the virus, rendering them ineffective. Live attenuated viruses are better able to induce both humoral and cell-mediated immunity than their nonlive counterparts. However, selectively passaged or even genetically constructed attenuated lentiviruses can revert to a more pathogenic state, eliminating this mode of immunization as a central platform. Relatively recent advances may facilitate the development of more effective attenuated vaccines while maintaining a high level of safety, but these remain years away from clinical evaluation.

One important focus has been the use of recombinant viral vectors. This approach has the ability to induce both humoral and cell-mediated immunity. Adenovirus as a recombinant platform, in particular, has been quite effective at eliciting both humoral and CTL-mediated immune responses whether used alone or as part of a heterologous prime-boost regimen. Importantly, in human clinical studies, these are the most potent recombinant viral platforms for inducting CTL responses against HIV. However, there are aspects of adenovirus-based vaccines that could be improved to make them more optimal as a platform for HIV vaccines. (1) Adenoviral vectors elicit potent immune responses directed against vector proteins that may inhibit attempts to boost with a homologous virus; therefore, new recombinant strains could be developed to avoid this issue. (2) A significant portion of the population has preexisting immunity to adenovirus; therefore, the development of primate adenoviral vectors may be important in this study. (3) Production, storage, and shipment of recombinant viral vectors would likely require the presence of an intact cold chain for vaccine handling. Improvements in formulations and storage are important to development. Advances in these areas will further improve this platform as a stand-alone strategy or as part of a prime boost platform with DNA or other vector systems.

DNA-based immunizations offer conceptual advantages compared to recombinant viral vectors. Like viral vectors, they have been shown to elicit strong humoral and cell-mediated immunity in vivo in small animal models. Unlike viral vectors, they can be boosted in theory infinitely, and there is no issue with preexisting serology. DNA-based vaccines are inherently conceptually safe because of the absence of a live vector and simple quality control issues.

In addition, they are easily stored and transported in a lyophilized form breaking the requirement for a cold chain. However, their major drawback is that compared to recombinant adenoviral vectors, the best DNA vaccines are 1/2 as immune potent. Improving their potency is a now a major focus (Table 1). Various adjuvants can also be encoded in the DNA vaccine to provide additional immune stimuli. Considerable effort is now being invested in enhancing the efficacy of DNA vaccines in humans and nonhuman primates. A doubling in their immune potency would make DNA vaccines a very useful preferred vaccine platform.

One of the more popular methods of immunization involves the use of multiple strategies in a heterologous prime-boost regimen. While immunization with DNA vaccines alone has had limited immune potency, priming with 2–3 rounds of DNA immunizations followed by a viral vector boost has been demonstrated to generate enhanced immune responses over that seen with homologous viral vector prime-boost regimens alone [76]. Researchers have reported long-term (greater than 200 weeks) viral control and maintained immune function in SHIV- (a hybrid virus composed of an SIV core and an HIV envelope) infected animals immunized using the DNA/viral vector prime-boost regimen prechallenge [74]. Recent results demonstrate the use of a heterologous prime-boost regimen consisting of three DNA immunizations encoding SIV*gag/pol* followed by immunization using a replication deficient vaccinia virus expressing SIV*gag/pol* induced higher levels of IFN-γ-secreting T cells and neutralizing antibodies against SIV resulting in better control of viral loads and higher $CD4^+$ T-cell counts compared to DNA or recombinant viral immunizations alone [90]. In a separate study, DNA and a replication deficient Ad5 vector, both encoding SIV*gag*, also demonstrated greater induction of SIV-specific T cells and better control of viral replication [26].

The addition of protein to heterologous prime-boost regimens has also been examined. These vaccines have consisted of a *D*NA prime, a *v*iral boost, and a second boost consisting of *p*rotein mixed with adjuvant (D–V–P). One study demonstrated the ability of D–V–P immunizations to induce the secretion of antibodies at mucosal surfaces, particularly when the protein was conjugated to mannan [91], suggesting this vaccine approach may be particularly effective at preventing infection. However, preexisting immunity to the vectors in the population may still be problematic and clinical improvement is still important. Vaccination strategies would be simpler and more effective if we can improve the overall potency of DNA vaccines.

Table 1 Methods to improve the potency of DNA vaccines

	References
Delivery mechanisms	
BioJet	[1, 41, 98]
Electroporation	[44, 97, 101]
Gene gun	[35, 95, 98]
Microneedle injection	[71]
Skin abrasion	[96, 104]
Transcutaneous immunization patch	[39]
Design	
Backbone/selection elements	[40]
Codon optimization	[32, 99, 107]
Leader sequences	[99]
Promoters	[99]
RNA optimization	[89]
Formulations	
Bupivacaine	[86]
Costimulatory molecules	[18, 75]
Cytokines	[13, 15]
Death receptors	[27, 77]
DermaVir	[61]
Heterologous prime-boost protocols	Many
Lipids	[34, 106]
Maturation factors	[47]
PLG	[28, 45, 68]
Saponins	[88]
TLR Ligands	[51, 55, 66, 102]

