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REVIEW ARTICLE

RECOMBINANT VECTOR VACCINES FOR THE PREVENTION AND TREATMENT OF HIV INFECTION

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SUMMARY

A number of gene-based vectors have been tested to deliver HIV-1 antigens to immune systems as vaccines. While many challenges remain, a number of promising results have recently been observed to re-energize the HIV vaccine field. Naked DNA vaccines using plasmids as vectors have suffered from low efficiency, but have recently been improved using approaches such as electroporation to improve transfection. Another approach is to use viruses as gene delivery vehicles. These have the advantage of naturally evolved infection efficiency, but have issues with immune responses and being biohazards. Particularly promising viral vectors are those based on low seroprevalence adenovirus vectors and those based off of poxviruses. These naked and viral vectors have most effectively been applied as prime–boost strategies to avoid vector-induced immunity that occurs each time a virus vaccine is used. These immune evasion approaches include serotype switching, vector switching and shielding vectors with polymers like polyethylene glycol (PEG). Effective prophylactic HIV vaccines will likely need to provide barrier protection at mucosal sites of entry of the virus when there are fewer virions and they are at most risk from immune rejection. For mucosal protection to occur, better mucosal vaccines and challenge models will be needed.

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INTRODUCTION

HIV

HIV-1 is likely the most well-known virus in the world today. The reader is referred to many excellent reviews on HIV and HIV vaccines, of which the following are a subset (1-10).

HIV is an enveloped virus of the retrovirus family. These viruses are termed "retro", since their biology runs in reverse to the central dogma of genetics, wherein DNA begets mRNA which begets proteins. Retroviruses break this dogma by possessing RNA genomes that are reverse transcribed into DNA that is integrated into the host genome as a provirus. Once reinvented as DNA, HIV then enters the normal flow of genetic information in the host cell until it packages its progeny RNA genomes into new virions. HIV-1, HIV-2 and its species analogues (simian immunodeficiency virus [SIV], feline immunodeficiency virus [FIV], etc.) fall into the genera of retroviruses known as lentiviruses, or "slow" viruses. This is somewhat of a misnomer, as HIV can rapidly take over whole organisms, but disease progression can be slow.

The immune system

HIV infects and damages the immune system. To describe the virus and development of vaccines against it, we must first briefly discuss a simplified view of the immune system. The immune system is an overlapping battery of cellular and protein-based barriers to infectious agents and cancer. At one level, the immune system can be divided into the innate and adaptive immune response, although in most cases a productive adaptive immune response requires a robust innate immune response first.

The innate immune response is characterized by ongoing surveillance by proteins and cells that are geared to provide quick but nonspecific protection. The innate immune response evolved early in plants and invertebrates. Its ability to protect these organisms in the absence of an adaptive immune response is a testament to its potency. In humans, innate immune responses involve phagocytes, such as macrophages and neutrophils that can take up and destroy pathogens, natural killer cells and eosinophils that can ablate infected cells and parasites, and protein mediators, including complement, defensins, interferons and acute-phase proteins, that act on their own or in concert with the aforementioned cells.

The adaptive immune response is usually thought of as antibody and cellular immune responses. These responses evolved in verte-

brates and have been polished by evolution up to and including the development of the human immune system. These immunological tools give vertebrates a better ability to repel pathogens to which they have already been exposed. It is this function that underlies vaccination as a strategy to establish immunity against pathogens without actually having to suffer the disease.

The adaptive immune system involves a complex orchestra of cells including antigen-presenting cells (APCs), such as dendritic cells (DCs), macrophages and B cells, effector and memory B and T cells, and a spectrum of regulatory cytokines and proteins. As indicated by its name, the adaptive immune response adapts. In so doing, it is able to select for antigen- and pathogen-specific immune responses, which generally get stronger and more specific with each exposure to a given pathogen. While adaptive immune responses are generally the goal of most vaccines, the innate immune responses must also be activated to maximally drive adaptive immune responses.

One can model a basic adaptive immune response against a pathogen involving: 1) detection of infection (via danger signals and the innate immune response); 2) presentation of pathogen antigens to B and T cells; 3) expansion of antigen-specific B and T cells for effector functions; 4) retraction of effector cells; and 5) establishment of immune memory cells that can respond more rapidly if the same pathogen is encountered. During a natural infection, one gets the disease associated with the pathogen, and if they survive, they generate memory immune responses that usually protect them if they are exposed again. Vaccination aims to provide the same memory immune responses while avoiding disease and, of course, death.

In most natural infections, the immune system is so good that it will run through all five steps above and provide excellent protection against secondary exposure to a pathogen. With all of the effective vaccines that we currently get, if one feeds the immune system a good non-self-antigen, the immune system takes over and provides long-lasting, sometimes life-long immunity. However, the most difficult pathogens require that nearly every step of the adaptive immune response be polished or frankly re-engineered. HIV is not just difficult, but is a profound challenge for the native immune system or for a vaccine that must activate the immune system.

Immunological goal of vaccines

Antibodies

An ideal vaccine would prevent any cell from being infected by a virus. For this reason, the production of antibodies is the hope of almost any vaccine (reviewed in 2). Antibodies can bind the surface of the virion, blocking its ability to bind cells or go through later steps of its life cycle. While vaccines against viruses such as influenza and hepatitis B virus can generate neutralizing antibodies even with relatively simple antigens (inactivated viruses, recombinant protein), it has historically been very difficult to generate neutralizing antibodies against HIV because of its ability to evade the immune system and the vast number of viral variants in the human population.

Once a virus enters the cell, most viruses are largely protected from antibodies, since they generally cannot enter the cells. While this is the general goal of an antibody-producing vaccine, more recent data in nonhuman primates suggest that activities such as antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cell-mediated viral inhibition (ADCVI) may play a bigger role than was previously appreciated (11). Recent preliminary analysis of immune correlates in the RV144 HIV vaccine trial in humans suggests that antibodies that bind but do not neutralize virus may correlate with protection (reviewed in 12).

