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REVIEW

Combination DNA plus protein HIV vaccines

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Abstract A major challenge in developing an HIV vaccine is to identify immunogens and delivery methods that will elicit balanced humoral and cell mediate immunities against primary isolates of HIV with diverse sequence variations. Since the discovery of using protein coding nucleic acids (mainly DNA but also possible RNA) as a means of immunization in the early 1990s, there has been rapid progress in the creative use of this novel approach for the development of HIV vaccines. Although the initial impetus of using DNA immunization was for the induction of strong cell-mediated immunity, recent studies have greatly expanded our understanding on the potential role of DNA immunization to elicit improved quality of antibody responses. This function is particularly important to the development of HIV vaccines due to the inability of almost every previous attempt to develop broadly reactive neutralizing antibodies against primary HIV-1 isolates. Similar to the efforts of developing cell mediated immunity by using a DNA prime plus viral vector boost approach, the best antibody responses with DNA immunization were achieved when a protein boost component was included as part of the immunization schedule. Current experience has suggested that a combination DNA plus protein vaccination strategy is able to utilize the benefits of DNA and protein vaccines to effectively induce both cell-mediated immunity and antibody responses against invading organisms.

Keywords HIV · Vaccine · DNA · Protein

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Introduction

In the last few years, notable progress in HIV vaccine research has been made by eliciting protective cell-mediated immunity, mainly against Gag as the target antigen, as demonstrated in the rhesus macaque model with SHIV challenge [1–3]. Although these studies have achieved protection by reducing the long-term viral setting point and avoiding the decline of $CD4^+$ T cell, none have induced sterilizing immunity, which demands an early and complete blocking of viral infection to the target cells, a task traditionally requiring strong anti-viral neutralizing antibody responses [4, 5]. Despite efforts in the last two decades, there has been little progress in generating cross-reactive neutralizing antibody responses against primary HIV-1 through active immunization. The diversity of HIV-1 subtypes and the high frequency of viral mutations have made it difficult to develop vaccines based on a single HIV-1 envelope (Env) antigen [6]. Efforts to identify a common neutralizing structure through studies of fusion intermediates [7], oligomerization [8, 9], and, recently, the consensus sequences of HIV-1 Env glycoproteins [10] have not yet led to the production of a unique HIV-1 Env antigen that can be used in active immunization to induce broadly neutralizing antibodies against primary viral isolates of diverse genetic backgrounds.

The need for polyvalent formulations containing multiple primary Env antigens has been discussed [11], but this approach, which would require Env antigens from different subtypes of primary HIV-1 isolates, has not been fully explored. One major technical barrier is that recombinant Env proteins alone were not sufficiently immunogenic to elicit neutralizing antibody responses in human immunogenicity studies [12]. On the other hand, DNA immunization is an ideal approach to deliver a polyvalent formulation

because the manufacturing process of DNA vaccines is relatively standardized and mixing of plasmids coding for different HIV-1 Env antigens does not generate unnecessary inter-molecular interactions at the DNA level. The main weakness of DNA vaccines is the limited quantity of antigenic proteins that can be expressed in vivo, which leads to low immunogenicity in large animals and human subjects.

Therefore, the recombinant protein vaccine is useful to boost hosts already primed with DNA vaccines to mount a vigorous antibody response. Research results accumulated in the last several years have suggested that a combination DNA plus protein approach was effective in improving not only the magnitude of antibody responses primed by DNA immunization, but also the overall quality of such antibody responses (as measured by avidity or neutralizing activities), which were hard to achieve by recombinant protein vaccines alone. In addition, the ability of DNA immunization to elicit satisfactory cell-mediated immune responses was not compromised in such a combination vaccine approach. Therefore, the DNA plus protein vaccination strategy is ideal for inducing a balanced humoral and cell-mediated immune response with a focus on protective antibody responses.

The rationale for the prime-boost strategy

The prime-boost concept demonstrated its early promise for SIV and HIV vaccine studies by using recombinant vaccinia virus as prime followed by subunit protein boost [13]. The exact mechanism of why such a combination approach was better than any of the single vaccination modalities was never elucidated. Although only Env antigens from T-cell-line-adapted (TCLA) viruses were used in these early studies, the novel combination of two vaccine delivery methods for one vaccine development clearly influenced later development with various prime-boost strategies.