Delivery mechanisms

The initial step in optimizing the effectiveness of DNA vaccines is to choose the appropriate route of immunization. Several factors play into this decision including relative ease, cost, and target cells. Perhaps the simplest way to deliver DNA is by direct intramuscular (IM) injection. IM injections are relatively easy and typically result in transient transfection of muscle tissue around the injection site, but have also been shown to result in the direct transfection of APCs [27]. Transfected muscle cells express the encoded antigen(s) which is then acquired by resident APCs through a variety of mechanisms, including delivery through apoptotic or necrotic bodies, secretion of the antigen, low level transfection or resident APC, and direct transfer via "nibbling" (Fig. 1). Having acquired the antigen, resident APCs, primarily dendritic cells, subsequently stimulate immunity by migrating to draining lymph nodes where they can interact with antigen-specific T cells. In theory, T cells could also be stimulated at the site of immunization via presentation by transfected muscle cells. However, the codelivery of plasmids encoding costimulatory molecules would be important. IM injections of

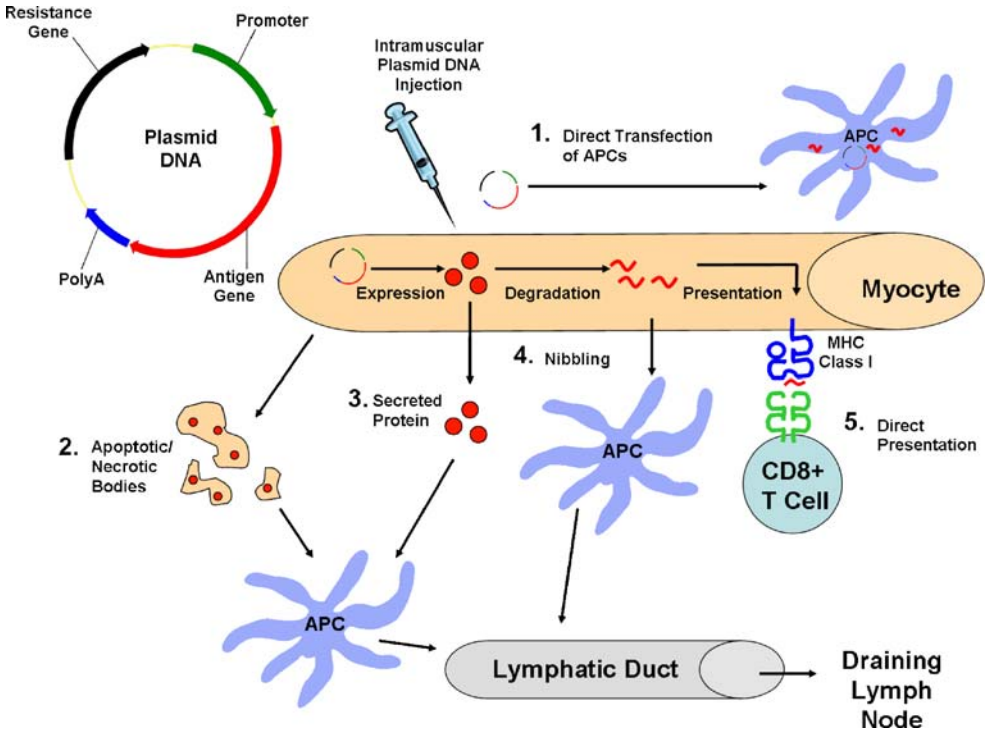

Fig. 1 Priming after intramuscular DNA injection. Intramuscular plasmid DNA immunizations have been demonstrated to result in the uptake of DNA by myocytes as well as resident APCs such as dendritic cells. $CD8^+$ T-cell priming can occur through direct transfection of APCs (*1*), the uptake of antigen-bearing apoptotic or necrotic bodies (*2*) or secreted protein (*3*) by APCs, direct transfer of antigens from healthy transfected myocytes to DCs (nibbling) (*4*), or by direct presentation of antigen on MHC class I by myocytes to T cells (*5*). Direct presentation would require the existence of coadministered, costimulatory molecules

plasmid DNA has been shown to stimulate cell-mediated immunity as well as antibody production in small animal models. However, naked DNA has been less effective than desired at inducing immune responses in humans and nonhuman primates, possibly due to low levels of transfection or the paucity of DCs in muscle tissues. It is important to improve the efficacy of this approach. One important area that has been explored is skin delivery.