T cells

If you cannot neutralize a virus before it infects the cells, you have to kill the infected cell or inactivate the virus inside the cells. Vaccines generally aim to achieve this by generating cytotoxic T lymphocytes (CTLs) or cytokine-secreting T cells (13, 14). For cellular immune responses, it is thought that DCs (of which there are many flavors) are the most potent APCs used to prime these responses. DCs are quite potent at presenting both major histocompatibility I (MHC I) and MHC II peptides to CD8 and CD4 T cells, respectively. DCs are effective at this process by virtue of their ability to also "co-stimulate" these T cells with secondary signals necessary for maturation and proliferation.

In the context of repelling pathogens, we generally think of CD8 T cells as CTL effector cells that are able to recognize virally infected cells by viral peptides displayed on MHC I on these cells. Once recognized, CD8 T cells can directly kill these cells (i.e., with CTLs) or secrete cytokines to affect cell biology or other immune responses. In contrast, CD4 T cells are generally referred to as T helper cells that are thought to be involved in supporting the maturation and expansion of CD8 T cells, as well as the B cells against most antigens. Notably, these CD4 cells are also the target for HIV infection and killing, explaining in part the difficulty in generating good vaccines, as well as raising the unique problem that a vaccine against HIV may also provide the virus more cells to infect.

Early work to develop HIV vaccines focused on producing the simplest of T cell immune correlates, that being CTLs (15, 16). More recent work has attempted to correlate multifunctional T cells that produce more and more cytokines (17, 18). As our understanding of the biology of T cells has improved, instead of being called simple effector and memory cells they are now classified as "effector memory" and "central memory" cells. Effector memory (Tem) cells are thought to mediate peripheral effector functions. Central memory (Tcm) cells are thought to have weak antiviral functions themselves, responding to reinfection by dividing and providing massive numbers of new effector cells with potent antiviral activities (reviewed in 19, 20). As their name implies, these immune correlates are hoped to "correlate" with protection. However, it is frequently unclear if a given correlate observed in an animal model will actually translate into real protection in humans. Indeed, this is the ongoing question at the center of deciding what might constitute an effective human HIV vaccine (reviewed in 21).

Some guidance may be found in studies of "elite" HIV controllers who control the virus well in the absence of therapy (reviewed in 22). There are a number of factors that likely play a role in the controller phenotype. Relevant to vaccine development, CD4 cells that are critical in providing "help" to CTLs and for antibody responses may respond differently to HIV in elite controllers. For example, central memory CD4 cells appear to be maintained better in elite controllers and may be more multifunctional in their responses (22).

The profoundly immunoevasive HIV-1 virus

There are a number of aspects of HIV biology that make this virus a challenge for vaccines (reviewed in 21, 23, 24). First, HIV is tropic for several pivotal cells of the immune system by use of CD4 as a primary receptor and chemokine receptors CCR5 and CXCR4 as co-receptors. CD4 is primarily expressed on T helper cells of the immune system and it is these cells that are depleted over the course of infection to a level wherein their helper functions are insufficient to maintain proper immune function. When CD4 counts fall from the normal range down below 200 cells/mm^3 one is defined as having acquired immunedeficiency (AIDS) (1). CD4 cells are also expressed on a number of other cells, including DCs. Therefore, since DCs are pivotal to stimulating T cells and CD4 cells are pivotal to producing new CD8 T-cell and antibody responses against the burgeoning swarm of HIV, this pathogen handicaps and indeed destroys the ability of the immune system to cope with the virus.

A second profound effect of HIV biology on generating and evading protective immune responses is its hypermutability. HIV uses its reverse transcriptase to convert its RNA genome into DNA. Unlike high-fidelity host cell DNA polymerases, reverse transcriptase has low fidelity and is thought to induce high levels of mutations in the new viral genomes at each round of replication (25). This low fidelity is likely compounded by later steps of the viral life cycle that use host RNA polymerase, which is also thought to have low fidelity.

It is currently thought that most HIV-1 infections start by the infection of one or a few cells with one or a few virions (reviewed in 4). This initial "beachhead" of infection by a viral clone quickly becomes a swarm of billions of viruses that are much harder to control, even using antiviral drugs to repress these virions. Therefore, the earliest mucosal entry of HIV-1 should be our target for a prophylactic vaccine. In contrast, a therapeutic vaccine must deal with rampant systemic virus swarms.

When HIV spreads from the mucosa, it ravages the gut, and then goes systemic. In this process, billions of mutant viruses with varied infectious "fitness" are generated. These are a profound challenge, since with mutation comes the potential to evade drugs or natural or vaccine-induced immunity to the virus (26). Therefore, in one person, billions of viral variants are produced that can kinetically evade each protective wave of the immune system with a compensatory wave of viral variants that can evade these immune responses. For example, under pressure from antiviral drugs (i.e., treatment with reverse transcriptase or protease inhibitors), drug-resistant HIV mutants are able to repopulate an individual within 14 days after elimination of the majority of the virus (27). Selection of mutants with a proliferative advantage is also a driving force for mutation in these retroviruses, as well as lentiviruses. This is demonstrated most remarkably by SIV that has been attenuated by a 12-base deletion in its *nef* gene. When this attenuated virus was infected into primates, SIV variants were selected that had actually replaced the deletion with another sequence and then mutated that sequence to rebuild the original *nef* sequence (28).

Rampant production of HIV mutants is a profound problem for developing a single vaccine against HIV-1. This is suggested for antibody responses by observations that HIV-positive patients who rapidly progress towards AIDS may have antibodies against HIV, but tend to have low to no neutralizing antibodies against the virions present in their body (29-31). Some of these viral variants appear to have been directly selected for escape from neutralizing antibodies against the immunodominant V3 domain of gp160 (32), particularly by mutations affecting residues within V3 (33) or mutations outside of V3 that alter the conformation of the antigen (34, 35). There is some evidence for the virus using a decoy strategy with the immunodominant V3 loop by attracting neutralizing antibody responses to this domain. Having generated a robust response against this epitope, the immune system appears restricted to boosting only these existing responses to the detriment of generating new antibodies against new V3 mutants (reviewed in 36). However, in some cases the immune system is able to circumvent this inhibition and generate neutralizing antibodies against the new V3 mutants (37).