The discovery of DNA immunization in early 1990s has greatly expanded the use of prime-boost strategies in HIV vaccine development. In principle, DNA vaccines share the major advantages of live attenuated vaccines by making antigens in vivo. This antigen production step determined the following two important consequences: 1) such endogenously produced antigens can be effectively presented to the immune system by major histocompatibility complex (MHC) class I and class II molecules—a critical step for the induction of CD8+ and CD4+ T-cell responses, respectively; and 2) these newly synthesized antigens can be folded in their native conformation and go through a well regulated post-translational modification process such as glycosylation. For several years, scientists focused on the ability of DNA vaccines to induce CD8+ T cells which, no doubt, play an important role in controlling the HIV infection. At the same time, how to explore the ability of DNA vaccines to induce CD4+ T-cell responses for the benefit of antibody response has not received sufficient attention. Misunderstandings also exist regarding the ability of DNA vaccines to elicit antibody responses despite the fact that antigens produced by DNA immunization can preserve excellent conformation and other important structural features.

Although DNA vaccination has been shown to be capable of eliciting antigen-specific immune responses in both small animals and non-human primates, and is generally safe in human studies [14–18], the levels of immunity elicited by the current delivery systems for DNA vaccines have not been strong, especially in humans. To improve the strength of DNA vaccines, mainly for the purpose of cell-mediated immune responses, strategies that involve priming with the DNA vaccine and boosting with a recombinant viral vector have been developed [19]. Protection against SHIV was achieved when macaques were vaccinated with a DNA prime coupled with a boost using viral-vector-based antigens [1, 3]. Currently, additional prime-boost strategies using various forms of pox viruses [20–23], adenovirus [3, 24, 25], and other viral systems [26–28] are being pursued. Most of these research efforts have focused on the induction of cell-mediated immunity (CMI). It is not clear what is the immunologic mechanism supporting for such combination applications. Viral-vector-based vaccines were developed for the induction of better CMI responses. When used alone, many of such vector systems were not necessarily more immunogenic than the DNA vaccination approach. More studies are needed to understand why a prime-boost approach using two modalities (DNA plus viral vector) focusing on the same CMI responses do better than either modality alone.

The combination DNA plus protein strategy to be reviewed in this article was based on a completely different rationale. DNA vaccines and recombinant protein vaccines utilize different mechanisms to elicit antigen-specific responses. DNA vaccination is effective in inducing cell-mediated immune responses including its ability to elicit CD4+ T-cell responses, which are critical for the development of T cell-dependent antibody responses. We hypothesize, and are supported by an increasing number of experimental results, that DNA immunization is highly effective in priming antigen-specific memory B cells due to its ability to produce intracellular antigens, which is important for the induction of excellent CD4+ T-cell responses. The recombinant protein vaccine, on the other hand, is more effective in eliciting antibody responses than cell-mediated immune responses. It has to be processed into smaller peptides to stimulate T-helper function.

Frequently, a strong adjuvant is needed, and many adjuvants also provide carrier function to bring protein antigens inside antigen-presenting cells. However, recom-

binant protein is effective in directly stimulating antigen-specific memory B cells to differentiate into antibody-secreting cells. This is a critical step for the final production of a large quantity of antigen-specific antibodies and is a step that DNA vaccines may not be able to function well due to the limited quantity of antigens that can be expressed by a DNA vaccine. Therefore, the DNA prime plus protein boost is a complementary approach by combining two very different immunization mechanisms. It combines benefits from both types of vaccines and overcomes their shortcomings. When used properly, the combination DNA plus protein vaccine approach should be particularly useful for the induction of protective antibody responses.