DNA-based immunizations targeting DCs were postulated to be more effective if delivered to the skin, where Langerhans cells (LC) and dermal dendritic cells reside in high abundance. Several methods of skin immunization have been used including injection, topical application, and gene gun delivery. The gene gun in particular has been shown to elicit potent immune responses in small animals, generating Th2-type antibody responses as well as CTL responses. Biolistic gene delivery to the skin results in transfection of both keratinocytes and LC, with transfected LC playing a prime role in the presentation of antigen to $CD8^+$ T cells [70]. Condon et al. [29] demonstrated that gene gun immunizations were able to elicit protective antitumor immune responses and confirmed the presence of transfected LC in the draining lymph nodes of immunized mice. Similarly, Larregina et al. [60] demonstrated direct transfection of human LC after biolistic DNA bombardment in human skin organ cultures, resulting in the migration of LC capable of stimulating antigen-specific T cells. Importantly, the gene gun uses only microgram quantities of DNA, while injection requires 100–1,000 times that amount to elicit similar responses. The use of gene gun immunizations in primates has been interesting with some positive results and some issues that require more investigation. One issue relates to a thicker keratinocyte layer in primates than mice which may interfere with efficacy. However, useful levels of antibody responses have been observed in both nonhuman primates as well as human clinical studies. The levels of CTLS induced in these studies have not kept place with the induction of antibody responses. Gene gun skin immunizations have largely been shown to become biased toward Th2 responses in rodent models. The use of adjuvants may be necessary to skew these responses toward a Th1 response and improve the efficacy of the cellular immune responses to more effective levels.

One approach to improve in vivo plasmid delivery is to apply a specific electric current to the site of injection. By this approach, electroporation can improve the efficiency of genetic immunization in some animal models. However, most of these studies have focused on humoral responses while there has been much less work in the area of quantitative cellular immunity. More work in this area is clearly needed. The increase in potency is presumably through a transient increase in cell membrane permeability resulting in increased plasmid delivery ultimately resulting in greater expression in vivo. However, it has been suggested that the inflammatory cellular infiltration induced by electroporation may also play an important role in the increase in vaccine efficacy [7]. Vaccine response rates

have been enhanced to varying degrees using in vivo electroporation, augmenting both humoral and cell-mediated immunity [8, 9, 31, 67, 79, 80, 101, 105]. This method has been used to transfect multiple tissues and has been used to deliver a wide variety of immunomodulatory cytokine genes such as GM-CSF, IL-2, IL-12, IL-15, IL-18, and TNF-α [44]. Typical DNA immunizations require milligram quantities of DNA, greatly increasing their cost. The improvement in transfection efficiency and immunity seen with in vivo electroporation may help to reduce the quantity of DNA that is necessary and, therefore, the cost of DNA-based vaccines. However, there are concerns with this approach that are actively being worked on. These include the physical trauma to the patient of the shock used for delivery itself resulting in pain and trauma to the vaccinee. This is an important issue particularly in the prophylactic setting, where the risk benefit ratio is less favorable. In addition, higher integration rates have been reported for this approach than optimal. There are several studies in progress to address this important issue which will benefit the field.

Plasmid design

The design of the plasmid can greatly affect the level of gene transcription. Therefore, multiple strategies have been employed to maximize gene expression after genetic immunization. An important aspect of plasmid design involves the choice of promoter, which drives the expression of the encoded gene. Promoters that are effective in microbial gene expression are not necessarily optimal for driving mammalian gene expression. Typically, the hCMV promoter is used for expression in mammalian cells. However, tissue-specific promoters may be useful in restricting gene expression to specific cell types. Codon optimization is another valuable tool that involves changing the genetic code to reflect the code that is most often used in a particular species. Different species are known to utilize different codons to encode the same amino acids, with a preference for a particular codon sequence. By optimizing the genetic sequence to reflect these differences, gene expression can be greatly increased. Modifications should also target mRNA structure, resulting in greater stability of the mRNA in the nucleus as well as enhanced export of the mRNA from the nucleus to ribosomes, where it can be efficiently translated [65]. Furthermore, sequences should be examined to remove cryptic RNA splice elements or instability sequences which can generate mRNA instability lowering protein expression. Leader sequences also help to stabilize the mRNA and improve translation, resulting in greater gene expression. Constitutive transport elements are also used to increase the transport and stability of mRNA from the nucleus. Together, these techniques and others help to increase the efficiency of transcription and translation after genetic vaccination and can contribute to the overall performance of the vaccine through enhanced protein expression.

Molecular adjuvants

Perhaps one of the most interesting methods being evaluated for enhancing the efficacy of DNA vaccines is the use of genetically encoded molecular adjuvants. There are a wide variety of adjuvants currently being tested, including chemokines, cytokines, costimulatory molecules, and even molecules that induce cell death. These constructs are coinjected with the plasmid encoding the target antigen and can influence the immune response through multiple mechanisms. Chattergoon et al. demonstrated the expression of Fas was able to induce death of antigen-expressing cells, resulting in an increase in the efficiency of antigen transfer to APCs and a subsequent enhancement of antigen-specific immune responses. These constructs are coinjected with the plasmid encoding the target antigen and can influence the immune response through multiple mechanisms [27]. Toll-like receptor agonists are particularly attractive for adjuvanting DNA vaccines. Data suggests TLRs provide a molecular code by which the immune system senses what type of pathogen it is responding to. By understanding this code, we may be able to engineer vaccines to generate favorable immune responses while hindering the development of unfavorable responses. Importantly, TLRs have been demonstrated to act synergistically, enhancing immune responses over that seen with single TLR agonists alone [37, 46, 64, 100]. In mice, imiquimod, a mimic of ssRNA and ligand for TLR7, was shown to enhance immune responses, even when used in conjunction with gene gun immunization [102]. In one primate study, TLR ligands for TLR7/8, TLR8, or TLR9 in combination with a protein prime followed by and adenovirus boost resulted in significantly enhanced immune responses over that seen without the TLR agonists, elevating the frequency of responding $CD8^+$ T cells [102]. Together, the data indicate that TLR agonists can enhance several vaccination strategies, suggesting further evaluation is warranted in the DNA setting.