HIV mutants are also selected to escape CTL recognition (38). Patients with high levels of CTLs also have a higher proportion of viral variants with mutations in the specific epitopes recognized by these CTLs. More direct evidence for the role of mutational escape comes from observations in a newly infected patient (39). This patient had an essentially homogeneous HIV population 35 days after infection. At this time, the patient generated robust polyclonal CTLs against an immunodominant epitope in gp160 in the existing virus. Viral burden then fell precipitously within several days, presumably due to the activity of the observed CTLs. In the face of this CTL-based immunological pressure, viruses with mutations in the CTL epitope appeared within as little as 14 days and predominated over 120 days. Mutations within the CTL epitope were found at a 20-fold higher frequency than in adjacent sequences, suggesting that selective pressure was focused on this epitope. The selective pressure appeared to be imposed by the CTLs, since the virus mutant that ultimately replaced the original could not be killed by the original CTLs. Similar effects have been observed in other patients, where the appearance of CTL escape mutants coincided with progression (40). In this case, escape appeared to be focused on an immunodominant Gag CTL epitope in the virus population.

Over time, HIV-1 has evolved and varied into a diverse set of groups and subgroups (e.g., clades) that tend to predominate in different parts of the world. When HIV is passed to a second person, any one of these successful viral variants can be passed to this new host. Extrapolate this to the millions of humans who currently are infected with HIV and their own billions of viral variants, and one can appreciate why this virus (or this global swarm of viruses) is a challenge for vaccine development. Fortunately, HIV cannot mutate all of its proteins, so there are antigens shared in common between viruses, particularly within the same subgroup or "clade".

HIV VACCINES

A variety of vaccine approaches have attempted to repel HIV. These past and current efforts generally fall into protein-based and gene-based vaccines and will be discussed below. The text below describes general concepts and strategies pointing towards current applications of HIV vaccines in humans (Table I). Additional information can be found in a number of excellent vaccine reviews (1-10).

Protein vaccines for antibody and cellular immune responses

Most past protein vaccines have utilized native or modified HIV envelope as the primary immunogen with the rationale that this exposed protein can be targeted by neutralizing or inactivating antibody

responses (reviewed in 2, 36). Many of the neutralizing antibodies that are produced in patients and after vaccination are directed towards the third variable domain (V3) of gp160. This immunodominant and hypervariable epitope may act to focus and misdirect immune responses against the virus by preventing later responses against other epitopes in the virus and against other variants of gp160, making this antigen an even more difficult target. While many of the antibodies generated by vaccines can neutralize laboratory strains of HIV, it has subsequently been shown that many of these appear incapable of neutralizing primary isolates of the virus (41). While this was discouraging, subsequent work has demonstrated that primary isolate neutralizing antibodies can indeed be generated, particularly by immunization with oligomeric forms of gp160 (42). Early modifications of *env* immunogens included deletions of the gp41 domain to produce gp120 as a secreted antigen. Subsequent efforts aimed to generate oligomeric antigen that could mimic the native state of Env on virions, to delete distractive variable domains, or to construct consensus or centralized immunogens that might generate more broadly cross-reactive immune responses than any single *env* immunogen (43, 44). Of these, oligomeric gp140 forms of Env are currently most popular in trials to drive antibody responses. Most of these Env protein antigens are being applied in human trials as boosts for weaker antibody responses generated by gene-based vaccines. For example, in some cases, adjuvants are also being tested to better stimulate the immune system with these extracellularly delivered proteins. While *env* is a preferred protein immunogen, many current trials also test other HIV antigens to drive humoral or cellular immunity (Table I) (43, 44).

In addition to a role for antibodies in an HIV vaccine, there is some evidence to suggest that CTLs may also be effective. There are examples of people who have been exposed repeatedly to HIV but have not become infected (45, 46). These individuals are interesting, since they did not have anti-HIV antibodies but did have robust CTL responses. When infected individuals have been followed over time, CTLs appear to play a role in controlling HIV following primary inoculation of the virus (47) and during ongoing infection (48, 49) by destroying virus-infected cells.

CTL production generally depends on intracellular production of antigen. Therefore, intact protein vaccines have not been as actively pursued for these responses. Instead, most efforts to drive CTLs have focused on the use of DNA or viral vectors that can express antigens intracellularly. However, intact proteins can be "cross-presented" to T cells to produce CTLs and recombinant protein vaccines, including *gag* and *tat* immunogens, as well as *env* antigens, are being tested for these immune correlates. Another protein-based approach to drive T-cell responses is to use peptides that contain or are specific CTL or T helper epitopes (32, 50, 51). Peptide vaccines have been shown to be effective in a number of animal models, but, like intact proteins, usually require a robust delivery platform or adjuvant to enhance immune presentation to T cells (52-54).

Genetic immunization and gene-based vaccines

An alternate approach for CTL and antibody generation is genetic immunization (e.g., DNA vaccines, gene-based vaccines, etc.). Genetic immunization is a method for producing immune responses to a protein by delivering the gene encoding the protein into an animal rather than the protein itself (15, 55-57). As originally conceived, genetic immunization used simple plasmid DNA as the carrier for antigen genes. Genetic immunization is particularly attractive for vaccine development, because foreign proteins are produced intracellularly in a manner analogous to their production in virally infected cells. This intracellular localization makes these antigens substrates for MHC class I or class II presentation and activation of CTLs (56, 57).