Current experience with combination DNA plus protein vaccines

Initial efforts to use DNA prime plus protein boost approach with TCLA viral Env antigens

Studies with well selected HIV-infected patient sera and monoclonal antibodies have repeatedly confirmed the role of envelope glycoprotein (Env) of HIV-1 as the neutralizing target for vaccine development [11, 29]. However, it is also well-known that HIV-1 Env is a poor immunogen. Antibody responses elicited by either protein immunization [30, 31] or DNA immunization [32, 33] with Env antigen is usually transient; titers rise and fall with successive immunizations. While single or singly boosted DNA immuzations often elicit strong and long-lasting neutralizing antibody response comparable with those seen in virally infected and convalescent animals in non-HIV models [34–36], multiple DNA immunizations are typically required to elicit even modest titers of HIV-1 neutralizing antibody [32, 33, 37–48].

The utility of a protein boost to improve anti-Env antibody responses was first demonstrated by using Env antigen from T-cell-line-adapted (TCLA) virus [49]. The recombinant Env protein was able to boost New Zealand White rabbits immunized with DNA vaccines as shown by increased binding antibody titers and significantly improved levels of neutralizing antibody responses against the TCLA viruses [49]. It was interesting to see in the control group of this study that the same recombinant Env protein was less effective in eliciting anti-Env antibody responses if no DNA prime was given. This study also demonstrated that the DNA prime plus protein boost approach was more effective in improving the avidity of anti-Env antibody responses than the protein-alone approach.

However, in this landmark study, the DNA prime plus protein boost group received three DNA prime immunizations plus two protein boost immunizations, while the protein alone group only received two protein immunizations [49]. The question remained unanswered whether additional protein immunizations in the protein alone group would further increase the quality of anti-Env antibody responses. It is also important to ask if the same DNA prime plus protein boost strategy observed with the TCLA Env antigen can be applied to envelope glycoproteins collected from the primary HIV-1 isolates.

Potent protective anti-HIV immune responses generated by the DNA prime plus protein boost vaccination was also demonstrated in an early rhesus macaque immunogenicity and protection study against the challenge of a chimeric virus expressing Env from TCLA HIV-1 isolate HXBc2 on a simian immunodeficiency $virus_{mac}$ backbone (SHIV-HXBc2) [42]. In this pilot study, immunization of two monkeys with HIV-1 HXBc2-derived Env DNA vaccines followed by a final immunization with Env DNA vaccine plus the *o*-gp160 IIIB protein purified from the parental HIV-IIIB virus completely protected monkeys from the SHIV challenge with homologous HIV Env antigen. Extensive serology and molecular virology analyses confirmed the protection of this combination immunization regimen. Significant increase of anti-gp120 binding antibodies and neutralizing antibodies against SHIV-HXBc2 responses were also observed after the final protein boost.

However, the interpretation of this study was somewhat difficult due to the complicated study design. This study was initially designed as part of exploratory series of experiments [42]. Animals received a total of five DNA prime immunizations at 4-week intervals, rested for 1 year, received another series of four DNA immunizations at 4-week intervals, and finally after an additional 4-month rest, received a single boost with DNA and *o*-gp160 protein delivered together at two separate sites. In the two control groups, one received only *o*-gp160 protein and the other received empty DNA vector plus ovalbumin that was added at the time of the final protein boost and thus may not serve as true negative controls as animals in the control groups received fewer immunizations. Although this pilot protection study used low numbers of monkeys and only TCLA Env antigen was used, the high levels of antigen-specific immune responses and evidence of complete protection from infectious challenge was highly significant.

In another study using a cohort of neonatal macaques, the animals were given a series of DNA priming inoculations using plasmids encoding SHIV-IIIB antigens followed by booster inoculations with soluble HIV-IIIB gp160 [50]. Upon challenge with homologous, nonpathogenic SHIV-vpu+, four of 15 DNA prime/protein boost animals and three of four animals that received protein boosts only appeared either protected or limited viremia as shown by low or transient virus infection. In this study, the DNA

prime phase used five DNA plasmids expressing SIV gag/pol, SIV nef, HIV HXB-2 particle, HIV HXB-2 gp120 and gp140 antigens. This approach may have caused a dilution effect to the immunogenicity of individual DNA vaccine components, which may explain the low fraction of protection in DNA prime/protein boost group.