Chemokines have the ability to bring in specific cells to participate in the development and longevity of the immune response. In particular, chemokines have the ability to recruit a variety of cells, including professional APCs, to

the immunization site. Sumida et al. [94] demonstrated the ability of MIP-1α and Flt3L, either alone or in combination, to increase the number of dendritic cells at the immunization site. This strategy can be greatly beneficial for intramuscular injections because there is a relatively low level of APCs in muscle tissues. Other chemokines have been evaluated as well, including RANTES and IL-8, which were demonstrated enhance cell-mediated protective immunity in a herpes virus model [85]. With the discovery of new chemokines, such as dendritic cell- and monocyte-attracting chemokine-like protein (DMC) [69], the potency of DNA vaccines to attract APCs to the site of immunization may be increased, particularly if used in combination with other APC-attracting chemokines.

Cytokines are known to act in a variety of ways. Some cytokines, such as GM-CSF, can recruit dendritic cells as well as promote their survival. The addition of plasmid GM-CSF (pGM-CSF) in DNA immunization regimens has been demonstrated to enhance immune responses to the encoded antigens. Barouch et al. [16] demonstrated the ability of a bicistronic plasmid encoding gp120 and GM-CSF to enhance the proliferative responses of $CD4^+$ T cells as well as their ELISPOT responses in mice. However, in primates and human clinical studies of a Malaria vaccine, no clear increase of immune potency with human GMCSF to DNA vaccines was observed. The addition of pIL-2 is believed to promote natural killer (NK)- and T-cell survival and may support the generation of $CD8^+$ T cells in the absence of $CD4^+$ T-cell help. Similarly, pIL-12 has been shown to enhance the generation of Th1-mediated immunity which is believed to be critical for fighting HIV. Barouch et al. [14] utilized a DNA-encoded fusion protein of IL-2, IL-2/Ig, shown to have a longer half life, to augment DNA vaccines in rhesus macaques. The addition of plasmid IL-2/Ig in their DNA vaccine resulted in the elicitation of protective immunity capable of controlling viremia and preventing immunodeficiency after infection with SHIV-89.6p. IL-12 is known to promote Th1-mediated immune responses, particularly the generation of cytotoxic T lymphocytes. Several studies have demonstrated the ability of plasmid-encoded IL-12 to stimulate potent $CD8^+$ T cell-mediated immunity in macaques after DNA immunizations [20, 33] and was shown to enhance antigen-specific proliferative responses in chimpanzees [19]. In macaques, IL-12 as a DNA vaccine adjuvant increased the CTL response to between three and five times, the levels of CTLs normally associated with vaccination with viral vectors. This adjuvant could also lower viral load after SHIV challenge in rhesus macaques in a *Mamu-A**01 independent manner. Another important adjuvant has been engineered forms of interleukin 15. It is interesting to note that pIL-15 has been shown to promote the induction and proliferation of IFN-γ-secreting $CD8^+$ T cells [56]. Furthermore, pIL-15 was able to restore secondary immune responses in $CD8^+$ T cells even in conditions where $CD4^+$ T-cell help was suppressed, suggesting IL-15 may be particularly useful for use in HIV immunotherapy [56]. The inclusion of plasmid-encoded chemokines and cytokines in DNA vaccines ensures the recruitment and education of the cells necessary for enhanced vaccine efficacy and may even enable tailoring of vaccines to generate specific types of immune responses.

Typical DNA-based immunizations require the presence of professional APCs to process and present plasmid-encoded antigens to naïve T cells. In some instances, APCs can be directly transfected. This occurrence is very rare in muscle tissues where APCs are sparse. The probability of directly transfecting APCs is greatly enhanced by immunizing intradermally where there is an abundance of Langerhans cells and dermal dendritic cells. The vast majority of transfected cells, however, appear to be non-APC in lineage. Dendritic cells are known to acquire antigens in a variety of forms, including soluble antigens (such as secreted proteins) and particulate antigens. Importantly, DCs are also capable of sampling cell-associated antigens from live cells via a process termed “nibbling” [42, 82]. Antigen-loaded dendritic cells can then traffic to draining lymph nodes where they stimulate naïve T cells, providing the appropriate costimulation necessary for T-cell activation [10, 92]. It is likely this pathway of antigen capture is important for the antigen presentation seen after transfection with plasmids encoding nonsecreted antigens. Theoretically, the requirement of professional APCs for proper antigen capture, processing, and presentation may be bypassed through the use of genetically encoded costimulatory molecules as adjuvants. In this model, transfected cells can express the encoded antigen as well as costimulatory molecules, such as CD80 and CD86. Presentation of antigen-derived epitopes via MHC class I molecules in the presence of costimulation could potentially drive T-cell activation. The addition of plasmids encoding cytokines to the immunization protocol would provide a third signal to polarize immune responses. Other molecules such as ligands for Toll-like receptors could act directly on APCs, enabling direction of immune responses through APC programming.