Since its discovery in 1992, genetic immunization using simple plasmid DNA has been applied against an ever increasing list of pathogens, including HIV and SIV (15, 16, 58-62). Indeed, approximately half of the ongoing human HIV vaccine trials utilize some form of plasmid DNA (Table I). For the bulk of these pathogens tested in small animals, genetic immunization has been remarkably effective as a vaccine approach. By contrast, many HIV and SIV plasmid vaccines have been disappointing when upscaled into nonhuman primates and humans. A variety of strategies have worked to circumvent this problem by either increasing the efficiency of gene delivery by plasmid or by amplifying immune responses using cytokine plasmids (63-65), antigen targeting (66-69) and prime–boost strategies in which DNA is used to prime immune responses and protein or virus-like particles to boost these weaker responses (70-72). Therapeutic trials NCT01266612 and IMIRC1003 in HIV-infected volunteers aim to test boosting of plasmid vaccines with cytokine plasmid or cytokine proteins, respectively (Table IB).

Improving plasmid delivery by device injection or electroporation

The fundamental problem with scaling up genetic vaccines into nonhuman primates and humans is the low efficiency of transfection by plasmid DNA used by gene gun or intramuscular injection (73, 74). Plasmid did not evolve to infect nucleated cells, and therefore it is not very effective at doing this in mammals. Some efforts to improve genetic vaccine efficiency involved needle-free injection devices (75, 76) or in vivo electroporation for HIV vaccines (77-81). Despite conceptual issues with the thought of shocking vaccinees with electrodes, electroporation appears to be a frontrunner in moving naked DNA vaccines forward in humans (79, 80, 82). Prophylactic trials HIVIS 07 and RV262 will test needle-free injectors and/or electroporation of DNA in combination with poxvirus boosting (Table IA). Therapeutic trial HIV-001 will test DNA electroporation in HIV-infected individuals and therapeutic trial NCT012666016 will test DNA and IL-12 plasmids with and without electroporation (Table IB).

Improving antigen gene delivery using viral vectors

As the efficacy of naked DNA vectors waned in larger hosts, many investigators turned towards viral vectors to deliver gene-based vaccines including adenoviruses (Ads), poxviruses and a burgeoning mass of other viral vectors (83-88). The appeal of this approach is that evolution has done the heavy lifting in terms of developing a platform that can efficiently deliver nucleic acids into cells. This appeal is compelling enough that 75% of current HIV vaccine trials utilize some type of viral vector (Table I). Given this, the Mark Twain quote, "reports of my death are greatly exaggerated" applies well to zealous and dire predictions of the death of viral vectors in gene therapy and in HIV vaccines.

The two primary negative aspects of viral vectors are that they are more biohazardous than a simple plasmid and suffer from pre-existing immunity and vaccine-induced immunity against the vector. These vectors come in several formats: as fully wild replication-competent viruses, as attenuated replicating viruses or as replication-defective viruses. In the context of biosafety, a replication-defective vector has the appeal of less opportunity to spread and cause side effects during vaccination. Conversely, an inability to proliferate can reduce vaccine potential by limiting the number of cells that can be infected and express the antigen genes for the vaccine.

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Protein vaccines are often used to boost gene-based vaccines (Table I). The rationale for these prime–boost strategies is based on observations that gene-based vaccines are generally best for priming, but less robust for boosting, whereas protein vaccines are generally weak at priming, but best at boosting an already primed antibody response. Of the 36 ongoing human trials listed in Table I, 7 use protein vaccines; 4 use proteins with no gene-based vaccine and 3 use protein as a boost for gene-based vaccines. Thirty-two of the 36 trials use some form of gene-based vaccine (Table I). Three

Table IA. *Ongoing prophylactic HIV vaccine trials*.*

Trial ID	DNA	Adenovirus	Poxvirus	Virus	Protein	Routes	Comments	Phase
DCVax-001					Gag		DC-targeted Gag (via anti-DEC205) with adjuvant	I
HVRF-380-131004					Gag		Protein with polyoxidonium adjuvant	I
CN54gp140-hsp70 Conjugate					Env	Vaginal	gp140-HSP70 fusion protein	I
ISS P-002					Tat/Env	I.d., i.m.	I.d. and i.m. immunization	I
VRC 015 (08-I-0171)		Ad5				I.m.	Comparison needle and needle-free injection	I
HVTN 084		Ad5				I.m.	Test antigen competition: Gag/Pol ± envelopes	I/II
Ad26.ENVA.01		Ad26				I.m.	Evaluate innate and mucosal responses	I
Ad5HVR48.ENVA.01		Ad5/48				I.m.	Ad5 with Ad48 hexon dose escalation	I
VRC 012 (07-I-0167)		Ad5, Ad35				I.m.	Ad35 dose escalation ± Ad5 boost	I
HVTN 083		Ad5, Ad35				I.m.	Ad5, Ad35 with homologous or heterologous Envs	I
IAVI B003		Ad26, Ad35				I.m.	Placebo, prime–boost order comparison	I
PedVacc001 & PedVacc002			MVA			I.m.	Vaccinia vaccine in infants born to HIV+ mothers	I
HPTN 027			ALVAC			I.m.	Canarypox vaccine in infants born to HIV+ mothers	I
RV 138; B011			ALVAC			I.m., i.d., s.c.	Autologous DCs infected with canarypox vaccine	I
MV1-F4				Measles		I.m.	Dose-escalation, replication-competent vaccine	I
HVTN 090				VSV		I.m.	Dose-escalation, replication-competent vaccine	I
HVTN 076	+	Ad5				I.m.	Test in Ad5 seronegative hosts	I
HVTN 082	+	Ad5				I.m.	Test in Ad5 seronegative twins	I
VRC 016	+	Ad5				I.m.	Compare DNA/Ad vs. Ad in Ad5 seronegatives	Ib
HVTN 505	+	Ad5				I.m.	Test DNA + Ad5 in men at risk of HIV infection	II
HVTN 077	+	Ad5, Ad35				I.m.	DNA, Ad5, Ad35 in Ad5-naive and immune hosts	Ib
HIVIS 05	+		MVA			I.m.	DNA, MVA, MVA boost	I
HIVIS 07	+		MVA			I.m.	Test DNA ± electroporation with MVA boost	I
HVTN 073	+		MVA			I.m.	DNA, MVA	I
RV262	+		MVA			I.m.	Compare needle-free injection and electroporation	I
TAMOVAC-01-MZ	+		MVA			I.d., i.m.	DNA (i.d.), MVA (i.m.)	I
NCHECR-AE1	+		rFPV				DNA + fowlpox vector	I/II
HVTN 205	+		MVA				comparison of MVA vs. DNA + MVA	IIa
HVTN 078		Ad5	NYVAC			I.m.	Ad-poxvirus prime–boost in Ad5 seronegatives	Ib
IAVI B002		Ad35			+	I.m.	Ad35 + adjuvanted GSK investigational vaccines	I
Extension HVTN073E/SAAVI102	+		MVA		Env	I.m.	Protein boost of previous DNA/MVA	I