The DNA prime plus protein boost approach is effective in improving the magnitude and quality of antibody responses against primary HIV-1 isolates

A recently published study [51] using JR-FL Env as a model immunogen addressed those important questions raised from the earlier studies using TCLA viral Env proteins. In this report, both plasmid-based and recombinant protein envelope (Env) glycoprotein immunogens were derived from a primary HIV-1 isolate, JR-FL. Sera from rabbits immunized with either gp120 or gp140 DNA vaccines delivered by gene gun inoculation followed by recombinant gp120 protein boosting were capable of neutralizing JR-FL by either the traditional PBMC-based neutralization assay or the newly developed pseudovirus assay system. Neither the DNA vaccines alone nor the gp120 protein alone generated a detectable neutralizing antibody response against this virus. Neutralizing antibody responses were similar using gp120 DNA compared to gp140 DNA for priming. The results suggest that Env DNA priming followed by gp120 protein boosting provides an advantage over either approach alone for generating a detectable neutralizing antibody response against primary isolates that are not easily neutralized.

In this study, two control groups were included. One group received the same number of DNA inoculations with the empty plasmid vector without HIV-1 Env gene inserts, followed with two recombinant gp120 protein boosts. The other group received four recombinant gp120 protein immunizations without any prime. Repeated boosting in this protein-alone group was still less effective than the DNA prime plus protein boost groups because its peak titers of anti-Env IgG were approximately one log lower than the peak titers generated by Env DNA priming plus protein boosting [51].

Of more interest, sera from rabbits immunized with recombinant protein alone were able to neutralize SF162 (a relatively easy to neutralize HIV-1 isolate), to a degree similar to that immunized with the DNA prime plus protein boost, but protein-alone sera were not able to neutralize JR-FL isolates. This result indicated that DNA priming is important in generating antibody responses that recognize specific structure features unique to JR-FL Env. While JR-FL Env protein is able to boost JR-FL-specific-neutralizing antibodies primed with JR-FL Env DNA immunization, JR-FL Env protein-alone immunization was not able to elicit such high-quality neutralizing antibody responses. In confirming this hypothesis, a more sensitive assay using the Biocore system demonstrated that the DNA prime plus protein boost sera had a higher level recognition against JR-FL gp120 than other primary HIV-1 gp120 antigens [51].

This study provided direct evidence that the DNA prime plus protein boost approach was equally effective with the primary Env antigens. Protein immunization alone, even with multiple boosts, was not able to produce the same quality of antibody responses elicited by a DNA prime plus protein boost strategy. Another potentially important finding from this study is that gp120 antigen, in its monomeric form, is actually immunogenic in eliciting neutralizing antibodies against the JR-FL virus. Previous efforts to develop stabilized trimeric forms of JR-FL Env proteins (including both gp120 and gp41 components) did not lead to success in generating neutralizing antibodies against JR-FL [52, 53]. In a subsequent study published only 1 month apart from the above JR-FL gp120 immunogenicity report, the DNA prime and protein boost approach was again proven superior to the protein-alone approach when various stabilized JR-FL trimeric Env antigens were tested in this expanded study [54].

This later JR-FL Env study mainly focused on the immunogenicity of disulfide-stabilized, cleaved forms of the trimeric HIV-1 Env complex (SOSIP-R6 gp140) [54]. The study found that it was beneficial to use DNA priming as compared to immunization with subunit protein only. This study conducted more extensive analyses on the magnitude and quality of antibody responses elicited by different immunization schemes. Antibody responses in primed animals were more robust and less subject to decline between protein boosts. The results suggested that the advantage of DNA priming was related more to the rate at which antibody responses were generated after subunit boosting, and to their longevity than to the magnitude and breadth of the final neutralizing antibody responses. Even the most potent of rabbit antisera had a very limited ability to cross-neutralize heterologous strains. As there is only one JR-FL Env protein included in this study, it is not surprising that the positive rabbit sera could not broadly neutralize other primary HIV-1 isolates. Therefore, this study, on one hand, confirmed the utility of DNA priming and, on the other hand, raised questions regarding the idea that one unique trimeric structure can neutralize every known HIV-1 virus. In this trimeric JR-FL Env study, the generation of a low level of neutralizing antibody responses to the autologous strain required an intensive and prolonged immunization regimen that made it more difficult to apply to future human vaccine development. In contrast, the study with JR-FL gp120 monomer in the earlier report used more straightforward immunization scheme, and the quality of

neutralizing antibody responses elicited by monomeric form of JR-FL Env was at least comparable to the trimeric approach [51].