Inhibiting immune signaling may prove to be as critical for eliciting effective immune responses in chronically infected patients as the vaccines themselves. Recent evidence suggests the PD-1/PD-L1 pathway is active in chronic infections, with high levels of PD-1 expression in chronically stimulated (exhausted) T cells [11]. Signaling through PD-1 delivers an inhibitory signal to T cells, resulting in impaired immune function. The administration of antibodies blocking this signaling pathway in chronically infected mice resulted in restored function of exhausted T

cells [11]. Recently, Puaux et al. [72] demonstrated the ability of a heterologous prime-boost regimen to induce antigen-specific T-cell responses. However, after challenge, the reactivity of the CD4 T cells was impaired. The impairment may be due to direct infection by SHIV or even anergy as suggested by the authors [72]. While PD-1 expression on these cells was not examined, the work performed in chronically infected mice suggests the functional impairment seen by Puaux may be due to PD-1 expression inhibiting T-cell activity. Further research is necessary to determine whether this pathway is active in HIV infection. However, it is likely that administration of agents capable of blocking this and other inhibitory pathways may allow for more effective immune stimulation during vaccination.

Molecular adjuvants that induce cell death may also be beneficial for enhancing immune responses to DNA vaccines. In this scenario, transfected cells expressing antigens would die via apoptosis or necrosis, which have both been demonstrated to deliver antigens to APCs [2, 48, 52, 58, 78, 81]. However, apoptosis may not generate the necessary danger signals to stimulate dendritic cell maturation [52, 78]. Therefore, the use of apoptosis-inducing plasmids may require additional adjuvants to elicit optimal immune responses.

Poly(lactide-co-glycolide) (PLG) has been demonstrated to enhance oral administration of a rotavirus DNA vaccine in mice. Administration of the DNA vaccine encoding the outer capsid of rotavirus with PLG adjuvant resulted in an increased secretion of virus-specific IgA antibodies and protection from a rotavirus challenge compared to immunizations with naked DNA alone [45]. Importantly, PLG has also been shown to enhance the effectiveness of DNA vaccines to HIV*gag* and HIV*env* in rhesus macaques [68], demonstrating the usefulness of PLG as an adjuvant in primates. In particular, it appears to lower the dose of DNA necessary to achieve comparable levels of immunity. Clinical results have supported that these PLG formulated DNA's do produce CTL's in humans. Another group of adjuvants for DNA vaccines that have demonstrated some success in animal models is the use of heat shock proteins (HSPs). It is interesting to note that HSPs have been reported to enhance the ability to enhance immune responses of immunized animals on multiple levels. HSPs are known to bind Toll-like receptors and are able to stabilize cellular proteins, making them available for capture by APCs for a longer period of time. Hsu et al. [47] demonstrated the ability of HSP70 fused to the HPV antigen E7 to increase vaccine efficacy. Furthermore, the authors demonstrated that the vaccine encoding the fusion protein was capable of conferring protection against an E7-expressing tumor challenge far better than DNA vaccines encoding E7 alone. Lipid formulations have been studied for their ability to enhance DNA delivery both in vitro and in vivo with some success [34, 106]. Finally, saponins and block-copolymers, which are commonly used to permeabilize cells, have proved to be interesting as vaccine adjuvants in the DNA setting. They likely function through the formation of pores to allow antigens or DNA access to the cytosol of cells [88]; however, they may also induce danger signals that can stimulate APC's. Ongoing studies of these adjuvants in nonhuman primates and in the clinical setting are very important.

Many other molecular adjuvants have been used with varying success as detailed previously [23, 38, 57]. These include skin DC targeting formulations such as DermaVir, which is the subject of a separate section of this volume. The numerous potential molecular adjuvants, under investigation either individually or in combination, show the great interest in this important area. These formulations collectively represent diverse strategies for both the enhancement of DNA uptake by these vaccines as well as the manipulation of the types of immune responses induced by the immunization strategy.

Preclinical and clinical studies

Nonhuman primate (NHP) SIV and SHIV models, while not perfect, remain the best models for the preclinical evaluation of HIV vaccine efficacy due to a disease progression that is similar to that seen in humans. Understanding mechanisms of protection or viral control in NHPs may give insight for the development of vaccines for HIV. In 2005, Su et al. [93] reported five conserved Gag CTL epitopes using a SHIV model in rhesus macaques. The study demonstrated these epitopes are maintained long into viral infection and even appear to be relatively conserved in several strains of SIV. While some animals failed to elicit immune responses to these epitopes, the data suggest that vaccines designed to target immune responses to these conserved epitopes may enhance control of the virus. Barouch et al. [12] demonstrated the ability of DNA immunization to elicit CTL responses against both dominant and subdominant epitopes in a SHIV infection model, suggesting DNA may be useful for eliciting broad CTL-based immune responses which are believed to be desired for control of HIV infection. Boyer et al. [22] reported the ability of DNA vaccines to protect chimpanzees from HIV-1 challenge despite the variability in the immune responses generated in the animals, suggesting different immune responses may be able to provide cooperative protection.