Table IB. *Ongoing therapeutic HIV vaccine trials*.*

Trial ID	DNA	Adenovirus	Poxvirus	Virus	Protein	Routers	Comments	Phase
ISS T-002					Tat	I.d.	Tat immunization in Tat antibody-negative HIV+	II
GCHT01				SIV		S.c.	Replication-defective SIV vector in HIV+	I
HIV-001	+					I.m.	DNA electroporation in HIV+	I
NCT01266616	+					I.m.	DNA ± electroporation ± IL-12 plasmid in HIV+	I
IMIRC1003	+				IL-2, GM, GH	I.d., s.c.	DNA + cytokines and growth hormone in HIV+	I

*Extracted in part from:
AIDSinfo from the National Institutes of Health (NIH) http://www.aidsinfo.nih.gov/ClinicalTrials/search.aspx
The HIV Vaccine Trials Network, http://www.hvtn.org/science/trials.html
International AIDS Vaccine Initiative's (IAVI), http://www.iavireport.org/trials-db/Pages/default.aspx
AIDS Vaccine Advocacy Coalition, http://www.avac.org
*Studies listed as active, but with unknown recruitment status were excluded.
I.d., intradermal; i.m., intramuscular; s.c., subcutaneous; MVA, modified vaccinia Ankara vector; ALVAC, canarypox vector; rFPV, recombinant fowlpox vector; GM, GM-CSF; GH, human growth hormone; NYVAC, New York strain vaccinia virus; VSV, vesicular stomatitis virus.

use plasmid DNA with no other gene-based vaccine. Thirteen of the 36 use plasmid combined with 1 or more other viral gene-based vaccines. Thirteen of the 36 use Ad vaccines, 10 use poxvirus vaccines, 1 uses both Ad and poxvirus, and the remainder use measles, vesicular stomatitis virus (VSV) or SIV vectors. Therefore, DNA and Ad vaccines top the human vaccine trials, with poxviruses next, followed by protein-, measles-, VSV- and SIV-based vaccines.

ADENOVIRUS VECTORS HAVE A HISTORY OF BREAKING TRAIL FOR OTHER VIRAL VECTORS

As evidenced by past and current use in humans (Table I), Ad vectors are one of the most potent vectors for in vivo gene delivery and vaccination (62, 83, 89-92). Head-to-head comparison of plasmid DNA, poxvirus vectors and Ad5 vectors as vaccines in rhesus macaques demonstrated that replication-defective Ad vectors were most potent (62). From a vaccine production standpoint, the ability to purify Ad to concentrations of up to 10^{13} particles/mL is nearly unrivaled by other virus vaccines, particularly when compared to enveloped viruses. Furthermore, replication-competent wild-type Ad has actually been administered to thousands of military personnel as oral capsules to generate mucosal immune responses against Ad infections (93). This use of Ad vectors as HIV vaccines in the late 1990s actually harkens back to early vaccine development work using replication-competent Ad as HIV vaccines (86, 94-97). The vast majority of what is known about Ad vectors was learned with Ad serotype 5 (Ad5), but recent efforts have delved into a number of other human and nonhuman Ads to circumvent immunological challenges.

Adenovirus as the poster child for immunological side effects against viral vectors

It should come as no surprise that the immune system will react against viral vectors, since it was designed in part to repel these pathogens. Since Ad led in vivo gene therapy and viral vector vaccines for HIV, it should also be no surprise that it was the first to encounter problems and side effects due to immune responses against the vector. Many proponents of non-Ad vaccines jumped on these problems as ways to promote their own favorite vectors. Persistence by Ad investigators has fought some of the non-scientific backlash against the vectors and these viruses are again one of the most used in humans (Table I). Immunological problems observed with Ad will likely be seen with any other viral vector, so Ad has become the platform to first observe problems and then avoid them. Therefore, we briefly discuss some of the immunological hurdles that have confronted Ad and their solutions to provide guidance for the likely similar problems that will confront most if not all other viral vectors in use as HIV vaccines.

Innate immune responses to Ad

Detection of Ad virions by normal and immune cells activates the release of inflammatory cytokines, including IL-6 and TNF-α, as part of the innate immune response. This response occurs with minutes to hours of injection (reviewed in 98). This response produced by intravenous administration of large doses (e.g., 10^{13} virus particles/kg) can lead to lethal events in primates (99) and likely played a role in the unfortunate death of Jessie Gelsinger (100). While these events are a problem after intravenous administration, in contrast, administration for vaccination appears remarkably safe, as evidenced by the safe administration of live replication-competent Ad to thousands of military recruits (93).

T-cell responses against Ad vectors

Injection of any viral vector into the immune system will produce T-cell responses against the virus. Once innate responses resolve within a few days of injection, the host immune system mounts adaptive CD4 and CD8 T cells not only against the HIV antigen, but also against Ad vector proteins. Most work with Ad vectors has utilized first-generation Ad5 vectors (FG-Ad5). These are rendered replication-defective due to deletion of the pivotal early gene *E1*. Because these vectors still bear most of the other Ad genes, these Ad proteins are also expressed in vaccine-transduced cells where their peptides can be presented by MHC I and MHC II. The produc-

tion of CTLs against gene-modified cells results in the destruction of these cells within 10-14 days after vector administration (101, 102).