The DNA prime plus protein boost approach provides an efficient tool to design and test modified HIV-1 Env antigens

Analysis of the crystal structure of HIV-1 gp120 envelope protein indicated that neutralizing epitopes are primarily clustered in one face of this protein, which is naturally occluded within the oligomeric envelope form [55, 56]. Several reports have indicated that specific modifications including deglycosylations and loop deletions introduced in the envelope proteins of HIV and SIV may increase the exposure of neutralization epitopes and possibly the immunogenicity of Env antigens [45, 57–60]. It is anticipated that more innovative structure modifications will be conducted to induce stronger and better quality of neutralizing antibody responses against HIV-1 primary viral isolates circulating in different regions of the world.

Testing of these structurally modified Env antigens represents a major challenge to the traditional practice of developing subunit-protein-based vaccines that need multiple steps of cloning, expression, and purification of Env proteins. Certain structural modifications make the production process even more difficult. For example, it is not possible to purify a naturally cleavable gp160 (full-length) or even gp140 (the ecto-domain) of HIV-1 envelope protein. There are also considerable lot-to-lot variations in the purity and conformation of purified Env proteins. In this regard, DNA immunization can serve as a valuable tool to allow scientists to quickly try different ideas. Based on the results from initial DNA immunization studies, the design of modified Env proteins can be finalized. Such modified proteins can then be used either alone or as part of the combination DNA plus protein vaccines.

The design of V2-loop-deleted Env antigens from the SF-162 isolate was a successful application of this strategy [61]. DNA vaccines expressing the wild-type SF162 and V2-deleted SF162 gp140s with an intact cleavable site between gp120 and gp41 were tested first in a rabbit model. By using these non-codon optimized Env gene sequences, at the end of DNA immunization ELISA titers in the order of 10^5 and 10^6 were recorded in both animal groups against both antigens. Although the modified Env was as effective as the wild-type Env in eliciting binding antibodies, sera collected from rabbits immunized with the modified Env had higher neutralization activity against both SF162 and SF162dV2 viruses than sera collected from animals immunized with the unmodified Env. Both the unmodified SF162 and modified SF162 gp140 immunogens elicited neutralizing antibodies against several heterologous primary HIV-1 isolates, but the potential of the modified immunogen to do so was greater [61].

After this rabbit study, a DNA prime and protein boost immunization study was conducted in rhesus macaques [61]. Animals received three DNA prime immunizations (expressing either the wild-type SF162 or the modified SF162 gp140 antigens) followed with the fourth DNA immunization in combination with matched recombinant gp140 proteins produced from CHO cells. This last boost led to 30- to 300-fold increase in the binding antibody titers from the lower peak level antibody responses induced at the end of three DNA immunizations. Again, the V2-deleted gp140 immunogen was more effective than the unmodified gp140 immunogen in eliciting neutralizing antibodies against isolates expressing the parental SF162 envelope and heterologous envelopes. While the animal numbers included in the macaque study was not large enough to draw a conclusion based on statistical analysis, the combination of rabbit and non-human primate studies did present a clear pattern of higher immunogenicity with modified SF162 gp140 antigens. This study provided one of the first examples for a model system in which DNA immunization can be first tested in small animals to study or optimize the designs of protective antigens in subunit-based-HIV vaccine formulations, followed with a DNA prime plus protein boost approach in non-human primates or humans to overcome the lower immunogenicity issue of DNA vaccines in large animal or human hosts.

More studies with modified Env structures are expected to use the DNA prime plus protein boost approach. For example, the immunogenicity of trimeric forms of uncleaved Env stabilized with a synthetic trimer motif isolated from the fibritin (FT) protein of the T4 bacteriophage, with or without being fused with the complement protein C3d, were recently tested in a DNA prime plus protein boost immunization study in mice [62].