DNA vaccines have generally performed poorly at inducing antibody responses. This is likely due to the lack

of soluble antigen access to B cells. In July 2005, Rosati et al. [73] examined the use of a DNA vaccines encoding Gag and Env fused to either MCP3 or β-catenin (CATE) to target the transcribed antigens to the secretory or degradation pathways, respectively. As would be predicted, the animals immunized with the MCP3 fusion construct developed stronger antibody responses. However, the production of antibodies failed to correlate with control of viral load after challenge. It is interesting to note that animals immunized with both constructs maintained better control of viremia that appeared to correlate with IFN-γ ELISPOT responses and CD4 T-helper responses [73]. The data further support the design of vaccination strategies that induce cell-mediated immune responses for control of HIV/SIV. Another DNA vaccination approach in NHPs involved the use of the plasmid encoding $SHIV_{\kappa U2}$ with a deletion in the reverse transcriptase, rendering the virus noninfectious [87]. This approach allowed for the expression of nearly all viral genes. The authors found the construct was able to induce cell-mediated immunity and neutralizing antibodies against $SHIV_{\kappa U2}$, but failed to elicit neutralizing antibodies that were cross-reactive with the challenge virus, SHIV89.6P. Of four immunized animals, one animal failed to survive the SHIV89.6P challenge despite the elicitation of the highest IFN-γ ELISPOT rates. However, the remaining three animals continued to survive with undetectable viral loads [87]. The results support that viral control can be achieved by plasmid vaccination encoding an attenuated form of SHIV and that control appeared to be partially dependent on the generation of cell-mediated responses. However, while DNA delivery was used in this study and provides important information regarding immune control, such an attenuated viral genome likely poses significant risks for clinical evaluation.

There has been particular interest in the use of heterologous prime-boost strategies for HIV/SIV. Boyer et al. [21] reported partial control of SIV239 after a DNA/*Listeria* prime-boost regimen. The combination of DNA prime and *Listeria* boost resulted in enhanced cellular immunity to virus over that seen with either agent alone [21]. Perhaps the most common prime-boost regimens involve the use of a DNA prime with a recombinant viral boost, including the use of fowlpox virus [50], adenovirus [26, 76], vesicular stomatitis virus [33], and vaccinia virus [3, 72, 74, 90, 93]. Kent et al. [50] examined the effect of the route of immunization in addition to the use of a DNA prime-viral boost using fowlpox virus, examining the effect of systemic vs mucosal site boosting. They found much more IFN-γ spot forming cells and antigen-specific proliferation when both the DNA and viral injections were IM compared to that seen with IM DNA and mucosal viral boost. However, both methods appeared to control viral loads after infection with pathogenic SHIV challenge. DNA/MVA prime-boost regimens have been shown to be effective at eliciting immune responses capable of controlling infection [4, 5]. It is important to note that some of these macaque studies are complicated by the use of *Mamu-A**01 macaque haplotype animals. Such *Mamu-A**01-expressing macaques are known to be able to spontaneously control infection.

In a non-*Mamu-A**01 study of interest, Egan et al. [33] reported the use of plasmids encoding IL-12 and Gag to prime animals followed by a boost using a recombinant vesicular stomatitis virus expressing Gag/gp160. These animals developed exceptionally strong cellular immune responses which resulted in better control of viral load and maintenance of CD4 T cells after SHIV89.6P challenge. Importantly, the authors correlate protection with the ability of their vaccine to induce cell-mediated IFN-γ ELISPOT responses and the ability of the host to produce titers of neutralizing antibodies after challenge, providing an insight to what may be required for a prophylactic vaccine [33]. This data is consistent with the data reported by Amara et al. [3] who demonstrated loss of viremic control after depletion of $CD8^+$ T cells and suggested a supporting role of neutralizing antibodies in controlling infection. While the use of live viral vectors carries some inherent risks, their effectiveness when used after DNA priming in a heterologous prime-boost regimen is clear, resulting in very long-term control of viral loads and maintenance of health as shown by Sadagopal et al. [74].