Antibodies against Ad

Exposure of the immune system to a virus naturally or as a vaccine generates robust antibody responses against that virus. These include pre-existing antibodies and vector-induced antibodies that can neutralize the virus. These neutralizing antibodies are generally serotype-specific and can attenuate the level of gene delivery after subsequent administration of the vector if the same serotype of Ad is used (103). In addition to antibodies produced by vector administration, 27-100% of humans have previously been infected by wild-type Ad5 and they have pre-existing neutralizing antibodies. These pre-existing antibodies in humans can blunt (but not ablate) even the first inoculation of vector into patients.

Whereas pre-existing antibodies that are acquired by natural infection may reduce Ad5 vaccine efficacy, this is an even worse problem for viruses that have active vaccine programs against them (e.g., measles virus, poliovirus, smallpox, etc.). In this case, immunity may be 100% and may involve much higher neutralizing antibody levels against the vaccine vector than are even observed against Ad5. Therefore, while Ad5 has been a poster child for battling the immune system, it is likely that most other viral vaccines will suffer similar, if not worse, problems working in immune hosts.

STEP trial side effects

While it has been recognized that antibody and T-cell responses against Ad can attenuate gene therapy or vaccination, data from the STEP HIV vaccine trial in humans suggest that these responses may have unexpected side effects. In this phase II trial, FG-Ad5 vaccines expressing *gag*, *pol* and *nef* were tested as a T-cell-generating HIV vaccine strategy (104). Early data from the STEP trial suggested that uncircumcised vaccinated individuals with higher titers of antibodies against Ad5 had higher rates of HIV acquisition than volunteers with low Ad5 antibodies (104-107). Based on this, it has been hypothesized that antibodies against Ad may form immune complexes that enhance HIV infection (18). An alternate hypothesis is that T cells directed against HIV (or against a viral vaccine vector) may "feed" HIV T cells to be infected. It has been suggested that DNA prime followed by vaccinia virus boosting may be safer than FG-Ad vaccination (106). Considering that it is unlikely that HIV-1 and Ad have a special immunological evolutionary relationship to cause the STEP side effect, it is likely that prior immunological exposure to any virus followed by its use as a vaccine vector could generate similar side effects.

Other data suggest that the so-called STEP side effect may be an aberration. Follow-up of STEP vaccinees showed that the difference between groups with high or low antibodies versus Ad disappeared at later time points. This effect was not observed in the similar Ad5 vaccine Phambili trial (104). In addition, a recent case-control study shows that immunity to Ad5 does not pose an increased risk of HIV infection (108). Finally, thousands of military recruits have been vaccinated with live Ad4 and Ad7, two viruses that are nearly as seroprevalent as Ad5 (109), without overt associations with other infections. Indeed, these viral vaccines are considered so safe that they are again in production as replication-competent vaccines (110).

EVADING IMMUNE RESPONSES AGAINST ADENOVIRAL VECTORS

Serotype switching to evade vector-induced neutralizing antibodies

One approach to evade pre-existing neutralizing antibodies and vaccine-induced antibodies is to "serotype switch" the vector at each round of immunization. For example, administration of Ad2 induces potent neutralizing antibodies against Ad2 that reduce subsequent transduction if Ad2 is used again (111). However, if an Ad2 vector is used first and then the animal is immunized by serotype switching with Ad5, there is little reduction in transduction because the Ad2 antibodies do not strongly neutralize Ad5 (111, 112). This approach has been applied more recently for FG-Ad HIV vaccines and has demonstrated the ability to enable multiple rounds of immunization with potent Ad vaccines to markedly enhance vaccine responses (113-115). This approach is less available for monotypic viruses like measles virus or viruses with few serotypes (i.e. , poxviruses, herpes simplex virus [HSV], etc.). While serotype switching is very effective, from the production standpoint it does require two or more vector products to be produced for one trial rather than one vaccine product.

Using lower seroprevalance human adenoviruses and nonhuman adenoviruses to evade pre-existing neutralizing antibodies in humans

While serotype switching of human Ad vectors will likely evade responses in nonhuman primates and mice, many humans have previously been exposed to viruses like Ad5 and are already immune. Given this, there have been extensive efforts by several labs to identify and vector Ad serotypes with lower seroprevalence. This is feasible for Ad, since there are at least 55 serotypes of human adenoviruses and many other Ads that infect other species (reviewed in 116). Particular progress has been made in vectoring human Ad26 (117, 118) and Ad serotypes from nonhuman primates (109, 114, 115). Based on these results, a number of lower seroprevalence human Ads (Ad26, Ad35, Ad5/48) and low seroprevalence chimpanzee Ads (AdC6 and AdC7) are in process.

PEGylation to evade pre-existing neutralizing antibodies and reduce adaptive antibody and cellular responses against Ad vectors

Polyethylene glycol (PEG) is a shielding agent already in use in humans to protect protein therapeutics. Hydrophilic PEG is generally chemically conjugated to lysines on these proteins to reduce interactions with proteins and cells. Lysine-reactive PEG has also been used with Ad5 to shield the virus with as many as 15,000 PEG molecules. This protects Ad5 to some extent from neutralizing antibodies to allow multiple administrations (103, 119, 120). PEGylation also reduces the adaptive immune responses against the virus (103). In addition to these shielding effects, recent work has also shown that PEGylation of Ad increases its transduction after oral administration by protecting the vector from the low pH of the stomach and from proteolytic enzymes in the gut (121). These data suggest that PEGylation may be a robust shielding approach to enable a single viral vector to be used multiple times for vaccination. This approach may be more effective for non-enveloped viruses like Ad than for enveloped viruses, since much more of the virus surface is available for PEG conjugation.