The DNA plus protein immunization approach is successful in delivering polyvalent HIV vaccine formulations in small animals, non-human primates, and human volunteers

Although the vaccine delivery method is important in determining the type and magnitude of desired immune responses, the quality of protective immune responses is highly dependent on the antigen composition of vaccine formulations. To develop high-quality neutralizing antibodies against HIV-1, there are two major strategies. One is based on the hope to discover a unique Env structure, which can encompass all the HIV-1 isolates. Significant effort has been devoted to this approach and substantial knowledge has been learned to further enhance our

understanding of the clever designs of HIV-1 to escape from host's immune system [29]. The other strategy relies more on empirical methodology, which has been the traditional approach for vaccine development. One important area that has not received enough attention is the use of polyvalent Env antigen formulations [63]. The DNA prime and protein boost strategy offered a unique opportunity to study how to develop polyvalent HIV vaccines based on a vast collection of primary HIV-1 Env antigens.

In the last several years, our laboratory has started a systematic approach to develop effective polyvalent Env formulations. First, in a preliminary study using DNA immunization as a quick tool, we screened a large panel of primary HIV-1 Env antigens from almost every major clade of the HIV-1 M group. Part of this initial screening work was summarized in a recent review [63] in which we demonstrated that rabbit sera immunized with a combination of DNA vaccines expressing HIV-1 Env antigens from clades A to G were able to neutralize available viruses from multiple clades, but rabbit sera immunized with individual DNA vaccines usually were effective in neutralizing only the autologous viruses. However, the neutralizing activities elicited by DNA immunization alone were very low, making it difficult to do further analysis.

In a following study, the DNA prime followed by protein boost vaccination approach was used to study the efficacy of polyvalent Env formulations to induce neutralizing antibody responses against a wide range of primary HIV-1 isolates. New Zealand White rabbits were immunized with one, three, or eight primary HIV-1 gp120 antigens delivered first as DNA vaccines and followed by a protein boost using one or four homologous primary gp120 antigens produced from CHO cells [64]. Each animal received four DNA vaccinations by a gene gun and two protein boosts by intradermal injection.

At the end of DNA priming, substantial levels of antibody responses were induced in both monovalent and polyvalent gp120 DNA vaccine groups against the primary HIV-1 gp120 antigens as measured by ELISA. The overall immunogenicity of four individual gp120 DNA vaccines was very similar with anti-gp120 IgG titers above 1:100,000, even though the rabbit sera usually recognized the autologous gp120 antigen slightly better than the other primary gp120 antigens. For the rabbits that received polyvalent DNA vaccines, the preference for autologous gp120 antigen disappeared and the sera recognized all of eight primary gp120 antigens. DNA-immunized rabbits were further boosted with two protein immunizations. There was a quick elevation of anti-gp120 IgG responses after only one protein boost and the anti-gp120 titers reached peak levels with no more than two boosts.

The neutralizing antibody responses in these sera were analyzed by two types of neutralization assays. The first assay was performed using a single round of infection flow cytometric assay as previously described [65]. This assay detects HIV-1 infected T cells by intracellular staining for HIV-1 p24-Ag. A protease inhibitor is used to prevent secondary rounds of virus replication. At the end of the DNA priming phase, only low-level neutralizing activities were detected against one clade B primary virus, SF162. After one protein boost, significant levels of neutralizing activities were detected against multiple primary viruses including SF162, Bal, JRCSF (clade B), TV1 (clade C), and DJ263 (clade A). After the second protein boost, the strength and the breadth of neutralizing activities in rabbit immune sera were further improved. More rabbits became positive against several primary viral isolates included in the panel. Rabbit sera with previously positive neutralizing activities after one protein boost demonstrated improved neutralizing activities and expanded breadth of such neutralizing activities against additional primary viral isolates such as S007 and DU151 (clade C). The negative control rabbits, inoculated with the empty DNA vector in the priming phase, did not show neutralizing activities even after two protein boosts.