There have been many vaccine strategies designed to test elicitation of immune responses to HIV in humans, with more than 30 human trials currently underway (Table 2). Thus far, the two immunization methods demonstrating the highest level of immunogenicity include the use of recombinant Ad5 adenoviral vectors either alone or in combination with plasmid DNA in a heterologous DNA/Ad5 prime-boost regimen. In a related preclinical study, Casimiro et al. [26] demonstrated the ability of DNA/Ad5 prime-boost to reduce viral loads by up to 7-fold in *Mamu-A**01 monkeys compared to control *Mamu-A**01 monkeys. However, after 6 months, the monkeys began to show signs of viral escape, demonstrating the need to enhance and broaden the immune response. VRC DNA adenovirus vaccines have entered phase II trials. Studies have demonstrated the presence of neutralizing antibodies to injected recombinant viruses inhibits their effectiveness. Importantly, regions hit hard by HIV also demonstrate a high prevalence of reactivity to Ad5 vectors. Therefore, numerous strategies are underway to circumvent this issue, including the use of higher doses of virus and even using different, less common, adenoviral serotypes. One way to skirt the issue of neutralizing antibodies is to utilize two different viruses in a prime-boost regimen. One study examined the use of an adenovirus prime and a MVA or

Table 2 Current clinical trials of preventative HIV vaccines

	Vector	Antigen (Clade)	Organizer, sponsor, manufacturer
Phase I trials	DNA	env (A,B,C,D,E)	St. Jude, NIH
		env (A,B,C), gag (A,B), RT (B), rev (B)	Karolinska Institute, Karolinska Institute and SIIDC, Vecura
	DNA prime, Adeno boost	gag, pol, env (B); gag, pol, env (A,B,C)	WRAIR, VRC NIAID, VRC
		gag/pol Polyprotein, env (A,B,C)	HVTN, NIAID, VRC NIAID, VRC
	DNA prime, Adeno boost or Adeno alone	gag, pol, nef (B), env (A,B,C); gag, pol (B), env (A,B,C)	IAVI, NIAID, VRC
	DNA or Adeno prime, both with Adeno boost	gag, pol, env (B); gag, pol, env (A,B,C)	HVTN, VRC
	DNA prime, MVA boost	gag, pro, RT, env, tat, rev, vpu (B); gag, pol, env (B)	HVTN, VRC, Geovax
		env (E), gag (A), pol (E)	Karolinska Institute, Karolinska Institute and SIIDCl, US Military HIV Research Program
	DNA + IL-2/Ig DNA adjuvant	gag, pol, nef (B), env (A,B,C)	HVTN, NIAID, VRC
	DNA +/− IL-12 DNA adjuvant prime, homologous boost	gag (B); gag (B) or env, gag, nef (B)	HVTN, NIAID, Wyeth
	DNA +/− IL-15 DNA prime, DNA + IL-12 DNA or DNA + IL-15 DNA boost	gag (B); gag (B) or env, gag, nef (B)	HVTN, NIAID, Wyeth
	DNA alone or DNA prime with NYVAC boost	env, gag, pol, nef (C); env, gag, pol, nef (C)	European Vaccine Effort
	DNA/PLG and gp140/MF59 adjuvant	gag and env DNA/PLG (B); Oligomeric gp140 (B)	HVTN, NIAID, Chiron
	DNA, protein, or DNA + protein	gag, pol, vpr, nef (B); Protein containing T-helper epitopes from env, gag, pol, vpu (B)	HVTN, NIAID, Pharmexa-Epimmune
	Adeno	gag, pol (B), env (A,B,C)	HVTN, NIAID, VRC
	AAV	gag, protease, RT (C)	IAVI, Targeted Genetics
	Alphavirus	gag (C)	HVTN, NIAID, Alphavax
	MVA	env, gag, tat-rev, nef-RT (C)	IAVI, Therion
		gp160, gag, and pol (integrase-depleted and RT nonfunctional) (A,E)	WRAIR, NIH
		env/gag-pol, nef-tat (C)	IAVI, ADARC
	MVA and FVP alone or in combination	env, gag (B); tat, rev, nef, RT (B); env, gag (B); tat, rev, nef, RT (B)	HVTN, NIAID, Therion
	Protein + Therapore adjuvant	Anthrax-derived polypeptide LFn gag p24 protein (B)	WRAIR, NIAID
	CTL multiepitope peptide vaccine + RC529-SE +/− GM-CSF	CTL epitopes from env or gag	HVTN, NIAID, Wyeth
	Canarypox transduced autologous dendritic cells	env, gag, pol (B)	WRAIR
Phase II trials	DNA prime, Adeno boost	gag, pol, nef (B), anv (A,B,C); gag, pol (B), env (A,B,C)	NIAID, VRC, HVTN, Vical, GenVec
	Adeno	gag, pol, nef (B)	HVTN, NIAID, Merck
	AAV	gag, PR, RT (C)	IAVI, Targeted Genetics
	LIPO-5 (lipopeptides)	5 lipopeptides containing CTL epitopes from gag, pol, nef (B)	ANRS, Aventis
Phase III trials	Canarypox prime, protein boost	env (E), gag/pol (B), env (B/E)	WRAIR, Dept. of Community Disease Control, MOPH, TAVEG, AFRIMS

Modified from information at http://www.iavi.org.