Helper-dependent Ad (HD-Ad) vectors to evade vector-specific T-cell responses

One approach to reduce the immunogenicity of expressed adenovirus proteins is to delete every viral gene from the adenoviral genome to produce HD-Ad vectors (122-124). In HD-Ad vectors, all viral sequences are deleted from the vector with the exception of the inverted terminal repeats (ITRs) and packaging signal needed to replicate and package the vector. One benefit of HD-Ad vectors is that removal of all viral genes now allows sequences as large as 35 kbp to be packaged, allowing very large genes or multiple genes to be packaged into one vector (i.e., three HIV genomes could theoretically be packaged into one HD-Ad). Since HD-Ad vectors express no viral genes, the missing gene products need to be supplied in *trans* from a helper virus. HD-Ad vector production was significantly enabled by the generation of "floxed" helper viruses in which the packaging signal of the helper virus is flanked by LoxP sites (125, 126). One produces HD-Ad vectors with this system by transfecting the vector genome into 293 cells expressing the Cre recombinase. These transfected cells are then infected with the floxed helper virus. Under these conditions, the helper virus provides Ad proteins in *trans* to package the HD-Ad vector. Normally, these proteins would package both the helper virus and the HD-Ad vector. However, the Cre recombinase expressed in the 293 cells excises the floxed packaging signal from the helper to reduce its packaging by several orders of magnitude.

No adenoviral proteins are produced in HD-Ad vector-transduced cells because all of these genes have been removed. Because of this, HD-Ad vectors do not produce Ad antigens and evade vector-specific CTL responses that can kill transduced cells (122-124). In addition, HD-Ad vectors also produce markedly reduced liver damage that is observed 5 days after i.v. injection. This reduced immunogenicity and reduced liver damage allows for transgene expression in mice and in baboons over years (112, 127, 128).

Comparison of replication-competent Ad (RC-Ad), FG-Ad and HD-Ad vectors in mice

The vast majority of HIV vaccine testing has been performed with replication-defective FG-Ad vectors. By virtue of its ability to replicate 10,000-fold in infected cells, RC-Ad have been shown to be more robust than FG-Ad as an HIV vaccine in nonhuman primates (reviewed in 23). While potent, RC-Ad vaccines run the risk of causing Ad infection and disease. Due to deletion of all Ad open reading frames, HD-Ad is less immunogenic, has better safety and mediates extended expression of proteins when compared to FG-Ad (112, 127-129). Given this immune evasion, we performed the only head-to-head comparison of RC-Ad, FG-Ad and HD-Ad for transduction and immune responses in mice (130). Relevant to vaccination, RC-Ad and HD-Ad induced significantly higher levels of anti-luciferase antibodies than FG-Ad after i.m. or i.v. injection. Relevant to vaccine safety, RC-Ad generated the highest liver damage, whereas HD-Ad produced only background levels of ALT consistent with previous results. FG-Ad produced liver damage that was intermediate between RC-Ad and HD-Ad. These data suggest that an immunoevasive HD-Ad vaccine may have equal efficacy as an arguably more dangerous RC-Ad vaccine and that both are superior to current FG-Ad vaccines, at least in mice. These data suggest that HD-Ad vectors or other viral vectors lacking viral open reading frames may have better potency/safety profiles than current vectors.

Other viral vectors

Ad has provided much of the proof of principle and proof of problems for HIV viral vector vaccines. While Ads are robust, a number of other viral vectors hold great promise in this application. Considering current human testing, poxviruses, measles virus, VSV and SIV are all being tested. However, poxviruses are second only to Ad vaccines in current trials.

Poxvirus vectors

Poxviruses were the first viruses used as vaccines (reviewed in 131). Live vaccinia virus was used in the smallpox eradication efforts in the mid-1900s based on several different laboratory strains of the virus. These strains were quite potent and mediated near life-long immunity to smallpox. This potency is the foundation for their attraction as vectors for HIV vaccines. While potent, these live viruses have also been described as the most dangerous vaccine in use in humans due to their potential for sometimes lethal side effects. Based on this, attenuated forms of vaccinia virus were developed, including modified vaccinia Ankara (MVA) and vaccinia Tiantan strain (VTT), which are both in use in human HIV vaccine trials (e.g., MVA in NCT01418235 trial in South Africa and VTT in trials in China). In parallel, poxviruses from avian hosts have been developed for their lack of replication in human cells. These include the canarypox vaccine ALVAC-HIV that was used in the prime–boost vaccine study RV144 in Thailand and the fowlpox vaccine in the HVTN 055 trial. These vaccines appear to be largely safe and provoke robust immune responses in humans, particularly as part of a prime–boost strategy.

PROMISING RESULTS IN SUPPORT OF HIV VACCINES

RV144 trial in Thailand

Results from the RV144 canarypox envelope trial in Thailand (88) have re-energized the HIV vaccine community after the perceived setback of the STEP trial. This placebo-controlled efficacy trial included over 16,000 volunteers in Thailand who had a low risk of HIV infection. These volunteers were vaccinated four times with the canarypox vector vaccine ALVAC-HIV (vCP1521) and were then boosted twice with the previously used gp120 protein vaccine AIDSVAX B/E. Analysis of 16,395 volunteers demonstrated 31% vaccine efficacy in this heterosexual population. The jury is still out on which if any immune correlates are associated with a reduced risk in this trial. One recent preliminary analysis suggested that those that were protected might instead have higher levels of anti-Env antibody that bind the protein but that do not necessarily neutralize the virus (reviewed in 12). Whether these proposed V1/V2 binding antibodies have legitimate efficacy remains to be demonstrated.

While the protection in RV144 was modest, it was substantially better than in previous trials, therefore providing good proof of principle for further testing of this approach and other vaccines. Considering that this avian poxvirus platform is not thought to replicate in human cells, parallel efforts with replication-competent vaccines like VTT (132) in trials in China and with replication-competent Ad (133) may hold promise in driving even better responses in humans. Most important will be head-to-head comparisons of the most promising vaccine platforms rather than side-by-side tests by different groups.