The other assay used pseudoviruses expressing primary Env antigens in a high-throughput-assay system [66]. The sera after two protein boosts from the above study were tested with this assay. The panel of viruses included diverse primary Env antigens, six from clade B, two each from clades A, C, D and E. Results demonstrated that polyvalent sera generated broader neutralizing antibody responses against this panel of randomly selected primary viral isolates than the monovalent based on statistic analysis [64].

The feasibility of the DNA prime plus protein boost approach to deliver polyvalent Env antigens was further tested in a non-human primate model. Specifically, the relative effectiveness of gene gun inoculation, which was used for the rabbit studies, was compared with the conventional needle injection of DNA vaccines through intramuscular (IM) and intradermal (ID) inoculations [67, 68]. Gene gun inoculation was clearly more effective than the IM or ID DNA immunizations in eliciting higher levels of anti-Env antibody responses in rhesus monkeys at the end of DNA priming. However, with 1–2 recombinant gp120 protein boosts, the antibody responses quickly reached peak levels in all the monkeys, irrespective of whether the gene gun or needle injection was used during the DNA prime phase. The binding antibodies showed broad reactivity against primary Env antigens from different subtypes. Monkeys immunized with the polyvalent Env formulations delivered by DNA prime and protein boost were protected from a $SHIV_{Bal}$ challenge including sterilizing protection in several animals, whereas the negative control animals were all infected [67, 68].

A phase 1 clinical trial in healthy human volunteers was organized based on the above pre-clinical data [69]. The study was a randomized, three-group trial testing two dosing levels of DNA administered by ID or IM route, and one standard dose of protein administered by IM route, with adjuvant QS-21. Groups A and B received three DNA inoculations (weeks 0, 4, and 12), either ID or IM, respectively (1.2 mg total DNA divided equally among one gag and five primary gp120 genes). Protein boosts were administered at weeks 20 and 28, and contained the five recombinant gp120 proteins (375 μg total protein) matching the DNA prime (two in clade B, one each in clades A, C, and E). Group C receives a higher dose of DNA (7.2 mg DNA) followed by the same above protein boost.

Although it was encouraging to see for the first time in human studies positive antigen-specific antibody responses with DNA immunizations in volunteers who received a high-dose DNA prime, the antibody responses at the end of DNA prime were relatively low. However, the antibody titers went up quickly after just one protein boost. With 1–2 protein boosts, the anti-gp120 titers reached $1{:}10^5$ or above level, comparable to that seen in chronically infected HIV patients. Early assay data suggested positive neutralizing antibody activities against multiple primary HIV-1 isolates including those not in the Env vaccine formulation. At the same time, significant levels of anti-Env cell-mediated immunity were detected in these volunteers. Results from this interim report confirmed the immunogenicity of the DNA prime and protein boost approach in humans, especially with its potential for multi-gene polyvalent formulations. This will offer new opportunities to advance our efforts in developing an effective HIV vaccine.

Technologies and applications beyond the classical DNA prime plus protein boost method that has been used in early HIV vaccine studies

Once the general principle and efficacy of the DNA prime and protein boost approach is established, more creative applications of this approach are expected. For the delivery of the DNA prime, there are already reports using electroporation [54], cationic poly(lactide-co-glycolide) (PLG) microparticles [70], or the Bioject needle-free injection device [23] (unpublished data) to further improve the DNA plasmid uptake as part of the DNA prime plus protein boost studies. As to the nature of HIV-1 antigens to be delivered, the priming DNA plasmids can encode HIV viral particles in addition to individual HIV antigens [49, 71, 72].

The protein boosts can also be in different forms as well. Short peptides, focusing on neutralizing epitopes [73], may be effective in boosting antibodies against specific antigenic structures. These peptide sequences can also be delivered in the form of chimeric proteins, fused in the middle of an unrelated carrier protein to stabilize the peptide structure (Susan Zolla-Pazner and Lu, unpublished data). The boosting protein antigens can be delivered in the form of virus-like particles [71] or inactivated virus [72].