ADARC Aaron Diamond AIDS Research Center, *AFRIMS* Armed Forces Research Institute of Medical Sciences, *ANRS* Agence Nationale de Recherche sur le SIDA, *HVTN* HIV Vaccine Trials Network, *IAVI* International AIDS Vaccine Initiative, *MOPH* Ministry of Public Health Thialand, *NIAID* US National Institute of Allergy and Infectious Diseases, *NIH* US National Institutes of Health, *SIIDC* Swedish Institute for Infectious Disease Control, *St. Jude* St. Jude Children's Research Hospital, *TAVEG* Thai AIDS Vaccine Evaluation Group, *VRC* Vaccine Research Center at the US National Institutes of Health, *WRAIR* Walter Reed Army Institute of Research

ALVAC boost. It is interesting to note that priming with either of the poxviruses followed by boosting with adenovirus failed to elicit impressive immune responses. However, when the monkeys were primed with adenovirus and then received a poxvirus boost, the responses were robust [25]. Together, the data indicate that the immunization order plays a critical role in the subsequent ability to generate immune responses. In addition, other viral vectors are being investigated, including canarypox virus. Alvac, a canarypox virus-based vaccine, demonstrated an IFN-γ ELISPOT response in only 3.9% of patients, with 47% developing antibody responses against HIV-1. The use of high-dose Alvac alone increased the IFN-γ response to nearly 10%. Despite a response rate of less than 50%, an immunization strategy involving the use of recombinant canarypox virus and gp120 has entered phase III clinical trials. One of the better IFN-γ ELISPOT patient response rates seen in clinical trials this past year was with the Chiron DNA vaccine, which involves the administration of DNA with lipopeptides. The IFN-γ ELISPOT response rate was only 16%, demonstrating the need for more effective strategies for eliciting potent Th1 immunity. Clinical trials involving the use of low-dose recombinant Venezuelan equine encephalitis virus, peptides, or lipopeptides alone were ineffective at eliciting immune responses. Therapy studies are also revealing in this area. MacGregor et al. [62] demonstrated more effective control of transient viral escape, or viral blips, in patients on HAART, suggesting DNA vaccines are capable of providing some form of immune stimulation to assist in controlling viral replication even though the ELISPOT response rates have not been optimal. Furthermore, studies from the laboratory of Wahren have shown increase in immune induction in the area of immune therapy. Together, the data demonstrate the importance of developing more efficacious vaccination strategies. Perhaps some vectors that have proven ineffective in these clinical trials may prove more effective at higher doses. However, with increased dosage comes an increased risk to patient populations particularly when using live virus. Importantly, the results demonstrate that viral vectors, while still believed to be more potent stimulators of immunity, including poxviral vectors, AAV vectors, peptide vaccines, among others, are not inherently more effective at generating CTL responses than highly engineered DNA vaccines. The recombinant adenoviral platform remains the most potent current platform for the induction of CTL responses in a naive population. It is likely that genetically encoded molecules designed to adjuvant DNA immunizations, improved formulations, as well as enhanced methods of physical delivery, such as some form of in vivo electroporation, will enable DNA vaccines to compare favorably to viral vaccines in effectiveness and control of immune responses while maintaining their level of safety. There is a great deal to be done and these future studies are eagerly awaited.

Conclusions

Thus far, no correlates of protection have been found for HIV. However, there is strong evidence that $CD8^+$ T cells play an important role in controlling HIV infection. Currently, the most common method for assessing vaccine efficacy is the measurement of the number of IFN-γ-secreting T cells by ELISPOT.

Adenoviral vectors have performed well in primate models and can be further improved if an animal has been primed using a DNA vaccine. While DNA vaccines alone have proven to be relatively nonimmunogenic, the addition of encoded molecular adjuvants, such as optimized IL-15 and IL-12, has been almost as effective as viral vectors. It is likely that further exploration of potential molecular adjuvants may enhance DNA vaccines further, surpassing the immunogenicity of live viral vectors while maintaining a superior level of safety.

There are a variety of factors that favor the use of viral vectors. DNA vaccines are nonlive, nonreplicating, nonspreading systems that must be highly engineered to even approach the immune induction levels of live systems. We have learned that for DNA vaccines to compete effectively in the clinic, they must be highly manipulated to address each of these issues. For example, viral vectors are very good at inducing gene expression, with a high level of transduction compared to DNA transfection. Modern techniques have improved the efficiency of DNA transfections in vivo through the use of electroporation. Perhaps the primary reason viral vectors induce such potent immune responses is because they provide strong signals for the induction of innate immunity, while DNA vaccines are relatively silent. Adjuvants, whether provided in addition to the plasmid DNA vaccine or encoded in the vaccine, will need to be provided to fully activate the immune response against encode antigens. In addition, adjuvants that stimulate innate immunity also have the added benefit of directing the type of specific immune responses that are induced. In combination with other adjuvants, they may provide potent signals capable of elevating DNA vaccine efficacy above that seen with live vectors and increase the control over the subsequent immune response. It is interesting to note that in vivo electroporation is likely able to address two of the problems seen with IM injection of DNA. First, the efficiency is far greater with electroporation. Second, the tissue damage associated with electroporation may release several endogenous danger signals, including heat shock proteins and uric acid which have previously been shown to stimulate DC maturation [6,

36, 83, 84]. Further studies are clearly important and will continue to mold and change this evolving field.

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