Therapeutic vaccination

Most HIV vaccine trials are directed at prophylactic vaccination against the virus. One rationale for this effort is that if one cannot protect under conditions of a small initial infection, how can one protect during a massive ongoing infection wherein the immune system is already responding to HIV? While this pessimistic view has its merits, other work probing therapeutic vaccine effects has yielded promising results. In one recent example, HIV-1-infected volunteers on highly active antiretroviral therapy (HAART) were vaccinated with FG-Ad5 Gag during analytical treatment interruption. Under these conditions, the viral setpoints for vaccinees was 0.26 $\log_{10}$ lower than controls, although the *P* value of 0.07 did not reach the study's goal of a *P* value of 0.025 (134). These data suggest that HIV vaccine testing may proceed as therapeutic rather than prophylactic vaccines to optimize the cost/benefit ratio of the trial and to avoid the possible acquisition of side effects in uninfected volunteers. While this opens new opportunities, the presence of HIV already in the host makes extrapolation for prophylactic vaccination considerably more challenging.

Opportunity: establish barrier protection at the site of HIV entry – the mucosa

It is estimated that as many as 90% of HIV-1 infections occur by sexual transmission. In these cases, infection is thought to occur on mucosal surfaces in the vagina, rectum or urethral surfaces (reviewed in 135). It is also now thought that one or only a few HIV-1 virions successfully infect the host and that these few infectious events occur at the mucosal surfaces (4).

Combating the earliest events in HIV entry when only a few viruses are present therefore makes great strategic sense. Neutralizing or killing these few virions or infected cells at mucosal entry is numerically more achievable than combating the billions of viruses that arise later and are disseminated to the gut and beyond. Despite the recognized need for mucosal protection, most vaccination strategies against HIV-1, SIV or SHIV to date have been directed toward systemic immunization and not mucosal immunization. Indeed, survey of the 36 trials ongoing in humans for HIV vaccines reveals that only 1 vaccine approach delivers vaccine to the mucosa (CN54gp140-hsp70 Conjugate; Table I).

The vast majority of human HIV vaccines are delivered intramuscularly into the systemic immune system. This may be problematic, since in many cases, the generation of systemic immunity does not efficiently "cross over" onto mucosal sites (136, 137). While one can demonstrate crossover of immune responses from i.m. vaccination onto mucosal surfaces, this has in most cases been demonstrated in 20-g mice where one can "flood" the system with vector or antigen. One generally cannot scale up most vaccines 3,000-fold to flood the immune system of a 70-kg human. Therefore, robust generation of mucosal immune responses after intramuscular injection is not a foregone conclusion in humans, particularly when combating this worst-case virus, HIV.

Given this, there has been increasing interest in the development of vaccines that can generate robust antibody and cellular responses at mucosal surfaces (reviewed in 138). While preclinical testing has turned more towards gearing HIV vaccines towards mucosal immune responses, these efforts have to date not translated into most HIV trials (Table I). Efforts to actively generate anti-HIV immunity at the mucosa will likely be one of the next phases in human HIV vaccine testing. It will only be in the large and challenging immune system of humans that these vaccine hypotheses can be accurately tested.

CONCLUSIONS

Recent promising results in HIV vaccine trials have begun to correct the perhaps overzealous criticisms of previous trials like the STEP trial. This heavy lifting by basic researchers and those who have moved HIV vaccines into humans is now showing that production of prophylactic and perhaps therapeutic vaccines may be feasible. More potent vaccines and effective prime–boost strategies appear critical to efficacy. Likewise, controlling early mucosal infection where fewer virions are present seems to be a viable strategy.

When considering these vaccines as drugs there are several tradeoffs between ease of production, ease of approval and efficacy. Synthetic peptides do not require biological processes for production, but have relatively low efficacy. Plasmid DNA is easy to produce in massive amounts from bacteria, is scalable and one type of production can be applied for any antigen. While plasmid is easy, it is generally not potent in humans and so requires the addition of adjuvant plasmids (encoding cytokines) or physical methods of delivery (i.e., needle-free injection or electroporation), which might make delivery more cumbersome. Recombinant HIV proteins like Env that require accurate mammalian glycosylation typically must be produced from mammalian cells, which makes them more cumbersome to produce than some other vaccines. While more difficult, these protein vaccines appear to provide the most robust boosts of antibody responses, and so may well be pivotal for most HIV vaccine prime–boost strategies. Viral vectors vary widely in ease of production, with Ad vectors arguably being one of the simplest to produce in high yields. While robust, these vaccines still require mammalian production systems and stringent GMP purification to maximize safety.

An ideal HIV vaccine would be one that can be used more than once in the same host. At this moment, most HIV vaccines in human trials utilize more than one vaccine type, since prime–boost approaches appear to yield the highest efficacy in animal models and in humans. Therefore, an ideal one-agent HIV vaccine "drug" is not on the immediate horizon barring as yet unobserved exceptional results in humans.

Therefore, the earliest HIV vaccines will likely consist of more than one vaccine type and will need to be applied in two or more vaccine rounds. This requirement may be circumvented by an exceptionally potent nonviral vaccine system or perhaps more likely and sooner by vaccines that can mimic the robust effects of live virus vaccines, like vaccinia or measles virus vaccines. Promising candidates for these types of virus vaccines include replication-competent Ads, attenuated, but replication-competent poxviruses, and perhaps measles vectors and others. Success for these virus vectors will be challenged by pre-existing immunity in vaccinees (i.e., for Ad5 in adults, but not in children, for vaccinia in smallpox-vaccinated humans, for measles in measles-vaccinated hosts). Low seroprevalence viruses hold great promise in this regard. If boosting is needed, efforts to "stealth" viruses like Ad, vaccinia, measles and others may provide the means to deliver one vaccine multiple times, rather than having to produce multiple viruses for prime–boost strategies.

DISCLOSURES

The author states no conflicts of interest.

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