The utility of the DNA prime plus protein boost approach has been further supported by an increasing number of non-HIV vaccine studies. DNA priming–protein boosting enhanced both antigen-specific antibody and Th1-type cellular immune responses in a murine herpes simplex virus-2 gD vaccine model [74]. Protein boosting did not change the Th1-type cellular immune responses primed by DNA immunization. In contrast, protein priming–DNA boosting produced a Th2-type cytokine profile similar to that induced by the protein priming–protein boosting approach. This suggested that the DNA priming–protein boosting is a more preferred combination sequence by eliciting both potent Th1-type cellular immune responses and antibody responses.

In a similar study, immunization of mice with a DNA vaccine encoding ESAT6 protein of *Mycobacterium tuberculosis* followed by an autologous ESAT6 protein boost was more effective than either DNA- or protein-alone approaches in eliciting Th1 type cellular immune responses without compromising ESAT6-specific antibody responses [75]. The advantage of the DNA prime plus protein boost approach was also confirmed with the highly immunogenic hepatitis B surface antigen (HBs). In a well characterized study, mice were immunized with either HBs DNA vaccine [pVAX(S)] alone, recombinant HBs (rHBs) alone, pVAX(S) prime-rHBs boost, or rHBs prime-pVAX(S) boost [76]. The pVAX(S) prime-rHBs boost regimen induced the strongest antibody responses, and it was also able to maintain strong cellular immune responses.

The concept of the DNA prime/protein boost approach was further reinforced by studies using DNA prime followed by inactivated vaccines as was shown for rabies vaccines [77]. The DNA prime was able to overcome the poor immunogenicity of inactivated vaccines. This may offer a convenient combination approach because many inactivated vaccines have been proven safe in humans for many years. Such a DNA-prime-inactivated-vaccine boost approach may require less complicated regulatory review and clinical testing.

Future outlook for combination DNA plus protein vaccines

For the DNA plus protein vaccination strategy to become a major vaccine technology platform, many issues need to be further studied. In the near future, we can expect that this

approach will continue to show its utility in HIV vaccine development. At the same time, studies utilizing other non-HIV vaccines will also contribute to our understanding to the areas discussed below:

The most important question is: what are the immunologic mechanisms that may contribute to the benefits of combination DNA plus protein vaccines? We hypothesize that DNA immunization is effective in generating antigen-specific memory B cells, while the protein immunization is effective in stimulating the proliferation of memory B cells into antibody-secreting plasma cells. However, experimental evidence is needed to prove this hypothesis. More importantly, does antigen processing and presentation play any role in this process, and how are T-helper cells involved in regulating the B-cell functions?

The optimal DNA plus protein immunization schedule is not very clear. How many DNA primes and how many protein boosts are needed for the induction of maximum antibody responses? A general understanding is needed although this may be antigen- or animal-species dependent. Because the DNA plus protein vaccines require the production of two types of vaccines, the cost for manufacturing, formulation, and clinical administration will be higher than a single modality vaccine. Thus, it is important to define the most cost-effective immunization schedule including dosing and frequency for both the prime and boost components. Co-delivery of DNA and protein as boosts has not been used effectively in previous studies. Is it true that co-delivery of DNA and protein as prime is less effective than using DNA priming alone? How closely should the protein boost match with the DNA prime? This is important for polyvalent HIV vaccines. Our previous results suggested that heterologous recombinant gp120 proteins can be used to boost antibody responses in gp120-DNA-primed animals. Is it possible to use a fixed set of recombinant Env proteins to boost animals receiving different polyvalent Env DNA prime formulations?

Traditionally, recombinant protein-based vaccines require a strong adjuvant. Is this still needed for the DNA prime plus protein boost vaccines? If so, then what type of adjuvants will be most suitable to this new combination vaccine application?

The above questions aside, the encouraging data we obtained in our recent phase 1 clinical trial with a polyvalent DNA prime plus protein boost HIV vaccine provided direct evidence that this approach is effective in humans. Although the recombinant protein-based subunit vaccination approach has not been able to maintain its original momentum over the last 20 years, the discovery of DNA vaccination and the combination of DNA plus protein vaccines are timely and may provide a much-needed option to the development of effective subunit vaccines against HIV and other major human pathogens.

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